Antimicrobial activities of canine platelet lysate

by

Melikasadat Mollabashi

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Approved by

Maria C. Naskou, Chair, Assistant Professor, Department of Pathobiology
Scarlett M. Sumner, Assistant Professor, Department of Clinical Sciences
Laura Huber, Assistant Professor, Department of Pathobiology

Abstract

Platelet lysate (PL), an acellular product derived from lysed platelets, holds promise as a dual-function therapeutic with regenerative and antimicrobial properties. Rich in growth factors and antimicrobial peptides (AMPs), PL supports wound healing and disrupts bacterial membranes, offering a biologically based alternative or adjunct to antibiotics. While human and equine studies have demonstrated PL's efficacy against pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, its effects on bacteria common in canine wounds remain uncharacterized. Moreover, the impact of PL on bacterial growth kinetics in canine pathogens has not been previously investigated.

This study first looked at the effects of various preparation methods on the antimicrobial activity of canine PL, considering variations in leukocyte concentration, plasma content, and complement activity. Blood from eight dogs was processed into leukocyte-rich or -reduced PRP, followed by plasma depletion or heat inactivation before generating PL. Antimicrobial activity was tested against *E. coli*, *E. faecalis*, *S. pseudintermedius*, and *S. aureus*. PL significantly reduced *S. aureus* and *E. coli* counts, while plasma depletion and complement inactivation reduced efficacy against *S. pseudintermedius* and *E. faecalis*, respectively.

Next, we investigated how PL, alone or combined with amikacin, affects bacterial growth dynamics. PL (20% and 80%) with or without amikacin was tested against *S. aureus*, *S. pseudintermedius*, and MRSA. PL reduced bacterial yield and prolonged lag time in all strains, with stronger effects at 80%. The combination with amikacin further enhanced these effects, although μ_{max} remained unchanged.

These findings suggest that plasma components, including complement proteins, are important contributors to the antimicrobial effects of PL against certain bacteria, whereas

leukocyte concentration does not appear to play a major role. In addition, PL primarily limits bacterial proliferation and delays the onset of growth, while its combination with amikacin amplifies these effects without influencing bacterial replication speed.

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List of Abbreviations

ACD-A	Acid Citrate Dextrose Solution A
AIC	Akaike Information Criterion
AMK	Amikacin
AMR	Antimicrobial-Resistance
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
C3	Complement Component 3
C5	Complement Component 5
CBC	Complete Blood Count
CFU	Colony Forming Unit
EDTA	Ethylenediaminetetraacetic Acid Tubes
EPS	Extracellular Polymeric Substance
HCT	Hematocrit
IACUC	Institutional Animal Care and Use Committee

LPC	Leukocyte Rich Platelet Concentration
LPL	Leukocyte-Rich Platelet Lysate
LPPC	Leukocyte Rich Platelet Pellet Concentration
LPPL	Leukocyte Rich Platelet Pellet Lysate
L-PRP	Leukocyte and Platelet Rich Plasma
MRSA	Methicillin-Resistant-Staphylococcus-Aureus
OD	Optical Density
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PC	Pure Platelet Concentrate
PG	Platelet Gel
PL	Pure Platelet Lysate
PLT	Platelet Concentration
PPC	Platelet Pellet Concentrate
PPL	Platelet Pellet Lysate
PPP	Platelet Poor Plasma
PRF	Platelet-Rich Fibrin
PRP	Platelet-Rich Plasma
ROS	reactive Oxygen Species
TGF- β	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell

CHAPTER 1

LITERATURE REVIEW

1.1 Platelets:

Platelets are disc-shaped anucleated cells that derived from the megakaryocytes of bone marrow. Platelets play a profound role in blood clotting, and in canines, their half-life ranges from 5 to 7 days. Platelets consist of four zones which include the periphery zone, the sol-gel zone, the organelle zone, and the membranous zone. The peripheral zone is responsible for adhesion, aggregation and serving as a phospholipid surface for coagulation [1] . Actin and microtubules in the sol-gel zone help clot retraction, allow shape change during activation, and preserve platelet shape [2] . The membranous zone includes the open canalicular system (OCS) for substance exchange and the dense tubular system (DTS) for calcium storage and thromboxane A_2 generation [3].The organelle zone contains four types of platelet granules, notably the alpha granules (α-granules), the delta or dense granules (δ-granules), and the lysosomes. Alpha granules are indeed among the most important granules found in platelets, primarily due to their diverse contents and functions. They contain chemokines, coagulation factors, immunologic and adhesion molecules, and regulators of growth and angiogenesis [4]. Platelet-activating mediators like serotonin, calcium, adrenaline, ADP, and histamine are present in the dense granules. Lysosomes in platelets

contain a range of acid hydrolases, including cathepsins, acid phosphatase, arylsulfatase, and β-glucuronidase [5].

More precisely, platelets are a major source of serum-found growth factors, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1, attachment factors, and enzymes [6].

In both humans and dogs, platelets are essential for primary hemostasis. Von Willebrand factor (vWF), which connects subendothelial collagen to platelet glycoprotein Ib-IX-V receptors, is the main mechanism by which they adhere to the exposed subendothelial matrix following vascular injury [7]. Platelet activation caused by this adhesion results in cytoskeletal reorganization and the release of granule contents like thromboxane A_2 and ADP. These mediators improve platelet recruitment through autocrine and paracrine signaling [8]. The first platelet plug is then formed when activated platelets aggregate by cross-linking through fibrinogen binding to integrin GPIIb-IIIa (α_IIbβ_3) [7]. Dogs and humans share a high degree of conservation in these sequential processes: adhesion, activation, and aggregation [9].

Beyond their classical role in hemostasis and thrombosis, platelets have emerged as key regulators in tissue repair. Bioactive compounds that affect angiogenesis, cell division, migration, and wound healing are stored in their granules [10]. Platelets and their derivatives, like platelet lysate, have become important tools in translational and regenerative medicine because of their regenerative qualities [11].

Platelets can release their internal contents upon activation, a process critical for hemostasis, immune modulation, and tissue repair. *In vivo*, platelet activation occurs naturally in response to vascular injury or inflammation, where exposure to subendothelial matrix proteins (e.g., collagen, von Willebrand factor) and soluble agonists (e.g., thrombin, ADP, thromboxane

A_2) triggers platelet adhesion, aggregation, and degranulation [12]. In contrast, *in vitro*, various techniques have been employed to induce platelet activation or lysis to release growth factors and other bioactive substances. Commonly used methods include sonication, repeated freeze–thaw cycles, chemical activation using calcium salt solutions (such as calcium chloride or gluconate), and solvent/detergent treatments [4].

1.1.1 Biological products derived from platelets:

Platelet-derived products have been proposed as an alternative regenerative therapeutic mostly due to the bioactive molecules stored in the alpha granules of platelets, such as growth factors and cytokines [13]. These molecules are known to impact several stages of wound healing, such as regulating inflammatory reactions, promoting collagen production, and promoting the creation of granulation tissue [14]. As a result, they can increase tissue repair and regeneration [15]. The production of platelet-derived products varies greatly; research indicates that variations in cellular composition, levels of growth factors and inflammatory cytokines, and the presence of plasma proteins involved in tissue metabolism may affect the products' performance both in vitro and in vivo [11, 16]. These inconsistencies may ultimately impact the therapeutic efficacy of the final product. In the following paragraphs, the characteristics and preparation methods of various platelet-derived products are explained:

a. PRP: Platelet-rich plasma (PRP) is one of the most widely used products produced from platelets [17]. Platelet-rich plasma (PRP) can be produced using several methods, including the commonly used double centrifugation technique. In this method, anticoagulated whole blood undergoes an initial centrifugation to separate the plasma

from the buffy coat and red blood cells. A second spin allows the separation of platelet-poor plasma (PPP) from the platelet-rich fraction, after which the platelet pellet is reconstituted in a small volume of plasma to achieve the desired concentration [18]. However, PRP can also be prepared using alternative methods such as gravity filtration, plateletpheresis, and a variety of commercial PRP preparation systems, each with distinct protocols and efficiencies [19]. Currently, PRP is used as a therapeutic for healing wounds, soft tissue injuries, and a variety of musculoskeletal injuries both in human and veterinary patients [20, 21].

b. PL: Platelet lysate (PL) is a novel biological product rich in growth factors and antimicrobial peptides that have been shown to accelerate the wound healing process in both human and veterinary species. Platelet lysate is derived from ruptured membranes of platelets, resulting in the release of the platelet contents. The lysis of platelets usually occurs by performing 2-5 freeze-thaw cycles on PRP [16, 22]. The lack of membrane makes PL a product rich in growth factors, cytokines, and chemokines [23]. One of the remarkable features of PL is that the immunogenic reaction issues from non-autologous PRP are solved due to the elimination of platelet membranes [23]. Another advantage of PL compared to PRP is the prolonged storage life without compromising (losing) its therapeutic features. Additionally, PL may be obtained from a pool of several donors to minimize donor variability [24].

c. PRF: This is another second-generation product derived from PRP. Platelet-rich fibrin (PRF) is a naturally generated bioscaffold made of a thick fibrin matrix that traps leukocytes, platelets, and various growth factors. In this way, they will be released slowly at the tissue repair site [25]. To generate PRF, the blood should be collected in a tube without anticoagulants, unlike PRP and PL production. After that, a high-speed

centrifuge should be performed to activate the coagulation cascade and stimulate thrombin generation. This leads to the formation of a fibrin clot [26]. This fibrin matrix can slowly release the bioactive components such as cytokine and growth factors. Its temporal degradation profile, which ensures alignment with physiological healing timelines, maximizes its contribution to tissue regeneration and structural reconfiguration [27]. Nowadays, PRF is utilized in dental treatments for human medicine [28].

d. PG: When platelet-rich plasma (PRP) is mixed with an activator like thrombin or calcium chloride, platelet gel (PG) is created. It is a fibrin-based, semi-solid matrix that can trap platelets and the bioactive molecule they are associated with [11, 29]. PG is typically applied topically at the site of tissue injury, including skin wounds, surgical incisions, and chronic ulcers. After being applied, PG works as a biological scaffold, continuously releasing growth factors like PDGF, TGF-β, and VEGF over several days. This will support cell proliferation and matrix remodeling [11]. Prolonged release enables extended therapeutic activity following a single application of PG. Clinical results have shown that PG improves wound healing through increased collagen deposition, decreased local inflammation, and stimulated angiogenesis [29]. Due to these effects, PG is especially useful in regenerative applications in orthopedic surgery, dentistry, and dermatology, where topical or localized delivery is necessary to optimize healing potential. Furthermore, compared to liquid PRP formulations, its solid form offers practical advantages in handling, containment within the wound bed, and tissue adhesion [29].

1.2 Platelets and Microbial Defense

Platelets have been shown to play a crucial role in combating microbial infections through multiple mechanisms. They combat bacteria in direct and indirect ways [30]. Through certain surface receptors such as GPIbα, GPIIb/IIIa (αIIbβ3), TLRs, complement markers, FcRɣIIa, P2Y, and CD62P, platelets directly kill bacteria [31, 32].

Through receptor-mediated interactions and effector mechanisms that work both independently and in collaboration with plasma components, platelets play a variety of roles in direct bacterial elimination. Surface receptors such as integrins (GPIbα, GPIIb/IIIa), Toll-like receptors (TLR2, TLR4), complement receptors (gC1q-R/P33), and FcRɣIIa are the main mediators of their antimicrobial activity. These receptors help identify pathogens in various bacterial species, including *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Borrelia burgdorferi* [33]. This bactericidal action is supported by four main mechanisms [30]. First, bactericidal compounds contained in α-granules are released by platelets. This includes a) reactive oxygen species (ROS), β-defensins, and thrombocidins that make a rupture in bacterial membranes [34, 35], b) Immunoglobulins (IgG/IgM) and complement proteins (e.g., C3a, C5a, MAC complex) which increase opsonization [36, 37], c) granulysins, and proteolytic enzymes (e.g., cathepsin D) which lead to degradation of bacterial proteins [38]. Second, despite not being expert phagocytes, platelets capture bacteria through the integrins GPIIb/IIIa or TLR2 and confine them within phagocytic vacuoles [39]. Platelet TLR2 activation improves phagocytic coordination by inducing CD62P-mediated contacts with neutrophils [31]. Third, platelets immobilize bacteria by: a) aggregation mediated by complement receptors or fibrinogen-bound GPIIb/IIIa [31], b) Scavenging, which means platelet motility is increased by autocrine activation, which also removes free bacteria from the bloodstream [40, 41]. Fourth, platelets improve the neutrophil

extracellular trap (NET) activity. NETosis is accelerated when platelets attach to bacteria through TLR2, FcR IIa, or CD62P [31]. In addition, Thromboxane A2 (TxA2) promotes platelet aggregation and vascular permeability, which recruits neutrophils and boosts NET effectiveness [42].

1.2.1 Platelet-derived antimicrobial peptides:

According to human studies, seven groups of antimicrobial peptides released from platelets are responsible for the antimicrobial features of platelets [43]. Platelet basic protein, connective tissue activating peptide 3 (CTAP-3), fibrinopeptide A (FP-A), fibrinopeptide B (FP-B), platelet factor 4 (PF-4), thymosin b-4 (TB-4), and RANTES are released after stimulating platelets with thrombin. PF-4 has the broadest activity among these peptides since it is effective against gram-positive and negative bacteria. Moreover, CTAP was more plentiful compared to other peptides [31, 44, 45]. PF-4 attaches to various bacteria resulting in the expression of neoepitopes [46]. The expression of neoepitopes leads to the manufacture of anti-PF-4 antibodies. This antibody will opsonize the PF-4-coated bacteria and after that platelets cover the opsonized bacteria. The platelets destroy the ingested bacteria via the chemical substances in their granules [47]. The performance of those antimicrobial peptides can be different due to the PH; for instance, FP-A and FP-B demonstrated better performance against bacteria in an acidic condition. However, TB-4 worked better under alkaline PH [31, 44, 45].

1.2.2 Platelet-Leukocyte Interactions in Antimicrobial Defense:

Platelet-derived antimicrobial peptides can improve leukocytes' capacity to fight off microbes in an acidic environment [21-23]. Human platelet antimicrobial peptides (HPAPs) exert their effects through multiple mechanisms: they can directly kill or inhibit the growth of microbes by disrupting their membranes, and they also enhance the antimicrobial activity of immune cells by promoting

phagocytosis and modulating inflammatory responses [43]. Thrombin-activated platelets release mediators that amplify the ability of macrophages to do phagocytosis and produce IL-1β. Platelets inhibit the intracellular growth of bacteria after phagocytosis [48]. Several studies proved that leukocytes could increase antimicrobial features and other functions of platelets [49, 50]. This is due to the ability of leukocytes to release myeloperoxidase which kills bacteria [51]. The high concentration of leukocytes added to PRP demonstrated a promising effect in killing *C. acnes* [49].

1.2.3 The antimicrobial properties of canine and feline platelet-derived products

Platelet-derived products, including PRP, PRF, and PL, have been proposed as an alternative or adjunct therapy to conventional antibiotics to fight bacterial infections. These products derive from platelet concentrates and are rich in growth factors, cytokines, and antimicrobial peptides released from the platelet granules [52, 53].

Canine platelet-derived products have also demonstrated promising antimicrobial properties in recent studies. One of those products is canine platelet-rich fibrin (PRF). Platelet-rich fibrin (PRF) has demonstrated significant antimicrobial potential, attributed in part to its unique structural and biochemical properties [54]. The three-dimensional fibrin network created during PRF preparation is one of the main mechanisms behind this action. It has a direct role in capturing and limiting bacterial growth in addition to serving as a scaffold for the continuous release of bioactive molecules. At several time points, canine PRF showed strong antimicrobial action against Escherichia coli, with reactive oxygen species (ROS) being a key factor in this activity. ROS produced in the PRF matrix can cause oxidative stress in bacteria, jeopardizing the viability and integrity of their cell membranes [54]. The antimicrobial efficacy of PRF has also been supported in clinical settings [55]. When PRF clots from canine donors were applied to feline wounds in a

recent case series by Soares et al. (2024), the wounds healed successfully, and no infection developed. Interestingly, this result was obtained without the application of any topical antiseptics, highlighting PRF's inherent antimicrobial qualities and its potential as an independent wound care product [55]. According to these results, PRF not only acts as a regenerative scaffold but also contributes to infection control, which is particularly useful when using antibiotics is not advised or resistance is an issue. Additionally, PRF has been thoroughly investigated in human dentistry, where periodontal pathogens have been tested for their antimicrobial qualities [56, 57]. Castro et al. (2019) showed that leukocyte- and platelet-rich fibrin (L-PRF) effectively inhibits the growth of several oral microbes, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* [57]. The antimicrobial activity of injectable PRF (i-PRF) and L-PRF was also compared in another study. Both forms demonstrated bacteriostatic effects against oral pathogens, though the effectiveness varies depending on the formulation and microbial target [56]. Another important platelet derivative, platelet-rich plasma (PRP), has shown encouraging antimicrobial qualities against a variety of pathogens, including strains that are resistant to multiple drugs. The ability of PRP to combat methicillin-resistant Staphylococcus aureus (MRSA) is one of the most clinically significant findings. According to Farghali et al. (2019), in dogs with MRSA-infected skin wounds, topical administration of autologous PRP dramatically decreased wound size and improved bacterial clearance. This indicates both regenerative and antimicrobial benefits in a single treatment modality. The concentrated presence of growth factors, cytokines, and antimicrobial peptides released from activated platelets at the wound site is primarily responsible for these effects. In vitro investigations further support the antimicrobial efficacy of PRP against Gram-negative organisms. Using the Kirby-Bauer disk diffusion method, leukocyte-rich platelet gel from canine donors generated distinct zones of inhibition against *Klebsiella pneumoniae,*

Pseudomonas aeruginosa, and *Escherichia coli*. These findings highlight the significance of leukocyte inclusion, which improves antimicrobial qualities by releasing reactive oxygen intermediates like myeloperoxidase. A case report by Gemignani et al. (2017) supported these conclusions by demonstrating the effective application of heterologous canine PRP in the treatment of a contaminated feline cutaneous wound. Interestingly, even in the absence of systemic or topical antibiotics, PRP application alone prevented infection, suggesting that it may be useful as an independent treatment in specific clinical situations. Mechanistically, PRP appears to exert primarily bacteriostatic effects rather than bactericidal activity. PRP significantly decreases bacterial growth but may not totally eliminate pathogens, as multiple studies have shown. This emphasizes the significance of combining PRP with other antimicrobial techniques in cases of severe infections . Both the cellular (derived from platelets and leukocytes) and acellular (derived from plasma protein) components of PRP are necessary for its antimicrobial activity . Drago et al. (2014) emphasized that platelet activation is required for antimicrobial action; plasma components by themselves were not enough to trigger the release of potent antimicrobial factors unless they were combined with activated platelets.

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1.2.4 The antimicrobial properties of equine platelet-derived products

Due to their antimicrobial and regenerative qualities, platelet-derived products are being utilized more and more in equine medicine. Bacterial suppression is one of their most promising uses. Equine platelet lysate (ePL), derived from lysed platelets, has demonstrated antimicrobial effects against a variety of clinically relevant pathogens. For example, *Staphylococcus aureus*, a major cause of septic arthritis in horses, was significantly inhibited by ePL. A decrease in bacterial

growth rather than complete eradication suggested that this inhibition was bacteriostatic, implying that it interfered with bacterial proliferation rather than directly killing the bacteria [58]. Additionally, it was found that ePL delayed the onset of the exponential (log) growth phase in both *Pseudomonas aeruginosa* and *Escherichia coli*. This delay was dose-dependent, meaning that higher ePL concentrations resulted in greater growth inhibition. Interestingly, such concentration-dependence was not observed for *Enterococcus faecalis* and *S. aureus*, where growth suppression occurred regardless of the ePL dose used, implying potential saturation of antimicrobial components or distinct mechanisms of action [59]. In addition to lysates, other equine platelet-derived products, such as platelet-rich plasma (PRP) and leukocyte-poor gel (LPG), also demonstrated notable antimicrobial effects. PRP and LPG both had bacteriostatic effects on *methicillin-sensitive S. aureus (MSSA)* at 6- and 24-hour time points, according to in vitro tests. *Methicillin-resistant S. aureus (MRSA)*, on the other hand, showed reduced sensitivity, suggesting possible limits in effectiveness against resistant strains. The study highlighted the significance of plasma components in PRP's antibacterial qualities by attributing these antimicrobial effects in part to complement proteins derived from plasma [60]. The therapeutic synergy between PRP and conventional antibiotics has also been explored PRP and amikacin together dramatically decreased the intra-articular bacterial burden caused on by S. aureus in a clinically relevant equine model of infectious arthritis. Notably, compared to horses treated with antibiotics alone, the majority of horses receiving both PRP and antibiotics showed complete bacterial clearance after 7 days of treatment. Previous in vitro research further supported these findings by demonstrating that PRP lysate not only broke up *S. aureus* biofilms but also restored the effectiveness of antibiotics against bacteria embedded in synovial fluid biofilms, which are usually challenging to treat [61, 62]. In addition to musculoskeletal and postoperative infections, PRP has shown therapeutic potential in

managing infectious reproductive disorders in mares, particularly persistent breeding-induced endometritis (PBIE). Subfertility in broodmares is frequently caused by PBIE, which is usually linked to the presence of *Staphylococcus aureus* or *Escherichia coli* in the uterine lumen after artificial insemination or mating [63]. In the end, these bacterial infections can delay endometrial regeneration and jeopardize embryo survival by inducing prolonged inflammatory responses marked by neutrophil influx, cytokine release, and impaired uterine clearance [64, 65]. PRP has been suggested as a biologically based substitute for conventional therapies like antibiotics or uterine lavage. In a controlled study, mares provided PRP showed noticeably lower rates of bacterial culture in uterine samples than those given lactated Ringer's solution, indicating that the platelet-derived factors have direct antimicrobial activity [66]. Additionally, PRP treatment was linked to decreased neutrophil infiltration and a shorter duration of inflammation, suggesting that it has a dual function in regulating host immune responses and microbial burden. Beyond reproductive health, PRP has also been investigated for its antimicrobial activity in equine ophthalmology. *Staphylococcus sciuri*, one of the many microorganisms found on the ocular surface of healthy horses, is generally non-pathogenic but can become opportunistic in compromised conditions like trauma, surgery, or immunological suppression. According to in vitro studies, PRP can significantly inhibit *S. sciuri* growth for up to six hours. The degree of inhibition increases with PRP concentration, confirming a dose-dependent bacteriostatic effect [65]. This is likely mediated by the bioactive proteins and peptides contained in PRP, such as platelet factor 4, which has known antimicrobial activity. PRP may be useful as a supportive or adjunctive therapy in ocular wound healing or infection management, especially in situations where antibiotic resistance or epithelial toxicity from conventional antimicrobials is a concern, according to the short-term but effective inhibition.

Refrences

1. Gremmel, T., A.L. Frelinger, 3rd, and A.D. Michelson, *Platelet Physiology.* Semin Thromb Hemost, 2016. **42**(3): p. 191-204.
2. Geraldo, R.B., et al., *Platelets: still a therapeutical target for haemostatic disorders.* Int J Mol Sci, 2014. **15**(10): p. 17901-19.
3. Selvadurai, M.V. and J.R. Hamilton, *Structure and function of the open canalicular system - the platelet's specialized internal membrane network.* Platelets, 2018. **29**(4): p. 319-325.
4. Burnouf, T., et al., *Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation?* Biomaterials, 2016. **76**: p. 371-87.

5. Heijnen, H. and P. van der Sluijs, *Platelet secretory behaviour: as diverse as the granules ... or not?* J Thromb Haemost, 2015. **13**(12): p. 2141-51.
6. Heldin, C.H. and B. Westermark, *Mechanism of action and in vivo role of platelet-derived growth factor.* Physiol Rev, 1999. **79**(4): p. 1283-316.
7. Cortese, L., P.W. Christopherson, and A. Pelagalli, *Platelet Function and Therapeutic Applications in Dogs: Current Status and Future Prospects.* Animals (Basel), 2020. **10**(2).
8. Scridon, A., *Platelets and Their Role in Hemostasis and Thrombosis-From Physiology to Pathophysiology and Therapeutic Implications.* Int J Mol Sci, 2022. **23**(21).
9. Cremer, S.E., et al., *The canine activated platelet secretome (CAPS): A translational model of thrombin-evoked platelet activation response.* Res Pract Thromb Haemost, 2021. **5**(1): p. 55-68.
10. Martinez, C.E., P.C. Smith, and V.A. Palma Alvarado, *The influence of platelet-derived products on angiogenesis and tissue repair: a concise update.* Front Physiol, 2015. **6**: p. 290.
11. De Pascale, M.R., et al., *Platelet derivatives in regenerative medicine: an update.* Transfus Med Rev, 2015. **29**(1): p. 52-61.
12. Stalker, T.J., J.D. Welsh, and L.F. Brass, *Shaping the platelet response to vascular injury.* Curr Opin Hematol, 2014. **21**(5): p. 410-7.
13. Dos Santos, R.G., et al., *The regenerative mechanisms of platelet-rich plasma: A review.* Cytokine, 2021. **144**: p. 155560.
14. Locatelli, L., et al., *Platelets in Wound Healing: What Happens in Space?* Front Bioeng Biotechnol, 2021. **9**: p. 716184.
15. Cecerska-Heryc, E., et al., *Applications of the regenerative capacity of platelets in modern medicine.* Cytokine Growth Factor Rev, 2022. **64**: p. 84-94.
16. Lunardon, T., et al., *Growth factor and cytokine characterization of canine platelet lysate with variable leukocyte concentration, plasma content, and heat-sensitive proteins.* Front Vet Sci, 2024. **11**: p. 1408080.
17. Dhurat, R. and M. Sukesh, *Principles and Methods of Preparation of Platelet-Rich Plasma: A Review and Author's Perspective.* J Cutan Aesthet Surg, 2014. **7**(4): p. 189-97.
18. Akhundov, K., et al., *Development of a cost-effective method for platelet-rich plasma (PRP) preparation for topical wound healing.* Ann Burns Fire Disasters, 2012. **25**(4): p. 207-13.
19. Marx, R.E., *Platelet-rich plasma: evidence to support its use.* J Oral Maxillofac Surg, 2004. **62**(4): p. 489-96.
20. O'Dowd, A., *Update on the Use of Platelet-Rich Plasma Injections in the Management of Musculoskeletal Injuries: A Systematic Review of Studies From 2014 to 2021.* Orthop J Sports Med, 2022. **10**(12): p. 23259671221140888.
21. Oneto, P. and J. Etulain, *PRP in wound healing applications.* Platelets, 2021. **32**(2): p. 189-199.
22. Klatte-Schulz, F., et al., *Comparative Analysis of Different Platelet Lysates and Platelet Rich Preparations to Stimulate Tendon Cell Biology: An In Vitro Study.* Int J Mol Sci, 2018. **19**(1).
23. da Fonseca, L., et al., *Human platelet lysate - A potent (and overlooked) orthobiologic.* J Clin Orthop Trauma, 2021. **21**: p. 101534.

24. Henschler, R., et al., *Human platelet lysate current standards and future developments.* Transfusion, 2019. **59**(4): p. 1407-1413.
25. Pavlovic, V., et al., *Platelet-rich fibrin: Basics of biological actions and protocol modifications.* Open Med (Wars), 2021. **16**(1): p. 446-454.
26. Su, C.Y., et al., *In vitro release of growth factors from platelet-rich fibrin (PRF): a proposal to optimize the clinical applications of PRF.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2009. **108**(1): p. 56-61.
27. Ghanaati, S., et al., *Advanced platelet-rich fibrin: a new concept for cell-based tissue engineering by means of inflammatory cells.* J Oral Implantol, 2014. **40**(6): p. 679-89.
28. Pietruszka, P., et al., *PRP and PRF-Subgroups and Divisions When Used in Dentistry.* J Pers Med, 2021. **11**(10).
29. Piccin, A., et al., *Platelet gel: a new therapeutic tool with great potential.* Blood Transfus, 2017. **15**(4): p. 333-340.
30. Tokarz-Deptula, B., et al., *What Function Do Platelets Play in Inflammation and Bacterial and Viral Infections?* Front Immunol, 2021. **12**: p. 770436.
31. Yeaman, M.R., *The role of platelets in antimicrobial host defense.* Clin Infect Dis, 1997. **25**(5): p. 951-68; quiz 969-70.
32. Kerrigan, S.W., *The expanding field of platelet-bacterial interconnections.* Platelets, 2015. **26**(4): p. 293-301.
33. Kerrigan, S.W. and D. Cox, *Platelet-bacterial interactions.* Cell Mol Life Sci, 2010. **67**(4): p. 513-23.
34. Micota, B., B. Sadowska, and B. Rozalska, *[The role of blood platelets in infections].* Postepy Hig Med Dosw (Online), 2015. **69**: p. 624-32.
35. Kraemer, B.F., et al., *Novel anti-bacterial activities of beta-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation.* PLoS Pathog, 2011. **7**(11): p. e1002355.
36. George, J.N., et al., *Immunoglobulin G is a platelet alpha granule-secreted protein.* J Clin Invest, 1985. **76**(5): p. 2020-5.
37. Mezger, M., et al., *Platelets and Immune Responses During Thromboinflammation.* Front Immunol, 2019. **10**: p. 1731.
38. Krijgsveld, J., et al., *Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines.* J Biol Chem, 2000. **275**(27): p. 20374-81.
39. White, J.G., *Platelets are covercytes, not phagocytes: uptake of bacteria involves channels of the open canalicular system.* Platelets, 2005. **16**(2): p. 121-31.
40. Gomez, R.M., A.O. Lopez Ortiz, and M. Schattner, *Platelets and extracellular traps in infections.* Platelets, 2021. **32**(3): p. 305-313.
41. Gaertner, F., et al., *Migrating Platelets Are Mechano-scavengers that Collect and Bundle Bacteria.* Cell, 2017. **171**(6): p. 1368-1382 e23.
42. Kerris, E.W.J., et al., *Platelets and platelet extracellular vesicles in hemostasis and sepsis.* J Investig Med, 2020. **68**(4): p. 813-820.
43. Tang, Y.Q., M.R. Yeaman, and M.E. Selsted, *Antimicrobial peptides from human platelets.* Infect Immun, 2002. **70**(12): p. 6524-33.
44. Yeaman, M.R. and A.S. Bayer, *Antimicrobial peptides from platelets.* Drug Resist Updat, 1999. **2**(2): p. 116-126.

45. Shafer, W.M., L.E. Martin, and J.K. Spitznagel, *Late intraphagosomal hydrogen ion concentration favors the in vitro antimicrobial capacity of a 37-kilodalton cationic granule protein of human neutrophil granulocytes.* Infect Immun, 1986. **53**(3): p. 651-5.
46. Krauel, K., et al., *Platelet factor 4 binds to bacteria, [corrected] inducing antibodies cross-reacting with the major antigen in heparin-induced thrombocytopenia.* Blood, 2011. **117**(4): p. 1370-8.
47. Freeze, H.H., *Mannose 6-sulfate is present in the N-linked oligosaccharides of lysosomal enzymes of Dictyostelium.* Arch Biochem Biophys, 1985. **243**(2): p. 690-3.
48. Ali, R.A., et al., *Platelets Mediate Host Defense against Staphylococcus aureus through Direct Bactericidal Activity and by Enhancing Macrophage Activities.* J Immunol, 2017. **198**(1): p. 344-351.
49. Prysak, M.H., et al., *Optimizing the safety of intradiscal platelet-rich plasma: an in vitro study with Cutibacterium acnes.* Regen Med, 2019. **14**(10): p. 955-967.
50. Parrish, W.R., et al., *Normal platelet function in platelet concentrates requires non-platelet cells: a comparative in vitro evaluation of leucocyte-rich (type 1a) and leucocyte-poor (type 3b) platelet concentrates.* BMJ Open Sport Exerc Med, 2016. **2**(1): p. e000071.
51. Lincoln, J.A., et al., *Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages.* Infect Immun, 1995. **63**(8): p. 3042-7.
52. Cl, K., et al., *Antimicrobial Effects of Platelet-Rich Plasma and Platelet-Rich Fibrin: A Scoping Review.* Cureus, 2023. **15**(12): p. e51360.
53. Farghali, H.A., et al., *Antimicrobial action of autologous platelet-rich plasma on MRSA-infected skin wounds in dogs.* Sci Rep, 2019. **9**(1): p. 12722.
54. Warin, R., et al., *In Vitro Antimicrobial Properties and Their Mechanisms in Relation to Reactive Oxygen Species of Canine Platelet-Rich Fibrin.* Animals (Basel), 2023. **13**(24).
55. Soares, C.S., et al., *Management of canine wounds using platelet-rich fibrin (PRF) biomaterial. A case series report.* Vet Med Sci, 2024. **10**(3): p. e1236.
56. Sindhusha, V.B. and J. Ramamurthy, *Comparison of Antimicrobial Activity of Injectable Platelet-Rich Fibrin (i-PRF) and Leukocyte and Platelet-Rich Fibrin (l-PRF) Against Oral Microbes: An In Vitro Study.* Cureus, 2023. **15**(9): p. e46196.
57. Castro, A.B., et al., *Antimicrobial capacity of Leucocyte-and Platelet Rich Fibrin against periodontal pathogens.* Sci Rep, 2019. **9**(1): p. 8188.
58. Avellar, H.K., et al., *In vitro antimicrobial activity of equine platelet lysate and mesenchymal stromal cells against common clinical pathogens.* Can J Vet Res, 2022. **86**(1): p. 59-64.
59. Gordon, J., S. Alvarez-Narvaez, and J.F. Peroni, *Antimicrobial Effects of Equine Platelet Lysate.* Front Vet Sci, 2021. **8**: p. 703414.
60. Lopez, C., M.E. Alvarez, and J.U. Carmona, *Temporal Bacteriostatic Effect and Growth Factor Loss in Equine Platelet Components and Plasma Cultured with Methicillin-Sensitive and Methicillin-Resistant Staphylococcus aureus: A Comparative In Vitro Study.* Vet Med Int, 2014. **2014**: p. 525826.
61. Gilbertie, J.M., et al., *A Platelet-Rich Plasma-Derived Biologic Clears Staphylococcus aureus Biofilms While Mitigating Cartilage Degeneration and Joint Inflammation in a Clinically Relevant Large Animal Infectious Arthritis Model.* Front Cell Infect Microbiol, 2022. **12**: p. 895022.

62. Gilbertie, J.M., et al., *Platelet-rich plasma lysate displays antibiofilm properties and restores antimicrobial activity against synovial fluid biofilms in vitro.* J Orthop Res, 2020. **38**(6): p. 1365-1374.
63. Canisso, I.F., L. Segabinazzi, and C.E. Fedorka, *Persistent Breeding-Induced Endometritis in Mares - a Multifaceted Challenge: From Clinical Aspects to Immunopathogenesis and Pathobiology.* Int J Mol Sci, 2020. **21**(4).
64. Christoffersen, M., et al., *Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis.* BMC Vet Res, 2012. **8**: p. 41.
65. Christoffersen, M., et al., *Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares.* Theriogenology, 2012. **78**(5): p. 991-1004.
66. Segabinazzi, L., et al., *Intrauterine Blood Plasma Platelet-Therapy Mitigates Persistent Breeding-Induced Endometritis, Reduces Uterine Infections, and Improves Embryo Recovery in Mares.* Antibiotics (Basel), 2021. **10**(5).

CHAPTER 2

PLASMA AND COMPLEMENT PROTEINS ARE ESSENTIAL FOR THE ANTIMICROBIAL ACTIVITY OF CANINE PLATELET LYSATE

Melikasadat Mollabashi[1,3], Alonza Klopfer[1], Thainá Lunardon[1,3], Nikolia Darzenta[1,3], Emily Davis[1], Matt Murray[1], Scarlett M. Sumner[2*,] Maria C. Naskou[1,3*]

[1]Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL, United States

[2]Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, Auburn, AL, United States

[3]Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL, United States

Abstract

Platelet-derived products have gained increasing attention as promising alternative biologicals for the treatment of canine wounds. Specifically, platelets play a crucial role during the inflammatory phase of wound healing due to the release of chemokines, proteins, cytokines, and growth factors. Additionally, platelets possess antimicrobial properties, which can be influenced by their manufacturing process, platelet and leukocyte concentration, activation method, and the presence of plasma and complement. The objective of this study was to assess how various preparation methods of platelet products affect their antimicrobial effect against bacteria commonly isolated from wounds. In this study, blood was collected from eight purpose-bred dogs, and platelet-rich plasma was produced using two methods of centrifugation, one leukocyte-enriching and one leukocyte-reducing. Some samples were processed for plasma depletion and platelet lysate was subsequently generated through freeze-thaw cycles. Additionally, portions of platelet lysate samples underwent heat treatment for complement inactivation. All treatment groups were tested against four common bacteria found in canine skin wounds: *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus pseudintermedius,* and *Staphylococcus aureus*. The antimicrobial effect of various lysate formulations was evaluated using a bacteria-spiking (time-killing) assay. Platelet lysate significantly reduced the number of *S. aureus* and *E. coli* after 3 hours compared to culture media. No significant differences were noted in the log reduction of bacteria between the centrifugation techniques. After depleting plasma, the log reduction of *S. pseudintermedius* was significantly less than before plasma depletion, whereas the opposite was seen for *E. faecalis* after 3 hours. Complement-depleted plasma led to a significantly lower log reduction for *E. faecalis* after 24 hours compared to platelet lysate. Therefore, the presence of

plasma and complement proteins in platelet lysate appear to play a critical role in inhibiting the growth of certain bacterial strains, whereas the leukocyte concentration does not have a significant effect. Further research is needed to identify the ideal formulation and dose of canine platelet lysate as an antimicrobial and wound healing treatment.

Introduction

Wound healing is a complex multicellular dynamic event characterized by several processes such as inflammation, coagulation, epithelization, tissue regeneration, and granulation tissue formation (1). Platelets play a crucial role during the inflammatory phase of wound healing due to their alpha granules releasing most of the chemokines, proteins, cytokines, and growth factors responsible for activating and recruiting other cells involved in inflammatory responses (2). Platelet-rich plasma (PRP) is a platelet-derived product containing an increased concentration of platelets, widely known to benefit wound healing processes and inhibit common pathogens found in wounds (3-7). Platelet concentrates, such as PRP, can be subjected to several manufacturing techniques that include lysis of platelets to release their chemotactic and growth factors (8). The resulting product is called platelet lysate (PL) (8).

PL is an acellular product containing platelet-derived growth factors that can serve as an off-the-shelf alternative to PRP (9). As it is acellular, issues of immunogenicity are reduced, which is another attractive attribute of PL as a therapeutic (9). Additionally, PL can be pooled from several donors to decrease donor variability and can be stored for extended periods in the freezer (9-11). Another attractive aspect of PL regarding wound healing is its antimicrobial potential. Specifically, the antimicrobial properties originate from the release of antimicrobial peptides (AMPs) and an array of growth factors (12-15). The activity of AMPs has been shown to initiate pore formation within bacterial membranes, which can evoke cell death (16). New studies have demonstrated that canine platelet-derived products display antimicrobial properties in wounds infected with antibiotic-resistant Gram-negative (17) and *Methicillin-resistant Staphylococcus aureus (MRSA)* (18), while equine PL was recently found to inhibit the growth of *Enterococcus*

faecalis and *Staphylococcus aureus* (8). Additionally, equine PL has shown both concentration-dependent and independent inhibition effects depending on the type of bacteria being treated(8). Therefore, PL has dual treatment potential, since it can be used for wound healing purposes, and as an alternative to traditional antibiotics for regulating wound-related bacterial infections, including those with antibiotic resistance.

PRP and PL can be manufactured in different ways, including various commercial systems, manual lab centrifugation methods, gravity filtration methods, and plateletpheresis (19). Final platelet concentrations of PRP vary greatly within the literature, even when using a single method of production (20, 21). Variability also exists regarding the leukocyte concentration (20, 21) and the method of platelet activation (22, 23). The variability of platelet products in the manufacturing processes and the inflammatory cytokine concentration, growth factors, and cellular composition can affect tissue metabolism in vitro and in vivo (7, 22-25). These factors can ultimately alter the efficacy of the final product.

Similarly, the antimicrobial potential of platelet products can be affected by the concentration of leukocytes; however, the results are controversial. Some studies showed that leukocytes were not essential for maintaining the antimicrobial properties of platelets (17, 26, 27). Specifically, leukocytes can produce pro-inflammatory protease and acid hydrolases that might induce an inflammatory response (28). On the contrary, some studies found that the presence of leukocytes in platelet products amplified their ability to inhibit bacterial growth (29, 30), because leukocytes produce myeloperoxidase that kills bacteria (31).

In addition to the platelets themselves, plasma can accelerate the wound healing process as well as provide antimicrobial properties (32). One specific component that likely contributes to its antimicrobial effect is the complement system. The complement system is comprised of unique

plasma proteins that interact to opsonize pathogens while employing inflammatory responses that correspond to invading infections (33) and act as an extracellular and intracellular defense mechanism (34). Certain complement proteins, such as C3 and C5, can initiate cell differentiation, maintain immunological tolerance, and activate cell mediate responses (34). The literature demonstrates that specific plasma proteins, specifically C3a, contribute to the direct killing of *Escherichia coli, Pseudomonas aeruginosa, and Enterococcus faecalis* by binding and inducing breaks in bacterial membranes (35). Finally, Burnouf et al. found that complement activation pathways are essential for the antimicrobial function of human plasma and platelet-derived products against certain bacteria such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella pneumoniae* (36).

This study aimed to compare the antimicrobial activity of canine PL generated by different methods, including a leukocyte-reduced and a leukocyte-rich method, against bacteria commonly present in canine skin wounds. Subsequently, we aimed to identify whether the presence of plasma and complement are responsible for the antimicrobial action of PL. We hypothesized that canine PL would display antimicrobial effects against common bacteria found in canine skin wounds compared to standard bacteria growth media and that plasma and heat-sensitive components- namely the complement system- would improve its effectiveness.

Materials and Methods

Canine blood donors

In this study, eight canine blood donors were recruited for whole blood donation. Dogs were purpose-bred beagles, four females and four males, with a mean age of 5.67 +/- 0.5 years and a mean weight of 14.83 +/- 2 kg. All donors were considered healthy based on history, preliminary blood work (complete blood cell count and chemistry panel), and physical examination. The study's protocol (2022-5085) was approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

Generation of platelet concentrates

Approximately 100 mL of blood was collected in ACD-A tubes from each canine donor. A portion of the blood was also collected into an EDTA tube for a complete blood count (CBC) using the Heska hematology analyzer (Heska Element HT5, Heska, Colorado, USA). Platelet rich plasma (PRP), leukocyte-rich platelet rich plasma (L-PRP), and platelet-poor plasma (PPP) were manufactured under a biosafety laminar flow cabinet (Thermo Scientific Biological Safety Cabinets) (37). Briefly, two different manual centrifugation methods were used to isolate platelets from whole blood. For PRP, whole blood was centrifuged at 1000 g for five minutes. The plasma layer was then collected and centrifuged at 1500 g for 15 minutes to pellet the platelets. For the L-PRP, whole blood was centrifuged at 180 g for 20 minutes. The plasma layer and buffy coat were then collected and centrifuged at 650 g for 15 minutes to pellet the platelets. For each method, the PPP was removed without disturbing the platelet pellet, leaving two milliliters remaining for pellet

resuspension. The platelet pellet was resuspended, and a platelet count was performed. All platelet concentrations were adjusted to 0.8 – 1 x 106 platelets/µl using PPP. Samples were stored at -80˚C.

Plasma depletion

Before storage at -80˚C, a portion of the PRP, L-PRP, and PPP samples were plasma depleted by centrifugation (Sorvall X Pro Series Centrifuge, ThermoFisher Scientific, Osterode am Harz, Germany) at 3000 g for 30 minutes. Plasma was removed without disturbing the pellet and the pellet was resuspended with an equal volume of phosphate-buffered saline without calcium and magnesium (PBS -/-, VWR, USA) to produce leukocyte-reduced platelet pellet concentrate (PPC) and leukocyte-rich platelet pellet concentrate (LPPC). Samples were then stored at -80˚C.

Generation of platelet lysates

PPP, PRP, LPRP, PPC, and LPPC formulations from eight dogs were thawed and pooled to generate three lots of each formulation, each containing lysate from three donors. All samples then underwent four additional freeze-thaw cycles using liquid nitrogen and a dry bath at 37˚C (Precision GP 20, ThermoFisher Scientific, Newington, NH). Samples were placed in a microcentrifuge (Fresco 21 Centrifuge, ThermoFisher Scientific, Osterode am Harz, Germany) at 20,000 g for 20 minutes. The supernatant was collected as the final products: processed PPP (PPP), leukocyte-reduced platelet lysate (PL), leukocyte-rich platelet lysate (LPL), leukocyte-reduced platelet pellet lysate (PPL), and leukocyte-rich platelet pellet lysate (LPPL). Samples were stored at -80˚C.

Heat treatment (complement inactivation)

Heat treatment was performed on a portion of the PL, LPL, PPL, and LPPL formulations via a high-heat dry bath at 56˚C for 30 minutes, followed by an immediate ice bath for 5 minutes (37). Samples were placed in a microcentrifuge at 10,000 g for 15 minutes at 4˚C. The supernatant

was collected to provide the final products: heat-treated leukocyte-reduced platelet lysate (hPL), heat-treated leukocyte-rich platelet lysate (hLPL), heat-treated leukocyte-reduced platelet pellet lysate (hPPL), and heat-treated leukocyte-rich platelet pellet lysate (hLPPL). Samples were stored at -80˚C.

Spiking Assay (time-killing assay)

The spiking assay was performed with four clinically isolated bacteria obtained from ATCC: *Escherichia coli* (*E. coli*; ATCC #25922), *Enterococcus faecalis* (*E. faecalis*; ATCC #19433), *Staphylococcus pseudintermedius* (*S. pseudintermedius*; ATCC #49051), and *Staphylococcus aureus* (*S. aureus*; ATCC #12600). Experiments were performed under a biosafety hood using an aseptic technique. First, one milliliter of brain heart infusion broth (BHI; Becton Dickinson, Sparks MD) was used to rehydrate the freeze-dried bacteria. Resuspended bacteria were incubated at 37˚C overnight with shaking at 200 rpm in a culture tube with a total of four milliliters of BHI broth. Following overnight incubation, the optical density (OD) was measured for each culture tube using duplicate aliquots in 96-well plates via Biotek Gen5 (Synergy HI Microplate Reader, Agilent Technologies Inc. Winooski, VT) at OD600. BHI broth was used as a positive control. Bacterial suspensions were adjusted to 0.3 OD, which corresponds to 108 CFU/ml (38) prior to the spiking assay.

Each treatment group (PL, LPL, PPL, LPPL, hPL, hLPL, hPPL, hLPPL) at 90% v/v was added to a sterile tube containing the appropriate volume (10%) of adjusted bacterial stock suspension (108 CFU/ml). BHI, PPP, and PBS were tested as positive and negative controls, accordingly. Treatments were incubated at 37˚C under constant rotation (200 rpm) for 3 and 24 hours. At each time point, cultures were serially diluted in PBS (-/-) (six 10-fold dilutions).

Nutrient agar plates were inoculated with 200 μL aliquots from each dilution and incubated overnight at 37˚C. Bacterial colonies were manually counted using a colony counter (Reichert Quebec Darkfield). Plates containing approximately 30-300 CFUs were selected for analysis from each experimental cohort.

Statistical analysis

To reduce individual variability, platelet lysates from three dogs were pooled per lot and a total of three lots were generated (11). A power analysis showed that a sample size of 3 would be sufficient to test the antimicrobial properties of lysates to achieve a statistical significance of $p<0.05$ and power=90%. Assessments between spiking assay results were based on the log reductions compared to bacteria cultures in BHI [Log reduction = Log (bacterial count of positive control/bacterial count of treatment)]. All data were imported into a statistical analysis program (Graphpad Prism; Graphpad Software Inc. San Diego, CA, USA). Normality was assessed by the visual examination of histograms of the residual, normal plots of residuals, and via utilizing the Shapiro-Wilks test. The equality of variances was calculated utilizing Levene's test and plotting residuals against the fitted value. Hypotheses were tested by repeated measures or 2-way analysis of variance (ANOVA). Tukey's test was used to adjust for multiple paired comparisons. All statistical analysis was done at $p<0.05$ level of significance. Continuous data were summarized and reported as mean ± standard deviation. Samples were analyzed in duplicates.

Results

Hematologic values

The mean concentration of platelets, white blood cells (WBC), and hematocrit (HCT), from the whole blood and platelet products are shown in Table 1. The mean (± SD) platelet concentration for whole blood was 239.375 ± 80.627 x 103/μL, the WBC concentration was 7.89 ± 1.64 x 103/μL, and the HCT was 53.32% ± 6.79. Compared to whole blood, there was an 11.8-fold increase in platelet concentration for PRP (2,837 ± 1335.605 x 103/μL) and a 9.46-fold increase for L-PRP (2,266.6 ± 1494.311 x 103/μL). The WBC concentration of PRP decreased 4.55-fold compared to whole blood (1.728 ± 6.69 x 103/μL) while L-PRP had a 2-fold decrease in WBC concentration compared to whole blood (3.926 ± 2.842 x 103/μL). There was minimal blood hemodilution for both PRP and L-PRP. The concentration of platelets, WBC, and HCT were negligible in all lysate formulation groups (PL, LPL, PPL, and LPPL; data not shown).

Spiking Assay

The effect of platelet concentration

The log reduction in growth of the *E. coli, E. faecalis, S. pseudintermedius,* and *S. aureus* after 3 and 24 hours of PL or PPP treatment is demonstrated in Figure 1. PL caused a significant log reduction of *S. aureus* (1.724 ± 0.7120, p = 0027) and *E. coli* (4.238 ± 0.8227, p = 0.0486) after 3 hours. However, this effect was not significant at 24 hours. PPP resulted in a significant log reduction of *E. faecalis* after 3 (0.7937 ± 0.1841, p = 0.0220) and 24 hours (1.215 ± 0.9646, p = 0.0149). A nonsignificant trend was noted for PL to achieve a greater log reduction than PPP for

E. coli, S. pseudintermedius, and *S. aureus* after both 3 and 24 hours. These findings suggest that platelets are essential for the antimicrobial action of PL against all bacteria except *E. faecalis*.

The effect of leukocyte concentration

The log reduction of bacteria treated with PL and LPL is shown in Figures 2. LPL caused a significant log reduction of *S. aureus* at 3 (1.576 ± 0.1900, p= 0.0237) and 24 hours (1.932 ± 0.2428, p = 0.0258). At 3 hours, LPL had a significantly greater log reduction of *S. aureus* than PBS (p = 0.0120). LPL (0.3728 ± 0.3514) caused a significant, weaker, log reduction of *E. faecalis* compared to PL (0.9720 ± 0.5482, p= 0.0045) and PBS (1.561 ± 0.2435, p= 0.0174) after 24 hours. LPPP (0.8369 ± 0.3485, 1.171 ± 1.058) and PPP (0.7937 ± 0.1841, 1.215 ± 0.9646) had a significant log reduction of *E. faecalis* at 3 (p= 0.0140, p= 0.0220) and 24 (p= 0.0192, p= 0.0149) hours. PL treatment exhibited a nonsignificant trend of a greater log reduction of *S. aureus* and *S. pseudintermedius* than LPL after 3 hours, and for *S. pseudintermedius* after 24 hours. The opposite trend, with LPL achieving a greater log bacteria reduction than PL, was noted for *E. coli* after 3 hours. Therefore, leukocytes do not appear to affect the ability of platelets to inhibit bacterial growth.

The effect of plasma content

The log reductions of bacteria in the presence of PL and PPL are illustrated in Figure 3. PPL caused a significant log reduction of *E. faecalis* at 3 (0.9623 ± 0.1493, p = 0.0396) and 24 hours (1.057 ± 0.2413, p = 0.0372). At 3 hours, the log reduction of *E. faecalis* was significantly greater with PBS than with PL (0.5296 ± 0.1331, p= 0.0260). In contrast, PL caused a greater log reduction of *S. pseudintermedius* at 3 (2.786 ± 1.377) and 24 hours (2.538 ± 1,275) compared to PPL (1.166 ± 0.4440, p = 0.0478 and 0.8274 ± 0.4030, p = 0.0325). A nonsignificant trend toward greater log reduction with PL compared to PPL was noted with *S. aureus* and *E. coli* at 3 hours

and *E. coli* at 24 hours (Figure 3A, B). These data demonstrate that plasma contributes to the antimicrobial properties of platelets.

The effect of complement inactivation

The log reduction of bacteria with PL and hPL to compare the effects of heat treatment is depicted in Figure 4. At 3 hours, hPL had a significant log reduction of *S. aureus* (2.049 ± 0.2612, p = 0.0267), which was significantly greater than the effect of PBS (0.7370 ± 0.1307, p = 0.0320). PL led to a greater log reduction of *E. faecalis* (0.9720 ± 0.5482) compared to hPL (0.3381 ± 0.3916, p = 0.0026) after 24 hours. No significant differences were noted between PL and hPL at either time point for *E. coli* or *S. pseudintermedius* (Figure 3A, B). This data suggests that heat-sensitive plasma proteins are necessary for the antimicrobial features of platelets.

The effect of complement inactivation combined with plasma depletion

The log reduction of bacteria with PL and hPPL to compare the effects of plasma depletion combined with heat treatment is shown in Figure 5. Treatment with hPPL led to a significant log reduction of *S. aureus* at 3 hours (1.819 ± 0.2719, p = 0.0368), which was also significantly greater reduction than PBS (0.7370 ± 0.1307, p = 0.0291), and at 24 hours (; 1.001 ± 0.09561, p = 0.0049). Treatment with hPPL also led to a significant log reduction *E. faecalis* after 3 (0.9620 ± 0.04092, p = 0.0012) and 24 hours (1.053 ± 0.3638, p = 0.0039). Conversely, the log reduction of *E. coli* after treatment with hPPL (1.645 ± 1.327) was significantly weaker compared to PPP (3.579 ± 1.566, p = 0.0282) after 3 hours. PL was not significantly different from hPPL at 3 and 24 hours, though trends were noted for PL to provide a greater log reduction of *E. coli* and *S. pseudintermedius*. Thus, the presence of plasma and its complement proteins is essential for maintaining the antimicrobial properties of platelets.

The effect of nutrient depletion

To evaluate whether the inhibition of the bacteria growth following the addition of platelet derived products could be partially due to nutrient deprivation we set up a serial of experiments evaluating bacteria growth in the presence of PL, PPP and PBS. Specifically, Bacterial colony counts for *S. aureus, S. pseudintermedius, E. coli* and *E. faecalis* after 3 and 24 hours of PL, PPP, and PBS treatment are demonstrated in Supplementary Figure 1. We found that, PL led to a nonsignificant inhibition of both *S. aureus* (Supplementary Figure 1A) and *S. pseudintermedius* (Supplementary Figure 1B) compared to baseline. However, PPP inhibited growth of S. *pseudintermedius* but supported the growth of *S. aureus* compared to baseline after 3 and 24 hours. Moreover, we found that the addition of PL, PPP, and PBS significantly inhibited the growth of *E. coli* after 3 hours compared to the baseline. Similarly, after 24 hours, PL, PPP, and PBS significantly decreased *E. coli* growth. PBS significantly supported the growth of *S. pseudintermedius* at 24 hours. The same nonsignificant trend was noted for *S. aureus* after the addition of PBS for two time points. Finally, PL significantly and PPP not significantly increased the growth of *E. faecalis* compared to baseline after 3 hours. The same trends were observed for PL and PPP after 24 hours. Even though PBS decreased its growth compered to baseline after 24 hours. Given that bacteria growth was encountered following the addition of PBS, it is not reasonable to believe that inhibition of bacteria growth is due to the lack of nutrients that are essential for bacteria growth.

Discussion

In this study, highly concentrated PRP was achieved in both the leukocyte-reduced and leukocyte-rich formulations. The recommended platelet concentration for platelet concentrates is at least 300 x 103 platelets/ μL for a therapeutic effect to occur based on the recommendation by the American Red Cross (22, 39). In this study, the number of concentrated platelets was consistent and exceeded the guidelines of the American Red Cross and previous studies (39). Due to the high concentration of platelets and standardization, the final concentration of all platelet products was standardized to 800 - 1000 X 103/μL prior to further processing. Additionally, leukocyte reduction in the leukocyte-reduced PRP was more successful compared to our previous work (37), which is in line with previous work by Shin et al. (22). However, leukocyte concentration was less successful in this study compared to previous studies using the leukocyte-rich method (17, 37). These variations are likely due to collection variations, donor variability, and processing time, particularly following the first centrifugation cycle.

When comparing PL and PPP, PL significantly reduced the growth of *S. aureus and E. coli*, and a trend was noted for PL to achieve a greater log reduction than PPP for *E. coli, S. pseudintermedius, and S. aureus*. These findings suggest that platelets are essential for the antimicrobial action of PL against *E. coli, S. pseudintermedius, and S. aureus*. Similar to PL, LPL demonstrated antimicrobial activity for *S. aureus, E. coli, and S. pseudintermedius* with a greater significance noted against *S. aureus* and *E. coli* at the 3-hour timepoint. Significant antimicrobial activity against *E. faecalis* was not identified with treatment of PL or LPL. Our findings are consistent with the previous findings reported in the literature, noting platelet preparations

inhibited *S. aureus* and *E. coli* (36), but not *E. faecalis* (40, 41). These results are also consistent with a previous study which proved leukocyte-reduced and leukocyte-rich platelet pellet concentrate have similar antimicrobial properties against five strains of bacteria (26). However, not all studies agree with this finding. Another study found the addition of neutrophils enhanced the antibacterial properties of platelet-rich plasma (30). Giusti et al. noticed that different leukocyte concentrations might have beneficial or detrimental impacts on the healing process, depending on the specific clinical circumstance (42). It is also worth noting that our results may be affected by the relatively low differential in leukocyte count between our formulations. Future studies should optimize the methodology used for leukocyte depletion during the manufacturing of platelet concentrates and guidelines may be needed for leukocyte concentrations depending on desired clinical applications.

Plasma has previously been noted to play a major role in the antibacterial properties of various orthobiologics (43). According to previous studies, the bactericidal properties of platelet products are mostly due to plasma factors (36). Other studies have also proved that the antimicrobial action of platelet products is related to the presence of plasma rather than platelets themselves (17, 36). Our results displayed that the depletion of plasma from PL (PPL) led to significantly less bacteria growth inhibition than PL after 3 and 24 hours for *S. pseudintermedius* and *E. coli*. In contrast, PPL was more effective than PL against *E. faecalis* after 3 and 24 hours and against *S. aureus* after 24 hours. This latter finding is not in accordance with the previous literature, which concluded the antimicrobial properties of PRP against *E. faecalis* are due to the synergistic effects of plasma and platelet-derived factors (44). Additionally, in this study, plasma depletion inhibited the growth of *S. aureus* after 24 hours. This might be due to the fact that *S. aureus* has a coagulase enzyme that converts fibrinogen to fibrin, which is used as an extracellular

matrix to protect itself (45). By removing plasma, the above protection mechanisms are not available, which makes *S. aureus* more susceptible to therapeutic invasions. Thus, we believe that plasma is necessary for the antimicrobial properties of PL. Plasma contains protease, which seems critical for the cleavage of proteins in platelets. It is worth mentioning that the amount of plasma is a factor that needs to be considered carefully. In our study, platelet concentrates were resuspended in 100% plasma. However other studies found that variable plasma concentrations can affect the antimicrobial properties of the product (15, 46). Specifically, one study found that 10% of plasma is the most efficient concentration for maintaining an adequate amount of protease (15). Furthermore, another study demonstrated that 10% plasma is efficient and higher than 50% plasma is detrimental to the antimicrobial properties of platelets (46). Future studies should evaluate meticulously how various plasma concentrations can affect the antimicrobial features of platelet-derived products.

Based on previous studies, the heat-sensitive complement proteins within plasma are critical in inhibiting bacterial growth (27, 36). Our results further support this finding as heat-treated PL (hPL) did not provide the same bacterial growth reduction in any bacteria tested compared to PL. Accordingly, the presence of the protein complements seems crucial for maintaining the antimicrobial activity of PL (47). Specifically, complement proteins use opsonin as labels on the bacteria to facilitate phagocytosis; in addition, they increase bacterial opsonization by antibodies since the antibodies kill the bacteria (33). Thus, our findings confirm the necessity of heat-sensitive complement proteins for maintaining the antimicrobial features of PL.

Combining plasma depletion and heat treatment was also evaluated. When plasma was depleted from PL, we found that plasma-depleted PL (PPL) was not efficient in inhibiting bacterial growth except for *E. faecalis*. Similarly, inactivating complements by utilizing heat had the same

effect on PL (hPL). When we performed plasma depletion and heat treatment simultaneously, we confirmed a decreased potential of that formulation in inhibiting bacterial growth. Future studies on platelet products should consider the effects of plasma proteins and processing, including heat exposure, as these may change the product's therapeutic potential.

To evaluate whether nutritional deprivation is responsible for the increased bacteria reduction in the presence of platelet-derived products, we evaluated the growth of a specific concentration of bacteria in the presence of PL, PPP, and PBS. We assumed that PBS would provide minimal notional support. According to our results, we confirmed that PL has a bactericidal effect against *E. coli* after both 3 and 24 hours since it decreased the colony-forming units of a certain bacteria concentration more than 3 log 10 CFU/ml (28). Our findings are aligned with Burnouf et al. since a similar bactericidal activity of platelet preparations against *E. coli* was observed (36). In addition, both PL and LPL showed bacteriostatic activity against *S. aureus*, and *S. pseud* which is also consistent with previous studies (28). Thus, the antimicrobial effect of PL cannot be contributed to a nutrient deprivation effect.

Limitations of this study include a low differential between leukocyte concentrations in our "leukocyte-reduced" and "leukocyte-rich" preparation. This was likely affected by our inability to solely collect plasma from the top layer of the buffy coats, as contamination of leukocytes from accidentally aspirating the buffy coat is suspected. Protocol adjustments or use of different techniques or equipment may aid in obtaining the desired leukocyte concentration. Additional limitations include a small sample size, limited time points, and using commercially available bacterial strains collected from a human healthcare setting. In this study we decided to pool lysates generated from eight donors to decrease individual variability as has been previously reported (11).

The preparation process of platelet lysates included four freeze-thaw cycles in liquid nitrogen. It is possible that the number of freeze/thaw cycles could affect the release of factors from platelet granules and subsequently their biological activity. However, various studies have demonstrated that performing 3-5 freeze/thaw cycles achieves the optimal concentration of growth factors from platelet granules (48). Regardless, future studies should evaluate the effect of various numbers and conditions of freeze/thaw cycles for the optimal release of growth factors and antimicrobial properties. Finally, the generation process of lysates included the addition of acid-citrate-dextrose solution A (ACD-A) as an anticoagulant. Even though the addition of dextrose could have an impact on the composition of the growth factors, ACD-A is a common anticoagulant used in transfusion medicine for the preparation of platelet-derived biologicals (49, 50). Specifically, a study evaluated the effect of various anticoagulants on the composition of growth factors and found that no differences were detected on PDGF concentration among the groups (49). However, a direct comparison has not been performed between ACD-A and acid-citrate to evaluate the effect of dextrose on factor composition.

Conclusion

Our results demonstrated that PL and LPL have antimicrobial properties independent of leukocyte concentration, specifically against *S. aureus, E. coli,* and *S. pseudintermedius*, but not *E. faecalis*. Plasma proteins and other plasma components seem to be critical for inhibiting the growth of some bacterial strains. In conclusion, platelet derived products have the potential to accelerate the wound healing process via the release of growth factors, anti-inflammatory and antimicrobial molecules. Future studies should investigate the most appropriate concentration of PL for targeted antimicrobial therapy and use in wound healing in dogs.

References

1. Pinto JM, Pizani NS, Kang HC, Silva LA. Application of platelet-rich plasma in the treatment of chronic skin ulcer - case report. An Bras Dermatol. 2014;89(4):638-40.

2. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008;16(5):585-601.

3. Alves R, Grimalt R. A Review of Platelet-Rich Plasma: History, Biology, Mechanism of Action, and Classification. Skin Appendage Disord. 2018;4(1):18-24.

4. Wroblewski AP, Mejia HA, Wright VJ. Application of Platelet-Rich Plasma to Enhance Tissue Repair. Operative Techniques in Orthopaedics. 2010;20(2):98-105.

5. Lynch MD, Bashir S. Applications of platelet-rich plasma in dermatology: A critical appraisal of the literature. J Dermatolog Treat. 2016;27(3):285-9.

6. Smith OJ, Wicaksana A, Davidson D, Spratt D, Mosahebi A. An evaluation of the bacteriostatic effect of platelet-rich plasma. Int Wound J. 2021;18(4):448-56.

7. Kaushik A, Kumaran MS. Platelet-Rich Plasma: The Journey so Far ! Indian Dermatol Online J. 2020;11(5):685-92.

8. Gordon J, Alvarez-Narvaez S, Peroni JF. Antimicrobial Effects of Equine Platelet Lysate. Front Vet Sci. 2021;8:703414.

9. da Fonseca L, Santos GS, Huber SC, Setti TM, Setti T, Lana JF. Human platelet lysate - A potent (and overlooked) orthobiologic. J Clin Orthop Trauma. 2021;21:101534.

10. Hagen A, Holland H, Brandt VP, Doll CU, Haussler TC, Melzer M, et al. Platelet Lysate for Mesenchymal Stromal Cell Culture in the Canine and Equine Species: Analogous but Not the Same. Animals (Basel). 2022;12(2).

11. Naskou MC, Norton NA, Copland IB, Galipeau J, Peroni JF. Innate immune responses of equine monocytes cultured in equine platelet lysate. Vet Immunol Immunopathol. 2018;195:65-71.

12. Avellar HK, Lutter JD, Ganta CK, Beard W, Smith JR, Jonnalagadda N, et al. In vitro antimicrobial activity of equine platelet lysate and mesenchymal stromal cells against common clinical pathogens. Can J Vet Res. 2022;86(1):59-64.

13. Tang YQ, Yeaman MR, Selsted ME. Antimicrobial peptides from human platelets. Infect Immun. 2002;70(12):6524-33.

14. Harman RM, Yang S, He MK, Van de Walle GR. Antimicrobial peptides secreted by equine mesenchymal stromal cells inhibit the growth of bacteria commonly found in skin wounds. Stem Cell Res Ther. 2017;8(1):157.

15. Gilbertie JM, Schaer TP, Schubert AG, Jacob ME, Menegatti S, Ashton Lavoie R, et al. Platelet-rich plasma lysate displays antibiofilm properties and restores antimicrobial activity against synovial fluid biofilms in vitro. J Orthop Res. 2020;38(6):1365-74.

16. Duong L, Gross SP, Siryaporn A. Developing Antimicrobial Synergy With AMPs. Front Med Technol. 2021;3:640981.

17. Attili AR, Iacoucci C, Serri E, Cuteri V, Cantalamessa A, Linardi M, et al. Antibacterial Properties of Canine Platelet-Rich Plasma and Other Non-Transfusional Hemo-Components: An in vitro Study. Front Vet Sci. 2021;8:746809.

18. Farghali HA, AbdElKader NA, AbuBakr HO, Aljuaydi SH, Khattab MS, Elhelw R, et al. Antimicrobial action of autologous platelet-rich plasma on MRSA-infected skin wounds in dogs. Sci Rep. 2019;9(1):12722.

19. Naskou MC, Sumner SM, Chocallo A, Kemelmakher H, Thoresen M, Copland I, et al. Platelet lysate as a novel serum-free media supplement for the culture of equine bone marrow-derived mesenchymal stem cells. Stem Cell Res Ther. 2018;9(1):75.

20. Franklin SP, Birdwhistell KE, Strelchik A, Garner BC, Brainard BM. Influence of Cellular Composition and Exogenous Activation on Growth Factor and Cytokine Concentrations in Canine Platelet-Rich Plasmas. Front Vet Sci. 2017;4:40.

21. Franklin SP, Garner BC, Cook JL. Characteristics of canine platelet-rich plasma prepared with five commercially available systems. Am J Vet Res. 2015;76(9):822-7.

22. Shin HS, Woo HM, Kang BJ. Optimisation of a double-centrifugation method for preparation of canine platelet-rich plasma. BMC Vet Res. 2017;13(1):198.

23. Perazzi A, Busetto R, Martinello T, Drigo M, Pasotto D, Cian F, et al. Description of a double centrifugation tube method for concentrating canine platelets. BMC Vet Res. 2013;9:146.

24. Carr BJ, Canapp SO, Jr., Mason DR, Cox C, Hess T. Canine Platelet-Rich Plasma Systems: A Prospective Analysis. Front Vet Sci. 2015;2:73.

25. Qian Y, Han Q, Chen W, Song J, Zhao X, Ouyang Y, et al. Platelet-Rich Plasma Derived Growth Factors Contribute to Stem Cell Differentiation in Musculoskeletal Regeneration. Front Chem. 2017;5:89.

26. Mariani E, Canella V, Berlingeri A, Bielli A, Cattini L, Landini MP, et al. Leukocyte presence does not increase microbicidal activity of Platelet-rich Plasma in vitro. BMC Microbiol. 2015;15:149.

27. Lopez C, Carmona JU, Giraldo CE, Alvarez ME. Bacteriostatic effect of equine pure platelet-rich plasma and other blood products against methicillin-sensitive Staphylococcus aureus. An in vitro study. Vet Comp Orthop Traumatol. 2014;27(5):372-8.

28. Anitua E, Alonso R, Girbau C, Aguirre JJ, Muruzabal F, Orive G. Antibacterial effect of plasma rich in growth factors (PRGF(R)-Endoret(R)) against Staphylococcus aureus and Staphylococcus epidermidis strains. Clin Exp Dermatol. 2012;37(6):652-7.

29. Bielecki T, Dohan Ehrenfest DM, Everts PA, Wiczkowski A. The role of leukocytes from L-PRP/L-PRF in wound healing and immune defense: new perspectives. Curr Pharm Biotechnol. 2012;13(7):1153-62.

30. Prysak MH, Lutz CG, Zukofsky TA, Katz JM, Everts PA, Lutz GE. Optimizing the safety of intradiscal platelet-rich plasma: an in vitro study with Cutibacterium acnes. Regen Med. 2019;14(10):955-67.

31. Lincoln JA, Lefkowitz DL, Cain T, Castro A, Mills KC, Lefkowitz SS, et al. Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages. Infect Immun. 1995;63(8):3042-7.

32. Powanda MC, Moyer ED. Plasma proteins and wound healing. Surg Gynecol Obstet. 1981;153(5):749-55.

33. Janeway CA Jr TP, Walport M, et al. Immunobiology: The Immune System in Health and Disease. edition t, editor2001.

34. Reichhardt MP, Meri S. Intracellular complement activation-An alarm raising mechanism? Semin Immunol. 2018;38:54-62.

35. Nordahl EA, Rydengard V, Nyberg P, Nitsche DP, Morgelin M, Malmsten M, et al. Activation of the complement system generates antibacterial peptides. Proc Natl Acad Sci U S A. 2004;101(48):16879-84.

36. Burnouf T, Chou ML, Wu YW, Su CY, Lee LW. Antimicrobial activity of platelet (PLT)-poor plasma, PLT-rich plasma, PLT gel, and solvent/detergent-treated PLT lysate biomaterials against wound bacteria. Transfusion. 2013;53(1):138-46.

37. Lunardon T, Sumner SM, Mollabashi M, Darzenta N, Davis E, Naskou MC. Growth factor and cytokine characterization of canine platelet lysate with variable leukocyte concentration, plasma content, and heat-sensitive proteins. Frontiers in Veterinary Science. 2024;11.

38. Shariati A, Moradabadi A, Azimi T, Ghaznavi-Rad E. Wound healing properties and antimicrobial activity of platelet-derived biomaterials. Sci Rep. 2020;10(1):1032.

39. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). Trends Biotechnol. 2009;27(3):158-67.

40. Bielecki TM, Gazdzik TS, Arendt J, Szczepanski T, Krol W, Wielkoszynski T. Antibacterial effect of autologous platelet gel enriched with growth factors and other active substances: an in vitro study. J Bone Joint Surg Br. 2007;89(3):417-20.

41. Mariani E, Filardo G, Canella V, Berlingeri A, Bielli A, Cattini L, et al. Platelet-rich plasma affects bacterial growth in vitro. Cytotherapy. 2014;16(9):1294-304.

42. Giusti I, Di Francesco M, D'Ascenzo S, Palumbo P, Rughetti A, Dell'Orso L, et al. Leukocyte depletion does not affect the in vitro healing ability of platelet rich plasma. Exp Ther Med. 2018;15(4):4029-38.

43. Nicol MJ, Brubaker TR, Honish BJ, 2nd, Simmons AN, Kazemi A, Geissel MA, et al. Antibacterial effects of low-temperature plasma generated by atmospheric-pressure plasma jet are mediated by reactive oxygen species. Sci Rep. 2020;10(1):3066.

44. Drago L, Bortolin M, Vassena C, Romano CL, Taschieri S, Del Fabbro M. Plasma components and platelet activation are essential for the antimicrobial properties of autologous platelet-rich plasma: an in vitro study. PLoS One. 2014;9(9):e107813.

45. Zapotoczna M, O'Neill E, O'Gara JP. Untangling the Diverse and Redundant Mechanisms of Staphylococcus aureus Biofilm Formation. PLoS Pathog. 2016;12(7):e1005671.

46. Trier DA, Gank KD, Kupferwasser D, Yount NY, French WJ, Michelson AD, et al. Platelet antistaphylococcal responses occur through P2X1 and P2Y12 receptor-induced activation and kinocidin release. Infect Immun. 2008;76(12):5706-13.

47. Zimmer J, Hobkirk J, Mohamed F, Browning MJ, Stover CM. On the Functional Overlap between Complement and Anti-Microbial Peptides. Front Immunol. 2014;5:689.

48. Strandberg G, Sellberg F, Sommar P, Ronaghi M, Lubenow N, Knutson F, et al. Standardizing the freeze-thaw preparation of growth factors from platelet lysate. Transfusion. 2017;57(4):1058-65.

49. Aizawa H, Kawabata H, Sato A, Masuki H, Watanabe T, Tsujino T, et al. A Comparative Study of The Effects of Anticoagulants on Pure Platelet-Rich Plasma Quality and Potency. Biomedicines. 2020;8(3).

50. Lei H, Xiao R, Tang XJ, Gui L. Evaluation of the efficacy of platelet-rich plasma in delivering BMSCs into 3D porous scaffolds. J Biomed Mater Res B Appl Biomater. 2009;91(2):679-91.

Table 1: Descriptive results of hematological data presented as mean ± standard deviation from whole blood, and platelet-derived concentrates. PLT, platelet concentration; WBC, white blood cell concentration; HTC, hematocrit; PRP, leukocyte-reduced platelet-rich plasma; L-PRP, leukocyte-rich platelet-rich plasma.

Hematology analysis			
Preparation	**PLT ($x10^3/\mu L$)**	**WBC ($x10^3/\mu L$)**	**HTC (%)**
Whole blood	239.375 ± 80.627	7.871 ± 1.644	53.312 ± 6.79
Pure platelet concentrates			
PC	2,837 ± 1335.605	1.728 ± 6.69	0
Leukocyte-rich platelet concentrates			
LPC	2,266.6 ± 1494.311	3.926 ± 2.842	0

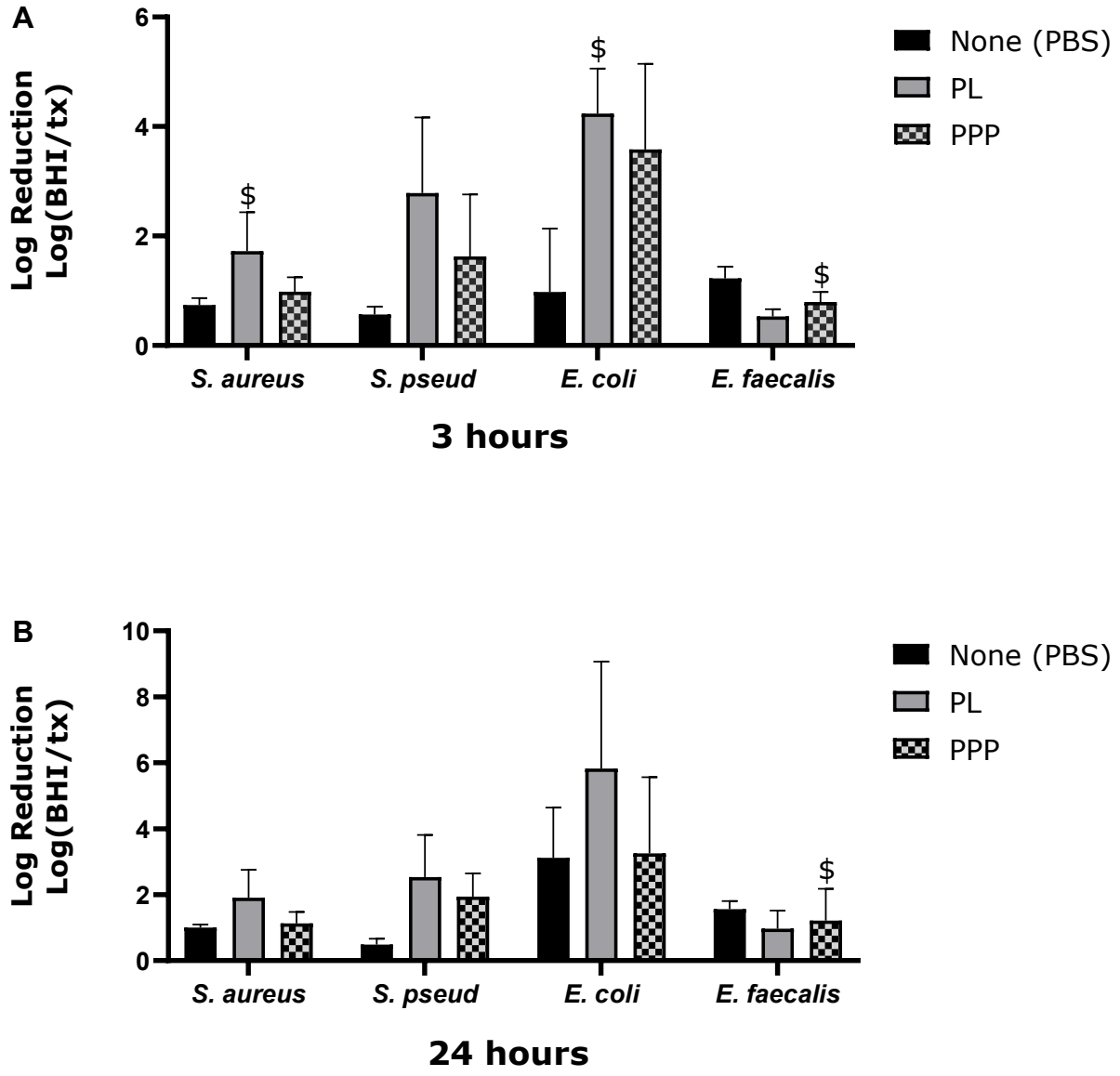


Figure 1: Log reduction of *S. aureus*, *S. pseudintermedius*, *E. coli*, and *E. faecalis* following the addition of PBS, PL, and PPP after 3 hours (A) and 24 hours (B). Data are presented as mean bacteria log reduction ± S.D normalized to bacteria grown in BHI according to the following formula: Log (BHI/treatment). $^{\$}$ $p < 0.05$ compared to BHI; n=3 lots of pooled platelet lysate. PBS, Phosphate Buffered Saline; PL, Platelet Lysate; PPP, Platelet-Poor Plasma; PPL, leukocyte-

reduced platelet pellet lysate; hPL, Heat-treated Platelet Lysate; BHI, brain heart infusion broth; LPL, Leukocyte-Rich Platelet Lysate; hPPL, Heat-treated leukocyte-reduced platelet pellet lysate.

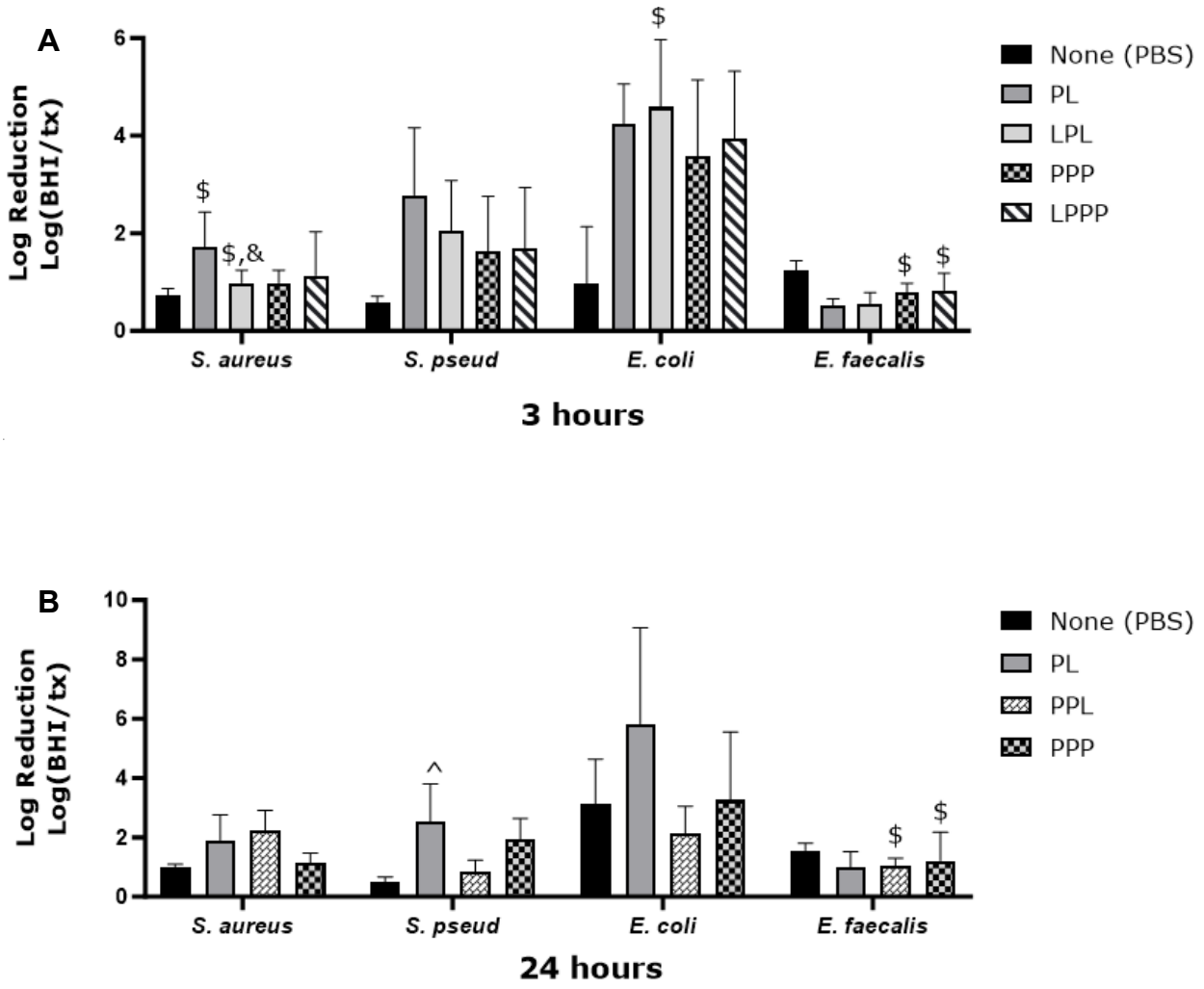


Figure 2: Log reduction of *S. aureus*, *S. pseudintermedius*, *E. coli*, and *E. faecalis* following the addition of PBS, PL, LPL, PPP, and LPPP after 3 hours (A) and 24 hours (B). Data are presented as mean bacteria log reduction ± S.D normalized to bacteria grown in BHI according to the following formula: Log (BHI/treatment). $^{\$}$ $p < 0.05$ compared to BHI; $^{\&}$ $p < 0.05$ compared to PBS; $^{\#}$ $p < 0.05$ compared to LPL; n=3 lots of pooled platelet lysate generated from 8 donors. PBS, Phosphate Buffered Saline; PL, Platelet Lysate; LPL, Leukocyte-Rich Platelet Lysate; PPP, Platelet-Poor Plasma; LPPP, Leukocyte-Rich Platelet-Poor Plasma; PPL, leukocyte-reduced platelet pellet lysate; hPL, Heat-treated Platelet Lysate; BHI, brain heart infusion broth; hPPL, Heat-treated leukocyte-reduced platelet pellet lysate.

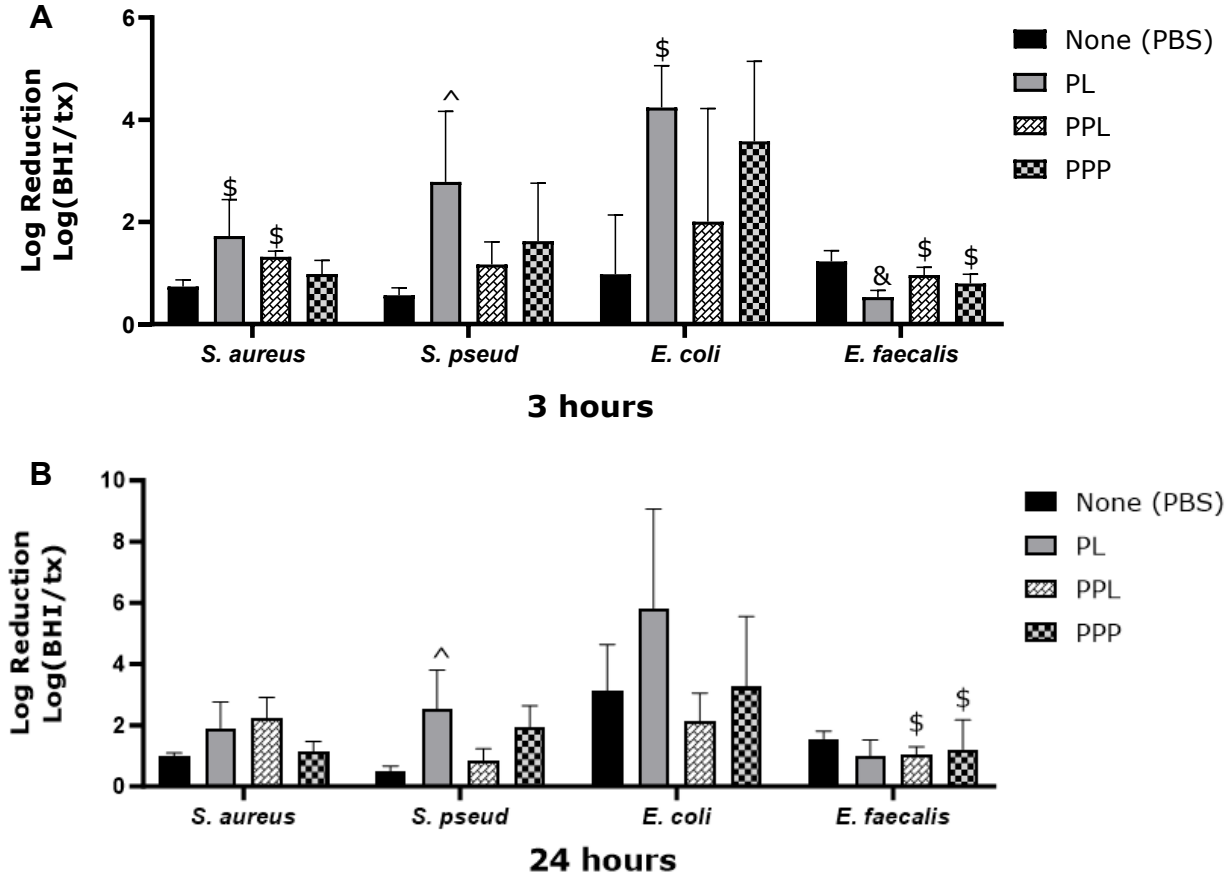


Figure 3: Log reduction of *S. aureus*, *S. pseudintermedius*, *E. coli*, and *E. faecalis* following the addition of PBS, PL, PPL, and PPP after 3 hours (A) and 24 hours (B). Data is presented as mean bacteria log reduction ± S.D normalized to bacteria grown in BHI according to the following formula: Log (BHI/treatment). $^{\$}$ $p < 0.05$ compared to BHI; ^ $p < 0.05$ compared to PPL; n=3 lots of pooled platelet lysate generated from 8 donors. PBS, Phosphate Buffered Saline; PL, Platelet Lysate; PPL, leukocyte-reduced platelet pellet lysate; PPP, Platelet-Poor Plasma; hPL, Heat-treated Platelet Lysate; BHI, brain heart infusion broth; LPL, Leukocyte-Rich Platelet Lysate; hPPL, Heat-treated leukocyte-reduced platelet pellet lysate.

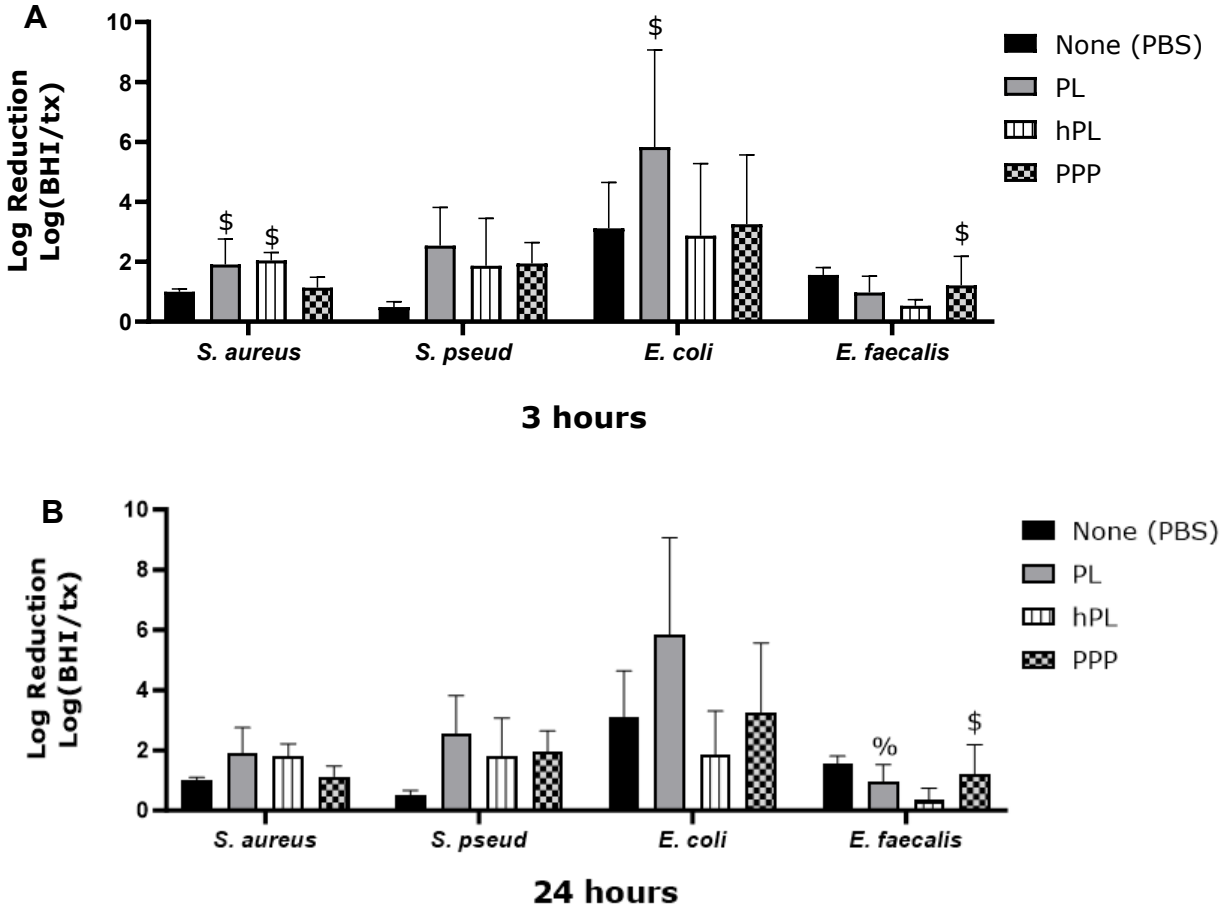


Figure 4: Log reduction of *S. aureus*, *S. pseudintermedius*, *E. coli*, and *E. faecalis* following the addition of PBS, PL, hPL, and PPP after 3 hours (A) and 24 hours (B). Data are presented as mean bacteria log reduction ± S.D normalized to bacteria grown in BHI according to the following formula: Log (BHI/treatment). $^{\$}$ $p < 0.05$ compared to BHI; $^{\%}$ $p < 0.05$ compared to hPL; n=3 lots of pooled platelet lysate generated from 8 donors. PBS, Phosphate Buffered Saline; PL, Platelet Lysate; hPL, Heat-treated Platelet Lysate; PPP, Platelet-Poor Plasma; PPL, leukocyte-reduced platelet pellet lysate; BHI, brain heart infusion broth; LPL, Leukocyte-Rich Platelet Lysate; hPPL, Heat-treated leukocyte-reduced platelet pellet lysate.

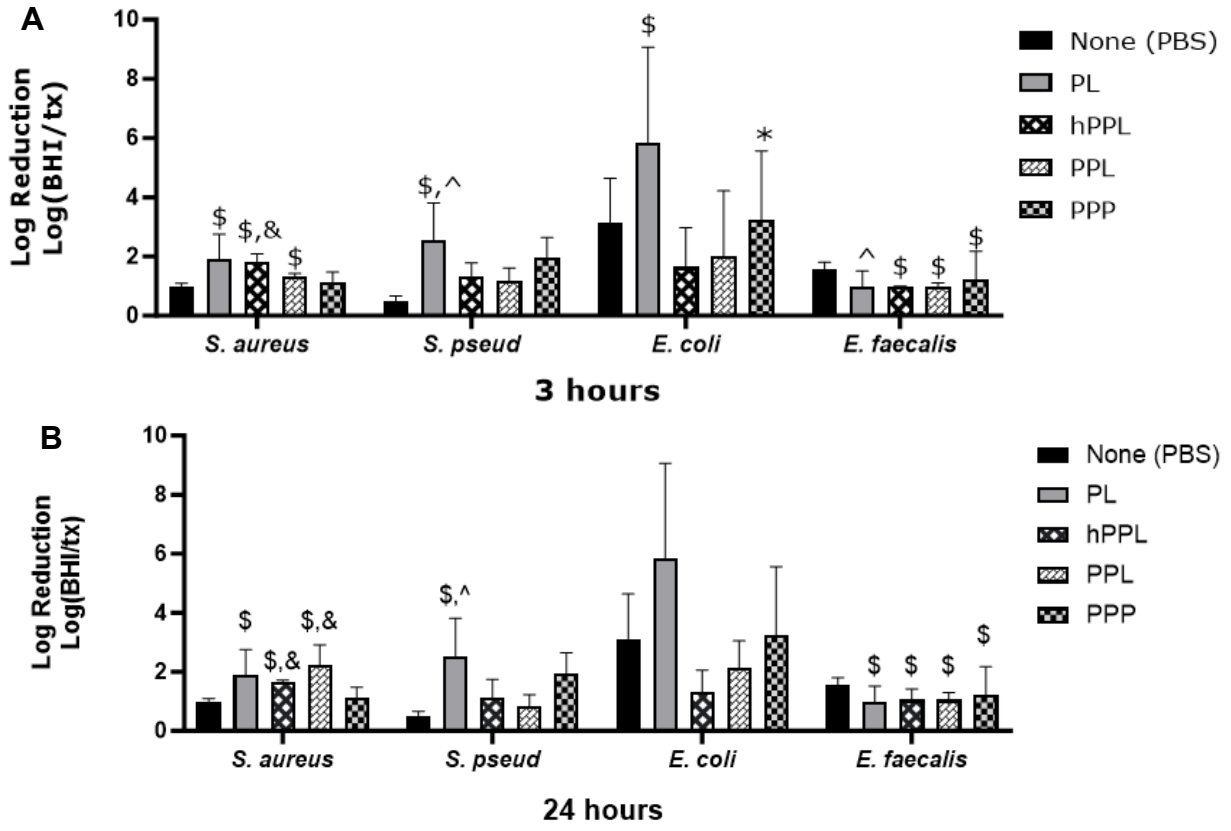
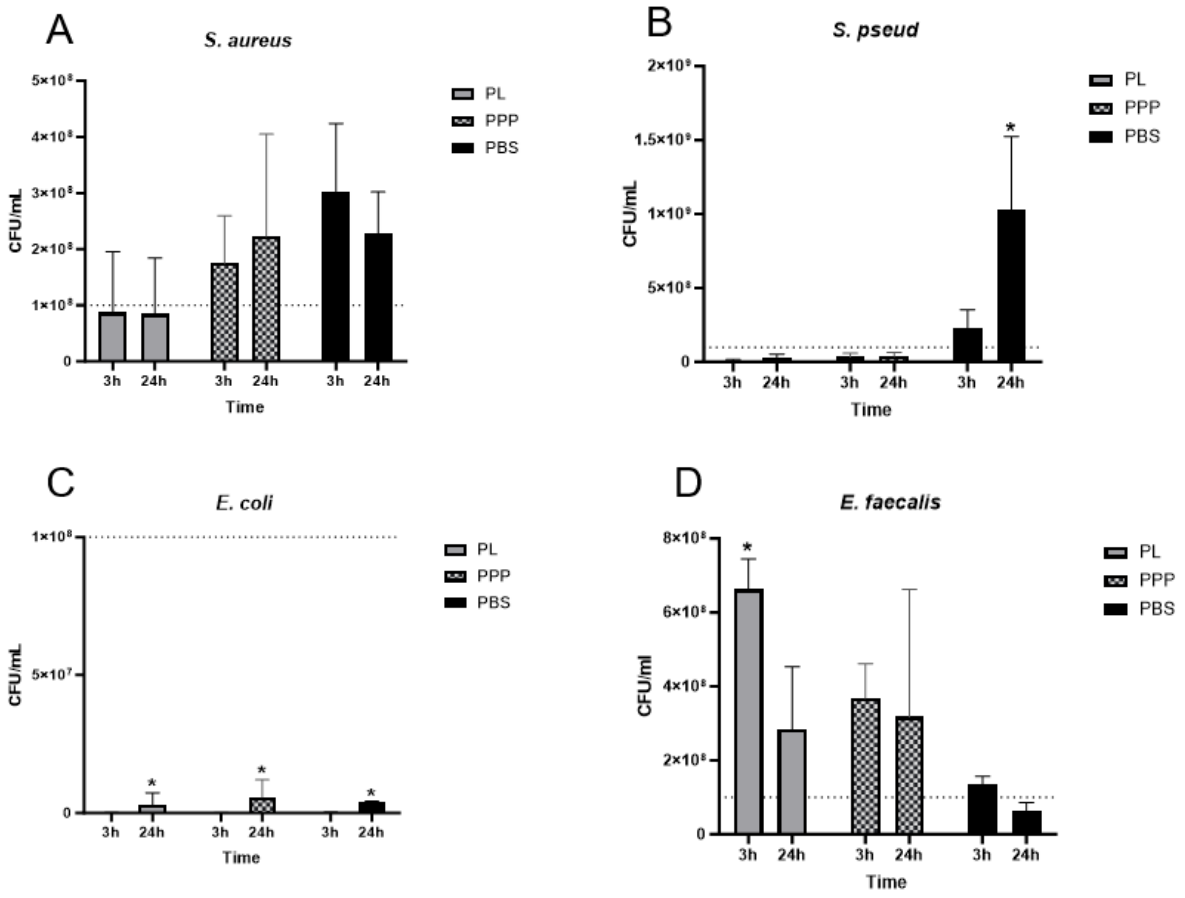


Figure 5: Log reduction of *S. aureus*, *S. pseudintermedius*, *E. coli*, and *E. faecalis* following the addition of PBS, PL, hPPL, PPL, and PPP after 3 hours (A) and 24 hours (B).Data are presented as mean bacteria log reduction ± S.D normalized to bacteria grown in BHI according to the following formula: Log (BHI/treatment). $^{\$}$ $p < 0.05$ compared to BHI; $^{\&}$ $p < 0.05$ compared to PBS; ^ $p < 0.05$ compared to PPL; * $p < 0.05$ compared to hPPL; n=3 lots of pooled platelet lysate generated from 8 donors. PBS, Phosphate Buffered Saline; PL, Platelet Lysate; hPPL, Heat-treated leukocyte-reduced platelet pellet lysate; PPL, leukocyte-reduced platelet pellet lysate; PPP, Platelet-Poor Plasma; hPL, Heat-treated Platelet Lysate; BHI, brain heart infusion broth; LPL, Leukocyte-Rich Platelet Lysate.



Supplementary Figure 1: Mean number of bacterial colonies following treatment with PL, PPP, and PBS after 3 and 24 hours for *S. aureus (A), S. pseudintermedius (B),* E. *coli (C), and E. faecalis (D).* Data are presented as mean Colony Forming Unit per milliliter. The initial inoculum population was 10^8 CFUs/mL, represented by the dotted line (* = $p < 0.05$). n=3 lots of pooled platelet lysate generated from 8 donors. PL, Platelet Lysate; PPP, Platelet-Poor Plasma; PBS, Phosphate Buffered Saline.

CHAPTER 3

ASSESSMENT OF GROWTH KINETICS IN BACTERIA TREATED WITH CANINE PLATELET LYSATE AND ANTIBIOTICS

Abstract

A major problem in veterinary wound care is the declining efficacy of traditional antibiotics due to the rising incidence of biofilm-associated infections and antimicrobial resistance (AMR). Bacterial resistance and persistence frequently result in poor healing outcomes in chronic canine wounds. A promising alternative therapy with both regenerative and antimicrobial potential is platelet lysate (PL), a bioactive substance abundant in growth factors and antimicrobial peptides. PL has been shown in earlier research to be effective against bacteria that are resistant to multiple drugs and to work in concert with antibiotics. This study examined the effects of canine PL on bacterial growth kinetics and its possible synergism with amikacin, with an emphasis on dose dependency. The whole blood was collected from eight healthy purpose-bred Beagle dogs to generate platelet-rich plasma (PRP), platelet-poor plasma (PPP), and platelet lysate (PL). Lysates were prepared via multiple freeze–thaw cycles and pooled into three lots per formulation. The antimicrobial activity of PL was assessed using a growth curve assay against *Staphylococcus pseudintermedius*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA). Bacteria were exposed to PL or PPP at 20% or 80% concentrations, with or without the addition of amikacin. Bacterial growth was monitored over 16 hours, and kinetic parameters were modeled using the Baranyi equation. Canine platelet lysate (PL) altered bacterial growth kinetics primarily by affecting the carrying capacity (K) and lag phase (λ), without significantly influencing the specific growth rate (μ_{max}). For *S. aureus*, platelet lysate (PL) at both 20% and 80% concentrations significantly reduced bacterial yield compared to untreated controls. In *S. pseudintermedius* and *MRSA*, only the 80% PL concentration resulted in a significant reduction in yield. Additionally, both PL concentrations increased the lag phase across all three bacterial strains. When combined

with amikacin, PL at both concentrations further reduced bacterial yield and extended lag time across all three strains. However, μ_{max} remained largely unaffected by PL alone or in combination. These findings suggest that PL impairs bacterial expansion and delays growth initiation, and its combination with amikacin enhances these effects without altering replication speed.

Introduction

Antibiotics have been utilized traditionally to treat infections caused by bacteria [1]. Even though, the discovery of antibiotics was a remarkable milestone in veterinary and human medicine, their wide usage can pose a concern to public health [2, 3]. Specifically, the overuse of antibiotics is one of the reasons for the emergence of antimicrobial resistance (AMR) [4, 5], while treatment of AMR infections can be challenging due to rising costs and the availability of efficient therapeutic treatments leading to increased mortality rates [6]. For instance, in 2019 only, there were 1.27 million deaths related to AMR infections. Additionally, based on reports from the Centers for Disease Control and Prevention (CDC), at least 23,000 people annually die in the US from antibiotic-resistant illnesses [7]. From an economic perspective, AMR-related medical expenses reach approximately 66$ billion yearly, while it is estimated that AMR will cost the world at least 300$ billion losses by 2050 [8]. Unfortunately, AMR is not the only reason for antibiotic failure. Biofilm formation and immunodeficiencies are other factors that can contribute to the reduced efficacy of antibiotics against bacterial infections [9]. Beyond their global impact on public health and economics, in localized infections like chronic wounds, biofilm formation, and antimicrobial resistance (AMR) pose especially significant problems [10]. In veterinary medicine, these problems are particularly noticeable [11], where standard treatments are often undermined by bacterial adaptation and persistence. Antimicrobial resistance (AMR) and biofilm formation are major challenges in the management of chronic canine wounds. While conventional wound care often includes topical antimicrobial agents and antiseptic solutions [12, 13], bacterial pathogens frequently develop resistance over time [14, 15], potentially leading to severe infections, systemic illness, and in extreme cases, limb amputation [16]. The development of bacterial biofilms, which consist of bacteria colonies

encapsulated in an extracellular polymeric substance (EPS) matrix, is a major factor that complicates even further clinical outcome [16-18]. This matrix not only physically shields the bacteria from antibiotic penetration but also supports the survival of metabolically dormant persister cells that are inherently less susceptible to antimicrobials [19]. Moreover, bacteria within biofilms have been shown to overexpress resistance-related genes [14, 15, 20] compounding the difficulty of eradicating these infections. Together, these factors underscore the urgent need for alternative or adjunctive antimicrobial strategies that can target biofilms and combat AMR in veterinary wound care.

Previous studies have demonstrated the emerging role of platelets as potential antimicrobials [21, 22]. Even though the major role of platelets is to impact hemostasis [23], they are also able to affect the healing process and reduce inflammation due to the presence of platelet-derived growth factors located in platelet granules [24]. However, recent evidence suggests that platelets contain antimicrobial peptides too, making them an attractive therapeutic alternative to conventional antibiotics.

Specifically, platelet lysate is such an example of a platelet-derived biological that is produced from concentrated platelets and contains all bioactive factors secreted from their alpha granules [25]. This product is rich in cytokines, growth factors, and a variety of proteins [26] including antimicrobial peptides (AMPs) such as platelet factor 4 (PF-4). After the activation of platelets, AMPs start eliminating bacteria by making pores in bacterial cell membranes, leading to their death [27]. Besides, these peptides can prevent the cell wall synthesis of bacteria by attaching to lipid II, a crucial factor in cell wall development [28]. The antimicrobial features of PL have been investigated in several studies across both human and veterinary medicine [29-31]. In veterinary medicine, equine PL has been shown to dramatically reduce the growth rates and

yields of both Gram-positive and Gram-negative bacteria, such as *Escherichia coli and Pseudomonas aeruginosa* in a concentration-dependent manner [30]. Similarly, canine-derived PL showed bacteriostatic effects against multidrug-resistant strains such as *Staphylococcus aureus* and *Klebsiella pneumoniae* [21]. In addition, some studies explored the potential synergistic effects between PL and conventional antibiotics. Specifically, a previous study found that human PL exhibited a synergistic effect when combined with specific antibiotics such as oxacillin and vancomycin against *Staphylococcus aureus* [31]. Moreover, equine platelet-rich plasma lysate (PRP-L) has been shown to enhance the efficacy of amikacin against *Staphylococcus aureus* biofilms, demonstrating a synergistic interaction that improves bacterial clearance in vitro [32]. However, similar interactions between amikacin and platelet-derived products have not been investigated in the canine species. Given the biological differences between equine and canine platelet products and the potential for species-specific antimicrobial mechanisms, it remains unclear whether canine PL exerts comparable synergistic effects when combined with amikacin.

In our lab, we have previously evaluated various formulations of canine platelet lysate (PL) and their antimicrobial potency against bacterial growth [33]. We have demonstrated that plasma and its complement proteins are essential for the antimicrobial activity of PL, whereas the presence of leukocytes had no significant effect on its efficacy against any bacterial species tested. While these findings clarified how specific components contribute to the antimicrobial potential of PL, the study did not examine how PL influences bacterial growth dynamics over time when also combined with specific antibiotics. In the current study, we filled this gap by examining bacterial responses to PL using kinetic growth models. Our specific goals were to: (1) assess the antimicrobial effect of canine platelet lysate on bacteria growth kinetics and 2)

determine the synergistic effect of canine platelet lysate when combined with a traditional antibiotic on bacteria growth kinetics. Finally, we wanted to evaluate whether the above effects of PL are dose dependent.

Methods

Canine blood donors

Eight purpose-bred adult Beagle dogs (four males and four females) were recruited as whole blood donors for this study. The dogs had a mean age of 6.0 ± 3.7 years and a mean body weight of 15.0 ± 4.6 kg. Based on physical examination, medical history, and baseline hematological and biochemical analysis results (complete blood count and serum chemistry panel), all animals were considered clinically healthy. The Institutional Animal Care and Use Committee at Auburn University examined and approved the study protocol.

Platelet Lysate Production

Platelet-rich plasma (PRP), platelet-poor plasma (PPP), and platelet lysate (PL) were prepared as previously described [34]. Briefly, 100 mL of blood was collected from each canine donor into ACD-A tubes, and a complete blood count was performed using the Heska Element HT5 hematology analyzer. PRP was generated by centrifuging whole blood at 1000 × g for 5 minutes, followed by centrifugation of the plasma at 1500 × g for 15 minutes. Platelets were resuspended in 2 mL of plasma and adjusted to 0.8–1.0 × 10^6 platelets/μL using PPP. Aliquots of PRP and PPP were frozen at –80 °C, thawed, and pooled into three lots per formulation. Lysates were produced by four additional freeze–thaw cycles (liquid nitrogen or 37 °C dry bath), followed by centrifugation at 20,000 × g for 20 minutes. Supernatants were collected as final PL and PPP products and stored at –80 °C.

Growth Curve Assay

Tree clinically isolated bacteria from ATCC were used in the growth curve assay: *Staphylococcus pseudintermedius* (*S. pseudintermedius*; ATCC #49051), *Staphylococcus aureus* (*S. aureus*; ATCC #12600) and *Methicillin-resistant Staphylococcus aureus* (*MRSA*; ATCC

#43300). Aseptic procedures were used to conduct experiments in a biosafety hood. First, the freeze-dried bacteria were rehydrated using one milliliter of brain heart infusion broth (BHI; Becton Dickinson, Sparks, MD). In a culture tube containing four milliliters of BHI broth, reconstituted bacteria were cultured overnight at 37˚C under continuous shake at 200 rpm. Subsequently, duplicate aliquots on 96-well plates were used to assess each culture tube's optical density (OD) at OD600 using a Biotek Gen5 (Synergy HI Microplate Reader, Agilent Technologies Inc. Winooski, VT). Before initiating the growth curve assay, bacterial suspensions were adjusted to 0.02 OD, or 107 CFU/ml.

1 mL aliquots of the adjusted bacterial suspensions in BHI were centrifuged at 3000 rpm for 10 minutes to pellet the cells. The pellets were washed with an equal volume of phosphate-buffered saline (PBS), followed by a second centrifugation at 3000 rpm for 10 minutes. The PBS was then removed, and 600 µL of treatment solution was added to each pellet. Treatments consisted of either platelet lysate (PL) or platelet-poor plasma (PPP) at final concentrations of 20% or 80%, with the remaining volume composed of BHI, with or without the addition of antibiotics. The antibiotic used in this study was amikacin (final concentration: 4 µg/mL; MP Biomedicals, Cat# 150342), selected based on CLSI resistance breakpoints [35]. Treated samples were plated in duplicate into 48-well microplates (250 µL per well). Growth was monitored over 16 hours at 37 °C with orbital shaking at 205 rpm, and optical density at 600 nm (OD_{600}) was recorded every 10 minutes using a microplate reader (Biotech Gen5, Agilent Technologies). For each treatment condition, a corresponding blank (no-bacteria) control was included, and its OD_{600} value was subtracted from the experimental readings. Gen5 software was used to extract additional growth parameters, including maximum bacterial yield (maximum OD_{600}), average growth rate (mean V), specific growth rate (μ_{max}), and lag time, enabling further analysis of bacterial fitness.

Kinetic parameters

In the context of bacterial growth, the carrying capacity (K) represents the maximum population size or biomass that the environment can support under specific conditions. A higher K value indicates that the medium or treatment environment is highly conducive to bacterial survival and proliferation. Consequently, a higher carrying capacity in the treatment group than the control group indicates that the therapy was insufficiently efficient in limiting bacterial growth, indicating poor antimicrobial efficacy. The other parameter is the maximum specific growth rate (μ_{max}), representing the fastest rate at which bacteria divide during the exponential phase and reflecting the efficiency of bacterial replication under given conditions. A higher μ_{max} in the treatment group compared to the control indicates that bacteria replicated more rapidly, suggesting the treatment did not inhibit bacterial growth. This may result from the presence of nutrients or growth-promoting factors within the treatment. Lastly, the first stage of bacterial growth, known as lag time (λ), is when cells adjust to their surroundings before starting active growth. A longer lag period usually indicates more stress or inhibitory effects given by the environment. Consequently, a longer lag time in the treatment group relative to the control group implies that the therapy delayed the onset of bacterial adaptation and growth, which may indicate antimicrobial efficacy.

Statistical analysis

Growth data from three bacterial strains were obtained under multiple experimental conditions, with three biological replicates per condition. Five primary growth models—Baranyi, Gompertz, modified Gompertz, Huang, and logistic—were evaluated for their goodness-of-fit using Akaike Information Criterion (AIC) and coefficient of determination (R^2). Among these, the

Baranyi model consistently demonstrated the best overall fit and was subsequently selected for final analysis. This model was refitted to estimate key kinetic parameters: maximum carrying capacity (K), maximum specific growth rate (μ_{max}), and initial physiological state (h_0), constrained within biologically plausible ranges. Model performance was further validated through visual comparison of fitted and observed values to ensure accurate representation of growth dynamics. To assess the effect of treatment conditions on kinetic parameters, the Kruskal–Wallis test was employed to detect overall group differences. Post hoc pairwise comparisons were performed using bootstrap resampling with Holm correction for multiple testing. Nonparametric methods were selected due to the limited sample size and distributional considerations.

Results

Growth curve assay

The effect of PL at two different concentrations on *S. aureus* kinetics is shown in **Figure 1**. PL20 (1.818 ± 0.631) and PPP 20 (1.391 ± 1.129) demonstrate significantly lower k value compared to PL80 (1.605 ± 1.200, p = 0.002), PPP80 (1.190 ± 0.726, p = 0.002), and BHI (2.450 ± 0.562, p = 0.002). Similarly, PL80 (1.605 ± 1.200) shows significantly lower K value compared to PPP80 (p = 0.002) and BHI (p = 0.002) (Figure 1A). Additionally, PPP80 (0.621 ± 1.076) shows significantly lower μ_{max} compared to BHI (7.221 ± 0.469, p = 0.014) (Figure 1B). Finally, PL20 (0.358 ± 0.081), PPP20 (0.261 ± 0.268), PL80 (0.632 ± 0.026) and PPP80 (0.862 ± 0.132) show significantly higher λ than BHI (0.747 ± 0.022, p = 0.00) (Figure 1C).

The effect of PL at two different concentrations on *S. pseud* kinetics is shown in **Figure 2**. PL80 (1.963 ± 0.463) shows significantly lower k value compared to BHI (1.001 ± 0.368, p = 0.002) (Figure 2A) while it had significantly higher μ_{max} (9.210 ± 1.367) than BHI (3.458 ± 1.143, p = 0.002) (Figure 2B). Moreover, PL20 (1.272 ± 0.677), PPP20 (0.609 ± 0.420), PL80 (0.513 ± 0.165), and PPP80 (0.663 ± 0.343) demonstrate significantly higher λ than BHI (0.894 ± 0.076, p = 0.00) (Figure 2C).

The effect of PL at two different concentrations on *MRSA* kinetics is shown in **Figure 3**. PL80 (3.246 ± 2.661) and PPP80 (3.435 ± 2.249) show significantly lower k value compared to BHI (0.796 ± 0.089, p = 0.002) (Figure 3A). On the contrary, PPP80 (3.244 ± 4.558) demonstrates higher μ_{max} than BHI (0.00 ± 0.00, p = 0.010), PL20 (3.726 ± 5.465, p = 0.091) and PPP20 (6.256 ± 3.743, p = 0.002). Furthermore, PPP20 (6.256 ± 3.743) illustrates significantly lower μ_{max} than PL80 (1.059 ± 0.976, p = 0.059) and BHI (p = 0.002) (Figure 3B). Eventually, BHI (0.925 ± 0.085,

p = 0.00) shows significantly lower λ than PL20 (0.733 ± 1.099), PPP20 (1.036 ± 1.001), PL80 (0.388 ± 0.163), and PPP80 (0.379 ± 0.051) (Figure 3C).

The effect of PL at two different concentrations and when combined with a specific antibiotic (amikacin) on *S. aureus* kinetics is shown in **Figure 4**. For 20% concentration of treatment, PL20 (1.818 ± 0.631, p = 0.003), PPP20 (1.391 ± 1.129, p = 0.003), and the combination of amikacin (AMK) with PL20 (AMKPL20) demonstrate significantly lower k value (0.470 ± 0.606, p = 0.016) compared to the combination of amikacin with BHI (AMKBHI; 0.710 ± 0.578,). Moreover, the addition of AMKPL20 shows a significantly lower k value (0.470 ± 0.606) than BHI (2.450 ± 0.562, p = 0.003) (Figure 4A). Moreover, the addition of AMKPL20 (3.333 ± 5.774) and AMKPPP20 (0.710 ± 0.693) show significantly lower μ_{max} (0.710 ± 0.693) than PL20 (3.676 ± 0.706, p = 0.003) and BHI (7.221 ± 0.469, p = 0.003). Similarly, AMKBHI (3.088 ± 2.509, p = 0.032) has significantly lower μ_{max} (3.088 ± 2.509, p = 0.032) than PL20 (Figure 4B). When evaluated the λ values, AMKPPP20 shows significantly higher λ (0.721 ± 0.718) compared to PL20 (0.358 ± 0.081, p = 0.09), PPP20 (0.261 ± 0.268, p = 0.00), AMKBHI (0.787 ± 0.081, p = 0.00), and BHI (0.747 ± 0.022, p = 0.00). Similarly, AMKPL20 has significantly greater λ (0.521 ± 0.174, p = 0.00) than AMKBHI (0.787 ± 0.081, p = 0.00) and BHI (0.747 ± 0.022, p = 0.00) (Figure 4C). For 80% concentration of treatment, AMKPL80 and AMKPPP80 show significantly lower k value (0.589 ± 0.591 and 0.784 ± 0.683, respectively) than PPP80 (1.190 ± 0.726, p = 0.003), AMKBHI (0.710 ± 0.578, p = 0.003) and BHI (2.450 ± 0.562, p = 0.003) (Figure 4D). For the μ_{max}, BHI demonstrated significantly higher values (7.221 ± 0.469, p = 0.003) compared to AMKPL80 (0.298 ± 0.516), AMKPPP80 (0.327 ± 0.566), and AMKBHI (3.088 ± 2.509) (Figure 4E). AMKPL80, AMKPPP80, and AMKBHI show significantly greater λ (0.551 ± 0.041, 0.564 ± 0.092, and 0.787 ± 0.081) than BHI (0.747 ± 0.022, p = 0.00) (Figure 4F).

The effect of PL at 2 different concentrations with antibiotics on *S. pseud* kinetics is shown in **Figure 5**. AMKPL20 (0.226 ± 0.295) and AMKBHI (0.835 ± 0.441) show significantly lower K value compared to PL20 (1.086 ± 0.917, p = 0.072, p = 0.003 respectively) and BHI (1.001 ± 0.368, p = 0.003) (Figure 5A). In comparison to BHI (3.458 ± 1.143, p = 0.003), AMKPL20 (3.333 ± 5.774) and AMKPPP20 (6.016 ± 5.301) exhibit significantly lower μ_{max} (Figure 5B). AMKPL20 (0.468 ± 0.132) and AMKPPP20 (0.918 ± 0.938) demonstrate significantly higher λ compared to AMKBHI (0.377 ± 0.087, p = 0.00) and BHI (0.894 ± 0.076, p = 0.00). Moreover, AMKPPP20 shows significantly greater λ than PL20 (1.272 ± 0.677, p = 0.07) and PPP20 (0.609 ± 0.420, p = 0.00) (Figure 5C). For the 80% concentration, AMKPPP80 (0.416 ± 0.297) shows significantly lower k value than PL80 (1.963 ± 0.463, p = 0.055) and BHI (1.001 ± 0.368, p = 0.003). Similarly, AMKPL80 (0.741 ± 0.796) has significantly lower k value than BHI (p = 0.003) (Figure 5D). PL80 (9.210 ± 1.367) exhibit significantly higher μ_{max} compared to AMKPL80 (p = 0.015), AMKPPP80 (p = 0.003), AMKBHI (p = 0.003). Additionally, AMKPPP80 (6.667 ± 5.774) show significantly less μ_{max} than PPP80 (7.687 ± 4.006, p = 0.003) and BHI (p = 0.044) (Figure 5E). AMKPL80 (0.346 ± 0.033) and AMKPPP80 (0.336 ± 0.009) show significantly higher λ than BHI (0.894 ± 0.076, p = 0.00) (Figure 5F).

The effect of PL at 2 different concentrations with antibiotics on *MRSA* kinetics is shown in **Figure 6**. AMKPPP20 (0.406 ± 0.307) demonstrates significantly lower k value than AMKBHI (0.874 ± 0.392, p = 0.003) and BHI (0.796 ± 0.089, p = 0.003) (Figure 6A). Furthermore, AMKPPP20 (0.863 ± 1.495) shows significantly less μ_{max} than BHI (0.000 ± 0.000, p = 0.09) (Figure 6B). AMKPPP20 (0.238 ± 0.014) and AMKPL20 (1.432 ± 0.983) show significantly greater λ compared to AMKBHI (0.811 ± 0.044, p = 0.00) and BHI (0.925 ± 0.085, p = 0.00). Moreover, AMKPPP20 (0.238 ± 0.014) demonstrates significantly higher λ than PL20 (0.733 ±

1.099, p = 0.04) and PPP20 (1.036 ± 1.001, p = 0.00) (Figure 6C). For 80% concentration, PL80 (3.246 ± 2.661) and PPP80 (3.435 ± 2.249) exhibit significantly less k value compared to AMKBHI (0.874 ± 0.392, p = 0.03) (Figure 6D). AMKPL80 and AMKPPP80 show significantly less μ_{max} than PPP80. Similarly, AMKPPP80 shows significantly less μ_{max} compared to BHI (Figure 6E). Eventually, AMKPL80 (0.705 ± 0.281), and AMKPPP80 (0.771 ± 0.322) exhibit significantly higher λ than BHI (0.925 ± 0.085, p = 0.00) (Figure 6F).

Discussion

This study aimed to investigate the antimicrobial effects of canine platelet lysate on bacterial growth kinetics and to compare its efficacy with that of a conventional antibiotic (amikacin). By applying mathematical modeling to kinetic data, we quantified the effects of PL and its combination with amikacin on three clinically relevant staphylococcal strains.

Amikacin was chosen as our comparator antibiotic because of its proven safety profile when applied topically and its potent bactericidal activity against both Gram-positive and Gram-negative pathogens, including *Staphylococcus* species [36]. A recent clinical study by Peng et al. showed that dogs with open wounds were treated with a topical amikacin hydrogel at a concentration of 45 mg/mL. With peak serum levels falling between 2.8 and 3.8 µg/mL, this treatment produced low systemic absorption and was significantly below the 5 µg/mL threshold linked to nephrotoxicity. According to these results, topical amikacin minimizes systemic exposure while achieving localized antibacterial effects, which makes it a clinically relevant standard for assessing the antimicrobial activity of canine platelet lysate.

A comparative look at a previous study performed on equine PL growth kinetics [30] reveals both parallels and distinctions relative to our findings on canine PL. In both studies, PL significantly reduced the overall bacterial yield of *S. aureus*, but the dose-response patterns differed. While our results showed that PL20 significantly reduced carrying capacity compared to PL80, indicating a concentration-dependent effect, the equine study reported no significant difference in bacterial yield across PL concentrations from 20% to 80%, suggesting a concentration-independent effect. Regarding lag time (λ), both studies found no significant difference between PL concentrations, indicating that the delay in bacterial adaptation is not dose dependent. In contrast to the equine study, which found no significant difference in lag time

between PL-treated groups and BHI, our study found that both PL20 and PL80 significantly increased lag time when compared to BHI. This implies that canine PL exerts a stronger and statistically significant delay in bacterial adaptation, even though the consistency of lag time across dosages is a common feature. Additionally, in our study, combining PL20 with amikacin (AMKPL20) further reduced carrying capacity and prolonged lag time compared to amikacin alone, indicating a potential synergistic effect on both growth suppression and adaptation delay. Our study showed no significant effect of PL, at either concentration, on the highest specific growth rate (μ_{max}) in relation to BHI when comparing bacterial growth rates. Amikacin and PL together, however, demonstrated stronger suppression: AMKPL20 and AMKPPP20 had noticeably reduced μ_{max} than PL20, suggesting that although PL by itself did not affect replication speed, its combination with antibiotics may increase their inhibitory effect on exponential growth. To the contrary, the growth rates were similar among various concentrations of equine PL [30], as they were all considerably lower than BHI. This suggests that equine PL might be able to inhibit bacterial replication speed more directly and independently [30]. Our data highlight interspecies differences and the impact of various platelet processing methods and bacterial strain variations on the antimicrobial activity of platelet-derived biologicals.

For *S. pseudintermedius*, although there was no significant difference in the reduction of carrying capacity between the two concentrations, PL80 exhibited a significantly lower carrying capacity compared to BHI, indicating a stronger suppressive effect relative to the untreated control. The combination of amikacin with both concentrations of PL, as well as amikacin alone, exhibited significantly lower carrying capacity than BHI. However, there was no significant difference in carrying capacity between the antibiotic-only and the combination treatments, suggesting that while PL possesses antimicrobial properties, it did not enhance the suppressive effect of amikacin

on *S. pseudintermedius* growth yield under these conditions. No significant effect on the maximum specific growth rate (μ_{max}) was observed for either PL20 or PL80 compared to BHI, indicating that platelet lysate alone did not meaningfully alter the replication speed of *S. pseudintermedius* during exponential growth. Likewise, amikacin alone (AMKBHI) did not differ significantly from BHI in terms of μ_{max}. Interestingly, among all tested conditions, only the combination treatment AMKPL20 resulted in a significantly lower μ_{max} compared to BHI, suggesting a potential interaction between PL20 and amikacin that modestly enhances bacterial growth suppression at the level of replication dynamics.

In the case of *MRSA*, PL80 significantly reduced the carrying capacity compared to both BHI and amikacin alone, indicating a notable suppressive effect. However, no significant difference in caring capacity was observed between PL80 and PL20, suggesting that the antibacterial impact of PL on *MRSA* yield was not concentration-dependent within the tested range. Neither concentration of PL produced a significant effect on the maximum specific growth rate of *MRSA* compared to BHI. It's interesting to note that, in comparison to the control, only PPP20 and AMKPPP20 produced noticeably lower μ_{max} values. Amikacin by itself did not demonstrate a statistically significant decrease in μ_{max} in comparison to BHI, indicating that, aside from certain PPP formulations, the majority of treatments, including PL and amikacin, did not affect the bacterial replication rate. Lag time (λ) analysis indicated that both PL20 and PL80 significantly increased lag time compared to BHI, indicating a delayed onset of bacterial growth under these conditions. Moreover, the combination treatments AMKPL20 and AMKPL80 exhibited even greater increases in lag time, with AMKPL20 showing a significantly higher λ than amikacin alone. Accordingly, PL could inhibit early bacterial adaptability and, when combined with

amikacin, may further postpone the bacterial entry into exponential growth. This suggests a potentially useful interaction at the level of priming inhibition or metabolic stress response [37].

The observed antimicrobial effects of PL, particularly its ability to reduce carrying capacity without significantly altering the maximum specific growth rate, may be explained by its complex mechanism of action. Numerous immune mediators and antimicrobial peptides are possibly present in PL that primarily exert a bacteriostatic rather than bactericidal effect against *staphylococcal* strains [33,38], limiting population expansion without directly impacting replication speed [39]. The fact that the maximum growth rate remained mostly unchanged is consistent with studies showing that PL's main impact is on bacterial membrane integrity and nutrient access rather than intracellular replication machinery [40]. The inverse dose relationship may reflect the concentration-specific release of antimicrobial peptides (AMPs) or plasma-derived factors known to inhibit bacterial replication, where lower concentrations may optimally release active peptides without the inhibitory effects of excess plasma proteins. Specifically, the extended lag time seen with PL and amikacin combinations indicates a potential delay in metabolic adaptation caused by oxidative stress or the need for membrane repair. This is consistent with previous literature [41, 42], demonstrated that platelet-released products can induce reactive oxygen species (ROS) and DNA damage in *staphylococci*. This supports the hypothesis that PL may enhance bacterial susceptibility to antibiotics by extending the period during which bacteria must cope with environmental stress. By delaying their ability to adapt and initiate replication, PL reduces the overall fitness and survival potential of the bacterial population, even if it does not directly cause rapid or extensive cell death.

This study has several limitations that should be acknowledged. First, the bacterial strains utilized were from human origin rather than canine, which may limit the direct applicability of the

findings, given that the PL were derived from canine donors. Host-specific interactions between bacteria and immune-derived products like PL could influence antimicrobial efficacy. Second, the evaluation was limited to two concentrations of PL (20% and 80%). Including a broader range of concentrations would have allowed a more detailed assessment of potential dose-response relationships and helped clarify whether PL's effects follow a linear or threshold pattern.

In conclusion, our findings demonstrate that PL exhibits dose-dependent antimicrobial activity against certain strains, such as *Staphylococcus aureus*. PL effectively alters key bacterial growth parameters, including carrying capacity and lag time, and in specific conditions, shows comparable or even synergistic effects when combined with antibiotics like amikacin.

Conclusion

In conclusion, our findings demonstrate that PL exhibits dose-dependent antimicrobial activity against certain strains, such as *Staphylococcus aureus*. PL effectively alters key bacterial growth parameters, including carrying capacity and lag time, and in specific conditions, shows comparable or even synergistic effects when combined with antibiotics like amikacin

References

1. Hauser, A.R., J. Mecsas, and D.T. Moir, *Beyond Antibiotics: New Therapeutic Approaches for Bacterial Infections.* Clin Infect Dis, 2016. **63**(1): p. 89-95.
2. Roberts, S.C. and T.R. Zembower, *Global increases in antibiotic consumption: a concerning trend for WHO targets.* Lancet Infect Dis, 2021. **21**(1): p. 10-11.
3. Salam, M.A., et al., *Antimicrobial Resistance: A Growing Serious Threat for Global Public Health.* Healthcare (Basel), 2023. **11**(13).
4. Kapil, A., *The challenge of antibiotic resistance: need to contemplate.* Indian J Med Res, 2005. **121**(2): p. 83-91.
5. Llor, C. and L. Bjerrum, *Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem.* Ther Adv Drug Saf, 2014. **5**(6): p. 229-41.
6. Dadgostar, P., *Antimicrobial Resistance: Implications and Costs.* Infect Drug Resist, 2019. **12**: p. 3903-3910.
7. Michaelidis, C.I., et al., *The hidden societal cost of antibiotic resistance per antibiotic prescribed in the United States: an exploratory analysis.* BMC Infect Dis, 2016. **16**(1): p. 655.
8. Pulingam, T., et al., *Antimicrobial resistance: Prevalence, economic burden, mechanisms of resistance and strategies to overcome.* Eur J Pharm Sci, 2022. **170**: p. 106103.
9. de la Fuente-Nunez, C., A. Cesaro, and R.E.W. Hancock, *Antibiotic failure: Beyond antimicrobial resistance.* Drug Resist Updat, 2023. **71**: p. 101012.
10. Mendhe, S., et al., *Impact of Biofilms on Chronic Infections and Medical Challenges.* Cureus, 2023. **15**(11): p. e48204.
11. Bell, S., *A guide to biofilm in veterinary wounds.* The Veterinary Nurse, 2024. **15**: p. 1-7.

12. Mickelson, M.A., C. Mans, and S.A. Colopy, *Principles of Wound Management and Wound Healing in Exotic Pets.* Vet Clin North Am Exot Anim Pract, 2016. **19**(1): p. 33-53.

13. Lux, C.N., *Wound healing in animals: a review of physiology and clinical evaluation.* Veterinary Dermatology, 2022. **33**(1).

14. Bowler, P.G., *Antibiotic resistance and biofilm tolerance: a combined threat in the treatment of chronic infections.* J Wound Care, 2018. **27**(5): p. 273-277.

15. Ciofu, O., et al., *Antibiotic treatment of biofilm infections.* APMIS, 2017. **125**(4): p. 304-319.

16. Smith, O.J., et al., *An evaluation of the bacteriostatic effect of platelet-rich plasma.* Int Wound J, 2021. **18**(4): p. 448-456.

17. Attinger, C. and R. Wolcott, *Clinically Addressing Biofilm in Chronic Wounds.* Adv Wound Care (New Rochelle), 2012. **1**(3): p. 127-132.

18. Percival, S.L., S.M. McCarty, and B. Lipsky, *Biofilms and Wounds: An Overview of the Evidence.* Adv Wound Care (New Rochelle), 2015. **4**(7): p. 373-381.

19. Wood, T.K., S.J. Knabel, and B.W. Kwan, *Bacterial persister cell formation and dormancy.* Appl Environ Microbiol, 2013. **79**(23): p. 7116-21.

20. Cetinkaya, R.A., et al., *Platelet-rich plasma as an additional therapeutic option for infected wounds with multi-drug resistant bacteria: in vitro antibacterial activity study.* Eur J Trauma Emerg Surg, 2019. **45**(3): p. 555-565.

21. Attili, A.R., et al., *Antibacterial Properties of Canine Platelet-Rich Plasma and Other Non-Transfusional Hemo-Components: An in vitro Study.* Front Vet Sci, 2021. **8**: p. 746809.

22. Warin, R., et al., *In Vitro Antimicrobial Properties and Their Mechanisms in Relation to Reactive Oxygen Species of Canine Platelet-Rich Fibrin.* Animals (Basel), 2023. **13**(24).

23. Scridon, A., *Platelets and Their Role in Hemostasis and Thrombosis-From Physiology to Pathophysiology and Therapeutic Implications.* Int J Mol Sci, 2022. **23**(21).

24. Nurden, A.T., *Platelets, inflammation and tissue regeneration.* Thromb Haemost, 2011. **105 Suppl 1**: p. S13-33.

25. Dohan Ehrenfest, D.M., et al., *Classification of platelet concentrates (Platelet-Rich Plasma-PRP, Platelet-Rich Fibrin-PRF) for topical and infiltrative use in orthopedic and sports medicine: current consensus, clinical implications and perspectives.* Muscles Ligaments Tendons J, 2014. **4**(1): p. 3-9.

26. Meftahpour, V., et al., *Platelet lysate: a promising candidate in regenerative medicine.* Regen Med, 2021. **16**(1): p. 71-85.

27. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nat Rev Microbiol, 2005. **3**(3): p. 238-50.

28. Koch, A.L., *Bacterial wall as target for attack: past, present, and future research.* Clin Microbiol Rev, 2003. **16**(4): p. 673-87.

29. Egli, P., et al., *Pilot study characterizing a single pooled preparation of equine platelet lysate for nebulization in the horse.* Front Vet Sci, 2024. **11**: p. 1488942.

30. Gordon, J., S. Alvarez-Narvaez, and J.F. Peroni, *Antimicrobial Effects of Equine Platelet Lysate.* Front Vet Sci, 2021. **8**: p. 703414.

31. Rozalski, M.I., et al., *Antimicrobial/anti-biofilm activity of expired blood platelets and their released products.* Postepy Hig Med Dosw (Online), 2013. **67**: p. 321-5.

32. Gilbertie, J.M., et al., *Platelet-rich plasma lysate displays antibiofilm properties and restores antimicrobial activity against synovial fluid biofilms in vitro.* J Orthop Res, 2020. **38**(6): p. 1365-1374.

33. Mollabashi, M., et al., *Plasma and complement proteins are essential for the antimicrobial activity of canine platelet lysate.* Front Vet Sci, 2025. **12**: p. 1605649.

34. Lunardon, T., et al., *Growth factor and cytokine characterization of canine platelet lysate with variable leukocyte concentration, plasma content, and heat-sensitive proteins.* Front Vet Sci, 2024. **11**: p. 1408080.

35. Clinical and Laboratory Standards Institute, *Performance Standards for Antimicrobial Susceptibility Testing*. CLSI supplement M100, ed. 33rd. 2023, Wayne, PA: Clinical and Laboratory Standards Institute.

36. Peng, Z.H., et al., *Serum amikacin concentrations in dogs with naturally occurring open wounds treated with topical amikacin in carboxymethylcellulose hydrogel.* Vet Surg, 2025. **54**(3): p. 453-460.

37. Cayron, J. and C. Lesterlin, *Multi-scale Analysis of Bacterial Growth Under Stress Treatments.* J Vis Exp, 2019(153).

38. Anitua, E., et al., *Antibacterial effect of plasma rich in growth factors (PRGF(R)-Endoret(R)) against Staphylococcus aureus and Staphylococcus epidermidis strains.* Clin Exp Dermatol, 2012. **37**(6): p. 652-7.

39. Tang, Y.Q., M.R. Yeaman, and M.E. Selsted, *Antimicrobial peptides from human platelets.* Infect Immun, 2002. **70**(12): p. 6524-33.

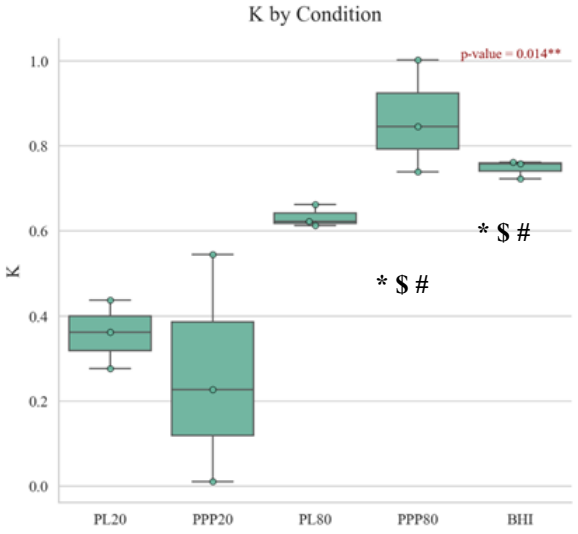
40. Cl, K., et al., *Antimicrobial Effects of Platelet-Rich Plasma and Platelet-Rich Fibrin: A Scoping Review.* Cureus, 2023. **15**(12): p. e51360.

41. Liu, E., et al., *Platelets Inhibit Methicillin-Resistant Staphylococcus aureus by Inducing Hydroxyl Radical-Mediated Apoptosis-Like Cell Death.* Microbiol Spectr, 2022. **10**(4): p. e0244121.

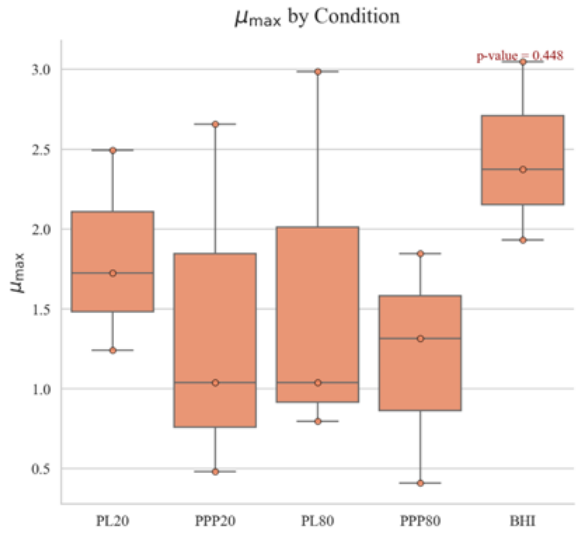
42. Xu, J., et al., *Platelets directly regulate DNA damage and division of Staphylococcus aureus.* FASEB J, 2018. **32**(7): p. 3707-3716.

.

A


K by Condition
p-value = 0.014**
K
1.0
0.8
0.6
0.4
0.2
0.0
* $ #
* $ #
PL20
PPP20
PL80
PPP80
BHI

B


μmax by Condition
p-value = 0.448
μmax
3.0
2.5
2.0
1.5
1.0
0.5
PL20
PPP20
PL80
PPP80
BHI

C

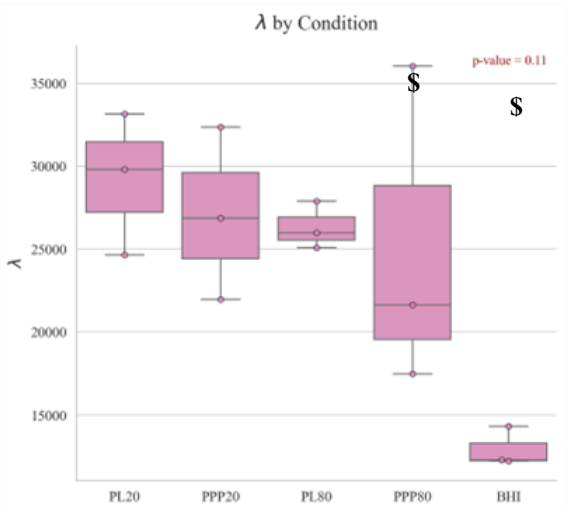
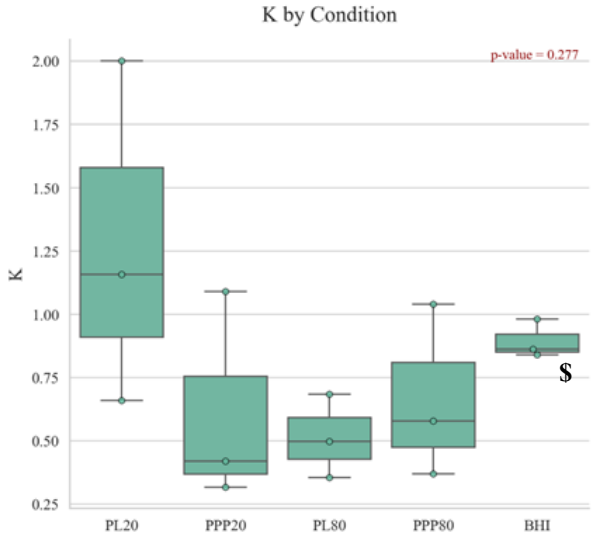
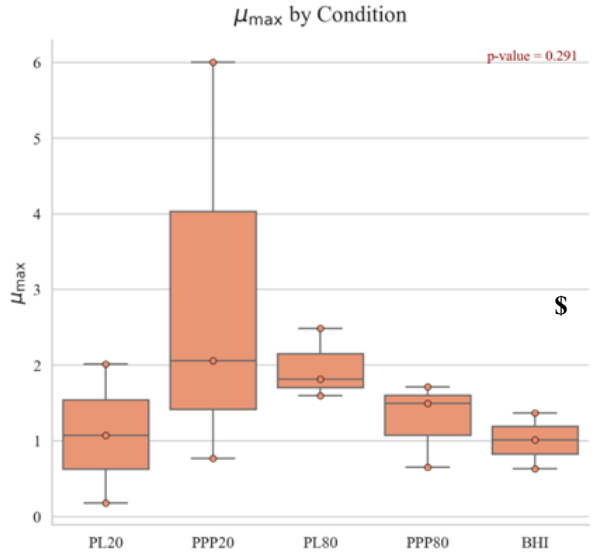

λ by Condition
p-value = 0.11
λ
35000
30000
25000
20000
15000
$
$
$
PL20
PPP20
PL80
PPP80
BHI

Figure 6: Effect of platelet lysate at two different concentrations on the growth kinetics of *Staphylococcus aureus*. (**A**) Carrying capacity (K), (**B**) maximum specific growth rate (μ_{max}), and (**C**) lag time (λ) of *S. aureus* under five treatment conditions: PL20, 20% platelet lysate; PPP20, 20% platelet-poor plasma; PL80, 80% platelet lysate; PPP80, 80% platelet-poor plasma; and BHI, brain heart infusion broth; as the control. Data from three biological replicates modeled using the Baranyi equation. Box plots show median, mean (circle), IQR, and range. Kruskal–Wallis test was used; p-values are shown in red. Significant differences ($p < 0.5$) were observed in panel A ($p = 0.0.014$), B ($p = 0.448$), and C ($p = 0.11$). $^{\$}$ $p < 0.5$ compared to BHI; * $p < 0.5$ compared to PL80; $^{\#}$ $p < 0.5$ compared to PPP80.

A


K by Condition
p-value = 0.277
K
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
PL20
PPP20
PL80
PPP80
BHI
$

B


μmax by Condition
p-value = 0.291
μmax
6
5
4
3
2
1
0
PL20
PPP20
PL80
PPP80
BHI
$

C

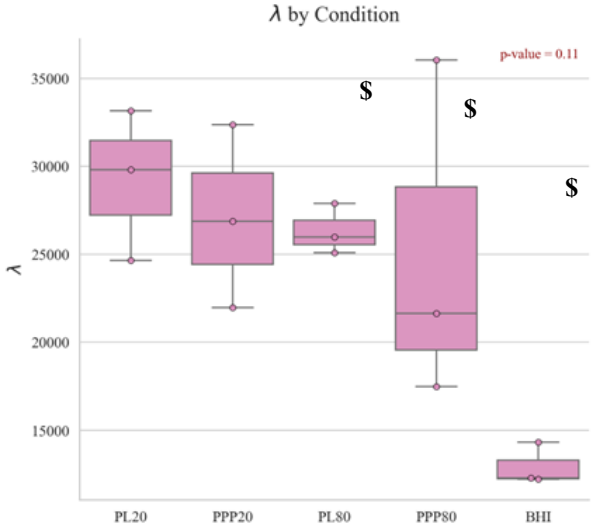

λ by Condition
p-value = 0.11
λ
35000
30000
25000
20000
15000
PL20
PPP20
PL80
PPP80
BHI
$
$
$
$

Figure 7: Effect of platelet lysate at two different concentrations on the growth kinetics of *Staphylococcus pseudintermedius*. (**A**) Carrying capacity (K), (**B**) maximum specific growth rate (μ_{max}), and (**C**) lag time (λ) of *S. pseudintermedius* under five treatment conditions: PL20, 20% platelet lysate; PPP20, 20% platelet-poor plasma; PL80, 80% platelet lysate; PPP80, 80% platelet-poor plasma; and BHI, brain heart infusion broth; as the control. Data from three biological replicates were modeled using the Baranyi equation. Box plots show median, mean (circle), IQR, and range. Kruskal–Wallis test was used; p-values are shown in red. Significant differences ($p < 0.5$) were observed in panel A ($p = 0.277$), B ($p = 0.291$), and C ($p = 0.11$). $^{\$}$ $p < 0.5$ compared to BHI.

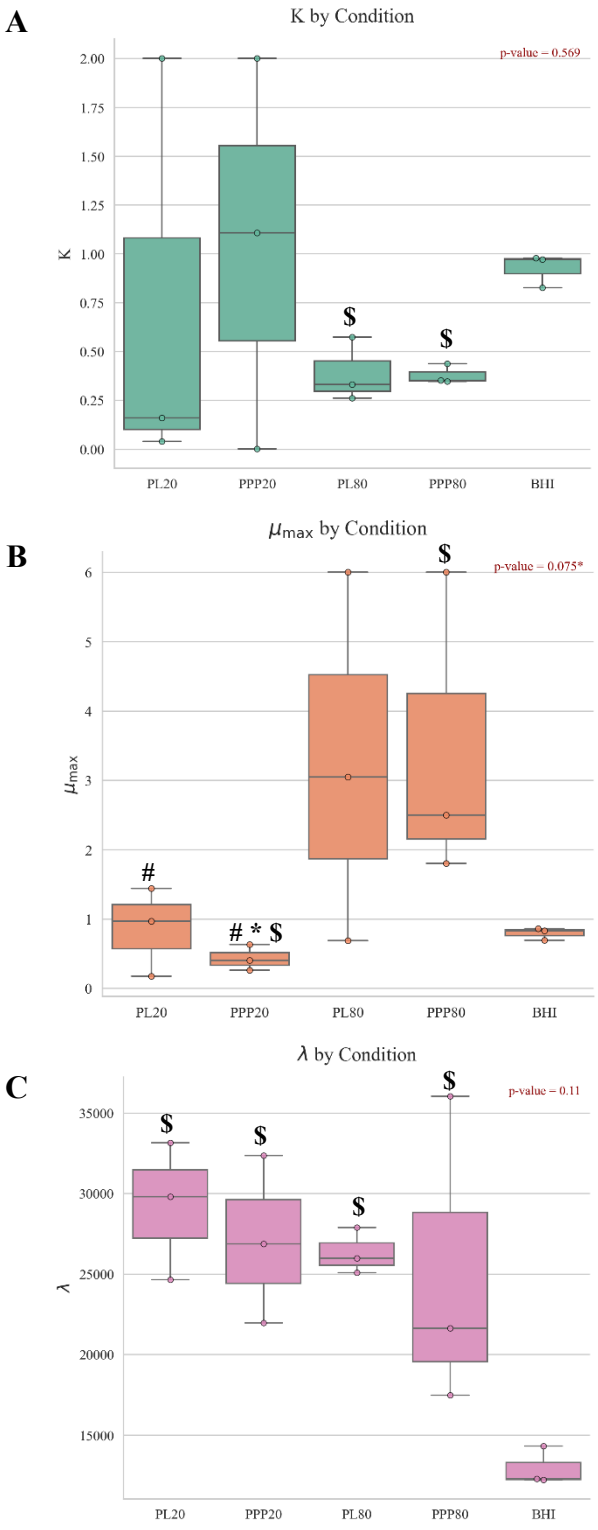
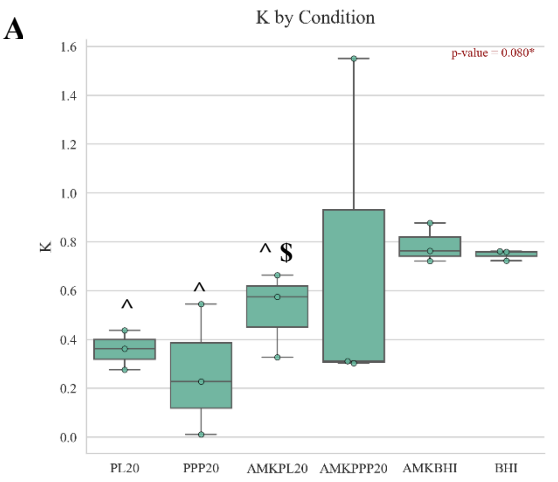
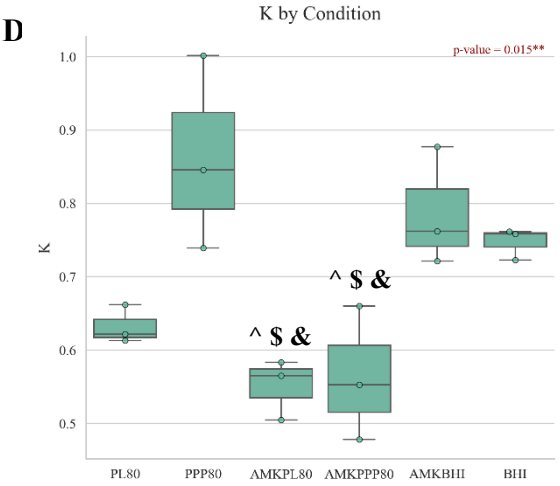
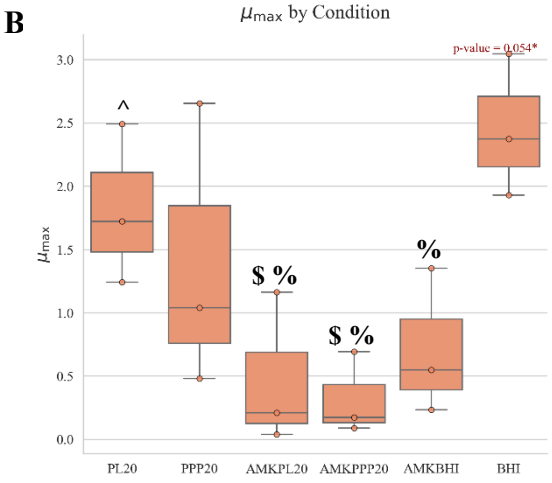
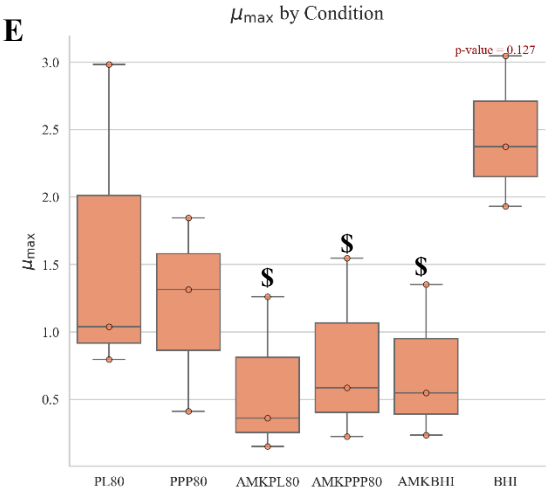

A
K by Condition
p-value = 0.569
K
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
$
$
PL20
PPP20
PL80
PPP80
BHI
B
μmax by Condition
p-value = 0.075*
μmax
6
5
4
3
2
1
0
$
#
* $
PL20
PPP20
PL80
PPP80
BHI
C
λ by Condition
p-value = 0.11
λ
35000
30000
25000
20000
15000
$
$
$
$
PL20
PPP20
PL80
PPP80
BHI

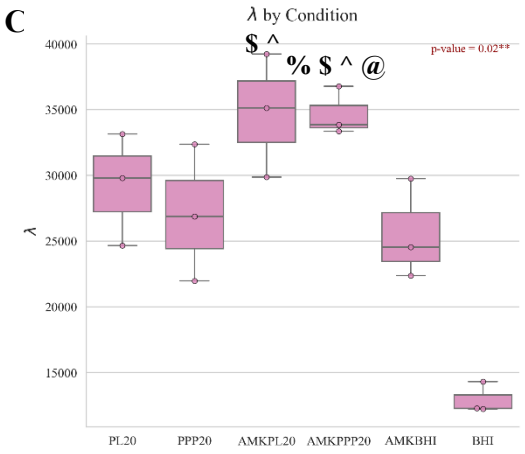
Figure 8: Effect of platelet lysate at two different concentrations on the growth kinetics of *Methicillin-Resistant Staphylococcus aureus*. (**A**) Carrying capacity (K), (**B**) maximum specific growth rate (μ_{max}), and (**C**) lag time (λ) of *MRSA* under five treatment conditions: PL20, 20% platelet lysate; PPP20, 20% platelet-poor plasma; PL80, 80% platelet lysate; PPP80, 80% platelet-poor plasma; and BHI, brain heart infusion broth; as the control. Data from three biological replicates modeled using the Baranyi equation. Box plots show median, mean (circle), IQR, and range. Kruskal–Wallis test was used; p-values are shown in red. Significant differences ($p < 0.5$) were observed in panel A ($p = 0.0569$), B ($p = 0.075$), and C ($p = 0.11$). $^{\$}$ $p < 0.5$ compared to BHI; * $p < 0.5$ compared to PL80; $^{\#}$ $p < 0.5$ compared to PPP80.


A
K by Condition
p-value = 0.080*
K
PL20
PPP20
AMKPL20
AMKPPP20
AMKBHI
BHI


D
K by Condition
p-value = 0.015**
K
PL80
PPP80
AMKPL80
AMKPPP80
AMKBHI
BHI


B
μmax by Condition
p-value = 0.054*
μmax
PL20
PPP20
AMKPL20
AMKPPP20
AMKBHI
BHI


E
μmax by Condition
p-value = 0.127
μmax
PL80
PPP80
AMKPL80
AMKPPP80
AMKBHI
BHI


C
λ by Condition
p-value = 0.02**
λ
PL20
PPP20
AMKPL20
AMKPPP20
AMKBHI
BHI

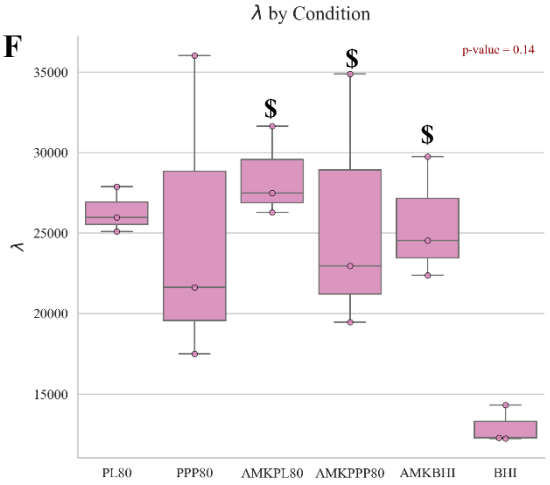

F
λ by Condition
p-value = 0.14
λ
PL80
PPP80
AMKPL80
AMKPPP80
AMKBHI
BHI

Figure 9: Effect of platelet lysate at two different concentrations with antibiotics on the growth kinetics of *Staphylococcus aureus.* Panels (A–C) show results for 20% concentrations (PL20 and PPP20), and panels (D–F) for 80% concentrations (PL80 and PPP80).(**A, D**) Carrying capacity (K), (**B,E**) maximum specific growth rate (μ_{max}), and (**C, F**) lag time (λ) of *S. aureus* under ten treatment conditions: PL20, 20% platelet lysate; PPP20, 20% platelet-poor plasma; AMKPL20, amikacin and 20% platelet lysate; AMKPPP20, amikacin and 20% platelet-poor plasma; PL80, 80% platelet lysate; PPP80, 80% platelet-poor plasma; AMKPL80, amikacin and 80% platelet lysate; AMKPPP80, amikacin and 80% platelet-poor plasma; AMKBHI, amikacin and brain heart infusion broth; BHI, brain heart infusion broth; as the control. Data from three biological replicates modeled using the Baranyi equation. Box plots show median, mean (circle), IQR, and range. Kruskal–Wallis test was used; p-values are shown in red. Significant differences ($p < 0.5$) were observed in panel A ($p = 0.080$), B ($p = 0.054$), C ($p = 0.02$), D ($p = 0.015$), E ($p = 0.127$), F ($p = 0.14$). $^{\$}$ $p < 0.5$ compared to BHI; * $p < 0.5$ compared to PL80; $^{\#}$ $p < 0.5$ compared to PPP80; $^{\%}$ $p < 0.5$ compared to PL20; $^{@}$ $p < 0.5$ compared to PPP20; $^{\&}$ $p < 0.5$ compared to PPP80; $^{\wedge}$ $p < 0.5$ compared to AMKBHI.

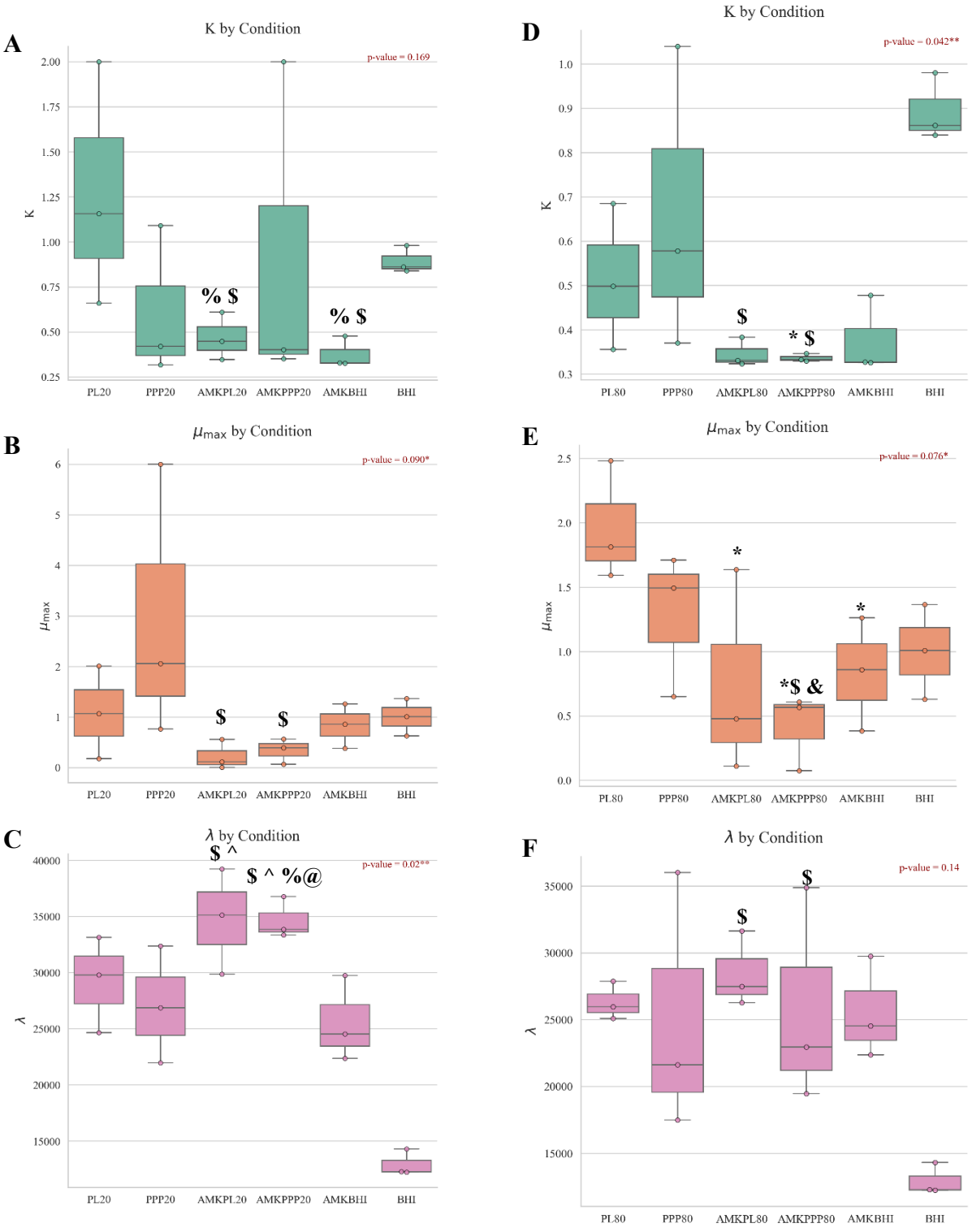

A
K by Condition
p-value = 0.169
K
PL20
PPP20
AMKPL20
AMKPPP20
AMKBHI
BHI
% $
% $
B
μmax by Condition
p-value = 0.090*
μmax
$
$
C
λ by Condition
p-value = 0.02**
λ
$ ^
$ ^ %@
D
K by Condition
p-value = 0.042**
PL80
PPP80
AMKPL80
AMKPPP80
$
* $
E
μmax by Condition
p-value = 0.076*
*
*$ &
*
F
λ by Condition
p-value = 0.14
$
$

Figure 10: Effect of platelet lysate at two different concentrations with antibiotics on the growth kinetics of *Staphylococcus pseudintermedius.* Panels (A–C) show results for 20% concentrations (PL20 and PPP20), and panels (D–F) for 80% concentrations (PL80 and PPP80).(**A, D**) Carrying capacity (K), (**B,E**) maximum specific growth rate (μ_{max}), and (**C, F**) lag time (λ) of *S. aureus* under ten treatment conditions: PL20, 20% platelet lysate; PPP20, 20% platelet-poor plasma; AMKPL20, amikacin and 20% platelet lysate; AMKPPP20, amikacin and 20% platelet-poor plasma; PL80, 80% platelet lysate; PPP80, 80% platelet-poor plasma; AMKPL80, amikacin and 80% platelet lysate; AMKPPP80, amikacin and 80% platelet-poor plasma; AMKBHI, amikacin and brain heart infusion broth; BHI, brain heart infusion broth; as the control. Data from three biological replicates modeled using the Baranyi equation. Box plots show median, mean (circle), IQR, and range. Kruskal–Wallis test was used; p-values are shown in red. Significant differences ($p < 0.5$) were observed in panel A ($p = 0.169$), B ($p = 0.090$), C ($p = 0.02$), D ($p = 0.042$), E ($p = 0.076$), F ($p = 0.14$)**.** $^{\$}$ $p < 0.5$ compared to BHI; * $p < 0.5$ compared to PL80; $^{\#}$ $p < 0.5$ compared to PPP80; $^{\%}$ $p < 0.5$ compared to PL20; $^{@}$ $p < 0.5$ compared to PPP20; $^{\&}$ $p < 0.5$ compared to PPP80; $^{\wedge}$ $p < 0.5$ compared to AMKBHI.

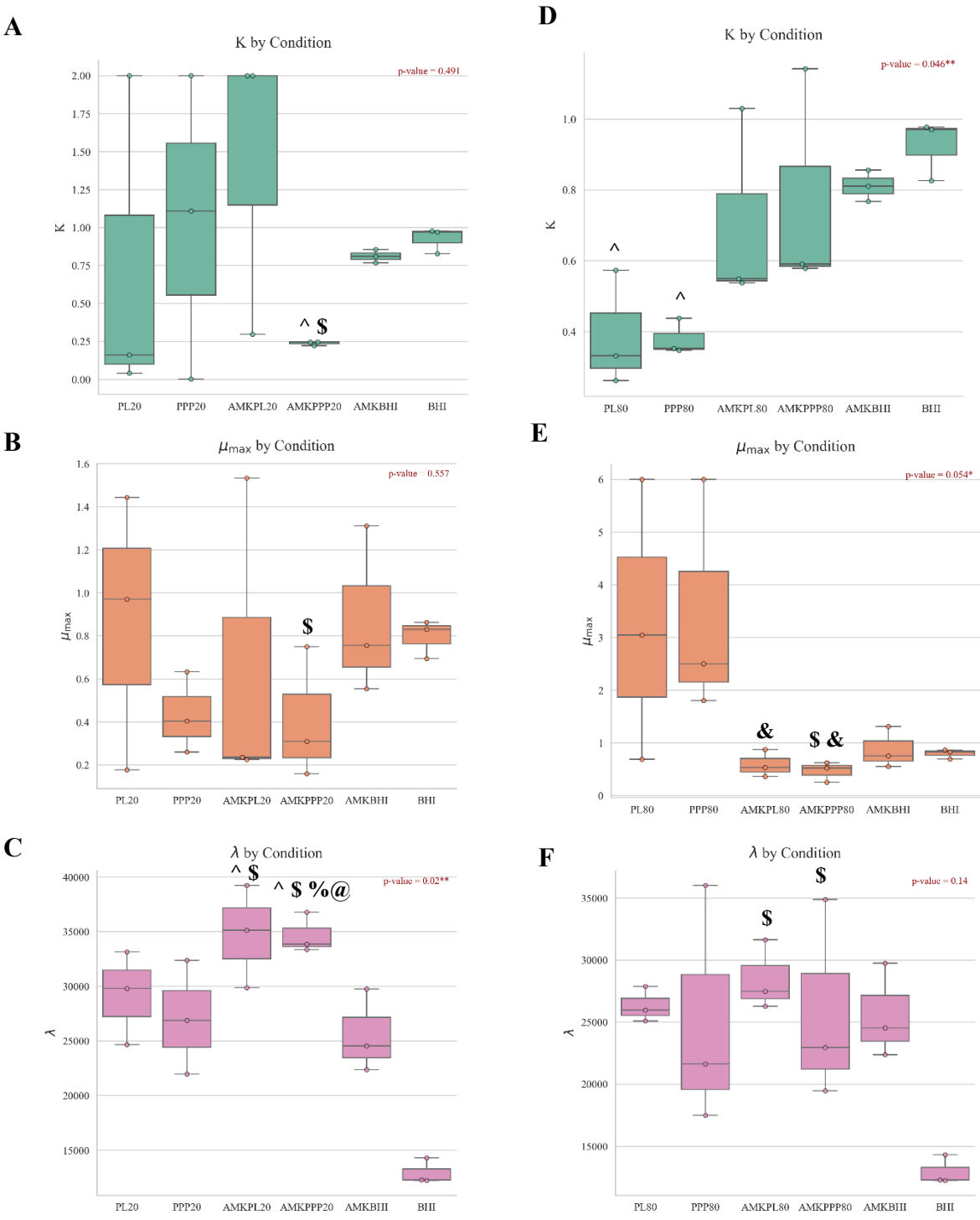

A
K by Condition
p-value = 0.491
K
PL20
PPP20
AMKPL20
AMKPPP20
AMKBHI
BHI
^ $
D
K by Condition
p-value = 0.046**
K
PL80
PPP80
AMKPL80
AMKPPP80
AMKBIII
BIII
^
^
B
μmax by Condition
p-value = 0.557
μmax
PL20
PPP20
AMKPL20
AMKPPP20
AMKBHI
BHI
$
E
μmax by Condition
p-value = 0.054*
μmax
PL80
PPP80
AMKPL80
AMKPPP80
AMKBHI
BHI
&
$ &
C
λ by Condition
p-value = 0.02**
λ
PL20
PPP20
AMKPL20
AMKPPP20
AMKBIII
BIII
^ $
^ $ %@
F
λ by Condition
p-value = 0.14
λ
PL80
PPP80
AMKPL80
AMKPPP80
AMKBIII
BIII
$
$

Figure 11: Effect of platelet lysate at two different concentrations with antibiotics on the growth kinetics of *Methicillin-Resistant Staphylococcus aureus.* Panels (A–C) show results for 20% concentrations (PL20 and PPP20), and panels (D–F) for 80% concentrations (PL80 and PPP80).(**A, D**) Carrying capacity (K), (**B,E**) maximum specific growth rate (μ_{max}), and (**C, F**) lag time (λ) of *S. aureus* under ten treatment conditions: PL20, 20% platelet lysate; PPP20, 20% platelet-poor plasma; AMKPL20, amikacin and 20% platelet lysate; AMKPPP20, amikacin and 20% platelet-poor plasma; PL80, 80% platelet lysate; PPP80, 80% platelet-poor plasma; AMKPL80, amikacin and 80% platelet lysate; AMKPPP80, amikacin and 80% platelet-poor plasma; AMKBHI, amikacin and brain heart infusion broth; BHI, brain heart infusion broth; as the control. Data from three biological replicates modeled using the Baranyi equation. Box plots show median, mean (circle), IQR, and range. Kruskal–Wallis test was used; p-values are shown in red. Significant differences ($p < 0.5$) were observed in panel A ($p = 0.491$), B ($p = 0.557$), C ($p = 0.02$), D ($p = 0.046$), E ($p = 0.054$), and F ($p = 0.14$). $^{\$}$ $p < 0.5$ compared to BHI; * $p < 0.5$ compared to PL80; $^{\#}$ $p < 0.5$ compared to PPP80; $^{\%}$ $p < 0.5$ compared to PL20; $^{@}$ $p < 0.5$ compared to PPP20; $^{\&}$ $p < 0.5$ compared to PPP80; $^{\wedge}$ $p < 0.5$ compared to AMKBHI.

Conclusion

The complex interaction between bacterial resistance mechanisms and compromised host responses makes wound healing, especially in the setting of chronic infections, a major clinical challenge in both human and veterinary medicine. Despite being revolutionary in the past, the widespread use of antibiotics has contributed to the alarming rise in antimicrobial resistance (AMR), a global health crisis linked to limited therapeutic options, increased mortality, and economic burden. Bacteria's capacity to form biofilms, which are organized microbial communities covered in protective matrices, exacerbates this problem by providing extra resistance to traditional antimicrobials and immune clearance. In veterinary wound care, where conventional topical treatments frequently fall short in eliminating resistant pathogens, these challenges are particularly troublesome.

Platelet lysate is an acellular product that can be offered as a promising substitute. It is due to its ability to provide a complex blend of growth factors, cytokines, antimicrobial peptides, and complement proteins in a low-immunogenic, stable, and affordable formulation. It has drawn attention due to its dual role in promoting wound healing and exerting antimicrobial effects in response to the growing need for alternative therapies. Growth factors, cytokines, and antimicrobial peptides (AMPs) are abundant in PL. They help platelets to damage bacterial membranes and obstruct the synthesis of cell walls

Previous studies demonstrated that the efficacy of PL can vary depending on its method of preparation, particularly in terms of leukocyte content and plasma retention**.** In Chapter 2, we showed that our canine platelet lysate regularly surpasses therapeutic thresholds and that, regardless of leukocyte concentration, plasma components, such as heat-sensitive complement

proteins, are essential for PL's antimicrobial activity against *S. aureus*, *E. coli*, and *S. pseudointermedius*. These results support and add to previous reports of PL's bacteriostatic and bactericidal properties.

Recent research has demonstrated that PL can inhibit the growth of multidrug-resistant strains like MRSA and may even have synergistic effects when combined with antibiotics in both human and veterinary contexts, including canine and equine models. In Chapter 3, kinetic modeling of bacterial growth further revealed that PL inhibits bacterial growth and delays the initial phase of growth, and when combined with amikacin, these effects are amplified without changing the rate of replication.