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Platelet enhancement of bacterial growth during room temperature storage: mitigation through refrigeration

Patrick M. Ketter,[1] Robin Kamucheka,[1] Bernard Arulanandam,[2] Kevin Akers,[1] and Andrew P. Cap[1]

INTRODUCTION: Due to high risk of septic transfusion reactions arising from bacterial contamination, US Food and Drug Administration regulations currently limit platelet storage to 5 days at room temperature (RT). However, blood culturing methods can take up to 7 days to detect bacteria, allowing transfusion of potentially contaminated units. Thus, cold storage (CS) may be a viable means of extending shelf life and improving safety.

STUDY DESIGN AND METHODS: Platelets and fresh plasma (FP) were collected by apheresis from healthy donors, aliquoted, and challenged with *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Staphylococcus epidermidis.* Aliquots were then stored at either RT or CS.

RESULTS: Significant ($p < 0.05$) bacterial growth was detected at RT for most bacteria as early as Day 1 after collection, with peak growth occurring between Days 3 and 4. Growth remained static during CS. Additionally, platelets appeared to enhance bacterial replication with growth significantly lower ($p < 0.05$) in FP relative to RT-stored platelets. Lactic acid promoted bacterial growth when added to FP at RT. Bacterial challenge also resulted in significantly increased platelet activation ($p < 0.05$) and significantly reduced platelet function ($p < 0.05$) in RT storage relative to uninfected controls by Day 5 after collection. Conversely, CS ablated bacteria growth, limited platelet metabolism, and preserved platelet function throughout the study.

CONCLUSION: These data suggest that CS presents an attractive alternative to RT to both extend storage life and reduce the risk of transfusion-related sepsis.

Septic transfusion reactions (STRs) remain a significant risk associated with platelet (PLT) administration.[1–4] Reports indicate that rates of bacterial contamination and STRs associated with PLT transfusions range from as low as 1 in 2000 transfused PLT units to as high as 1 in 750.[1,5,6] While PLTs are screened for bacterial contamination, there are significant deficiencies in bacterial detection.[1,2] Current AABB guidelines require only that blood banks and transfusion services collecting PLTs "have methods to limit and to detect or inactivate bacteria in all platelet components" (5.1.5.1) and that "detection methods shall either be cleared or approved by the FDA" (5.1.5.1.1).[2–4,6,7] However, many facilities only perform aerobic blood cultures on collected blood products for this purpose resulting in an inability

ABBREVIATIONS: CS = cold storage; FP = fresh plasma; RT = room temperature; STRs = septic transfusion reactions; TRAP-6 = thrombin receptor–activating peptide-6.

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This work was supported in part by the U.S. Army Medical Research and Materiel Command (USAMRMC), Combat Casualty Care Research Program and by appointment to the Postgraduate Research Participation Program at the US Army Institute of Surgical Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and USAMRMC.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

Received for publication January 11, 2019; revision received February 25, 2019, and accepted February 27, 2019.

doi:10.1111/trf.15255

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TRANSFUSION 2019;59;1479–1489

to detect anaerobic bacterial contaminants.[2,4] Furthermore, FDA-approved bacterial blood culturing systems often fail to detect contamination leading to STRs,[1,3,6,8] as there are no set standards for quarantine of blood products following collection to ensure sterility.[2] This has resulted in an increased number of STRs resulting from anaerobic bacteria such as *Clostridium perfringens*[8] and other slow-growing bacteria.[8–10] Although many blood centers and hospitals do quarantine PLTs for 12 to 24 hours before release,[1,2,4] many do not and release the units immediately after collection.[2] Even so, the 24-hour quarantine periods may be insufficient to adequately prevent STRs,[1] particularly in cases involving slow-growing bacteria[1,10] or bacteria that do not propagate well under PLT storage conditions.[11] In fact, a recent call for cases from The US government's Centers for Disease Control and Prevention Epidemic Information Exchange was released in July 2018 in response to two cases of *Acinetobacter baumannii* infections directly attributed to contaminated PLT units in California and Utah.

Currently, according to the FDA Code of Federal Regulations Title 21 (21CFR610.53), PLT storage is limited to 5 days at room temperature (RT; 21-24°C), creating a significant hurdle for modern bacterial blood culturing methodologies, which can take up to 7 days to detect potential contamination.[10] As a result, PLTs may be transfused before contamination is ultimately detected.[10] Recently, our laboratory has demonstrated that cold storage (CS; 4-6°C) of PLTs prolongs shelf life for periods in excess of 14 days[12] and preserves both PLT viability and hemostatic function,[12–18] allowing adequate time to quarantine units for the requisite 7 days necessary for bacterial culture. However, a longstanding criticism of PLT CS is that while it is assumed that CS would prevent bacterial growth, to our knowledge, no specific evidence has been reported for PLT components prepared by current procedures.

In this study, we addressed this concern by comparing the growth of five bacterial strains, commonly reported causes of STRs associated with PLT transfusions,[1] that were directly inoculated into apheresis PLTs and plasma samples, then held at either RT or refrigerated.

MATERIALS AND METHODS

Bacteria

The gram-negative bacterial isolates *A. baumannii* clinical isolate 79, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27317, were graciously provided by Dr. Bernard Arulanandam at the University of Texas at San Antonio. Dr. Kevin Akers at the US Army Institute of Surgical Research at Fort Sam Houston graciously provided the gram-positive bacterial isolates *Staphylococcus aureus* UAMS-1 and *Staphylococcus epidermidis* ATCC 49619. Frozen bacteria aliquots were cultured onto either LB-Lennox agar (US Biological Life Sciences) for culture of gram-negative isolates, or tryptic soy agar (US Biological Life Sciences) for culture of gram-positive isolates. Isolated colonies from each culture were then subcultured to matching broth media (US Biological Life Sciences) and grown overnight at 37°C. Overnight cultures were diluted 1:100 in fresh broth media and grown for 2 to 3 hours at 37°C. The optical density, measured at a wavelength of 600 nm (OD_{600}), of each sample was assessed, and samples were summarily diluted to an OD_{600} corresponding to desired bacterial concentrations.

Collection of platelets and fresh plasma

PLTs and fresh plasma (FP) were collected from healthy volunteers by apheresis using an automated blood collection system (Trima Accel, Terumo BCT) according to an approved US Army Institute of Surgical Research Standard Operating Procedure. All units were collected into acid-citrate-dextrose anticoagulant. FP and fresh frozen plasma were used interchangeably for experiments depending on availability. No personal identification was collected as part of this study.

Challenge assays

PLTs and FP were inoculated with indicated bacteria to deliver a final concentration of 1000 CFU/mL, approximately 100-fold higher than the limit of detection for bacterial enumeration by colony counting, to facilitate accurate and reproducible results. Once inoculated, 10 mL of either PLTs or FP were transferred to PLT storage minibags (pH SAFE, Blood Cell Storage, Inc.). PLT samples were incubated for 5 days at either 21°C ± 1°C (RT) with gentile agitation on an orbital shaker set to 60 rpm, or refrigerated at 4°C (CS) with no agitation. FP samples were stored at RT only. Sampling was performed on each sample on Day 0 and every day thereafter using a 1-mL syringe. Samples collected on Day 0 were collected prior to aliquoting. Approximately 0.6-mL samples were collected on Days 1, 3, and 5, while 0.3-mL samples were collected on Days 2 and 4 directly from pH Safe minibags. Thus, the total volume within the pH Safe minibag was reduced approximately 24% by Day 5 of the study. Dilution plating for bacterial enumeration was performed daily, as was lactate measurement by a point-of-care analyzer (iSTAT CG4+ test cartridge, Abbott Laboratories), and glucose monitoring by blood glucose meter (AimStrip Plus, Germaine Laboratories). Flow cytometry was performed on PLT aliquots collected on Days 0, 1, 3, and 5. Platelet aggregation assays using thrombin receptor–activating peptide-6 (TRAP-6) agonist (TRAP Test, Diapharma) were performed with a multiplate analyzer (Model No. MP0010, Diapharma) in parallel with flow cytometry.

Lactic acid add-Back

L-lactic acid (Sigma Aldrich) was dissolved in FP to achieve concentrations of 2, 1.5, 1, and 0.5 M. Lactic acid solutions were subsequently diluted 1:100 in FP to achieve the desired final concentrations of 20, 15, 10, and 5 mM,

respectively. Lactic acid concentrations were confirmed by iSTAT point-of-care analyzer through measurement of lactate as described previously. FP supplemented with lactic acid at the specified concentrations were then challenged with bacteria as indicated previously at concentrations of 1000 CFU/mL.

Flow Cytometry

PLT aliquots were diluted 10-fold in 0.1 μm filtered Hank's Buffered Salt Solution. Diluted PLTs were then incubated with an antibody cocktail consisting of anti-CD42b (Clone HIP1, BioLegend) to identify the PLT population and anti-CD62P (Clone AK-4, BD Biosciences) to assess PLT activation. Samples were run on a flow cytometer (FACSCanto II, BD Biosciences).

Statistics

Data presented are cumulative of at least four individual experiments, each performed in duplicate. PLTs and FP were collected from a single donor for each experiment assessing differences in bacterial growth in the presence or absence of live PLTs. Lactic acid add-back experiments were performed separately from PLT studies and required additional donors for each experiment. In total, 8 volunteers provided PLTs and FP for these studies.

For each individual experiment, all challenge groups were prepared in duplicate, and assessment of bacterial growth, plasma lactate, plasma glucose, and flow cytometry were performed on each as indicated. In an effort to limit sample volumes, approximately 250 to 300 μL was pooled from each duplicate sample for measurement of PLT aggregation.

Error bars represent standard error of the mean (SEM). Statistical differences between groups were assessed by t test. Pearson correlation coefficients were calculated where indicated. All statistical analyses were performed using computer software (Prism 7, GraphPad Software).

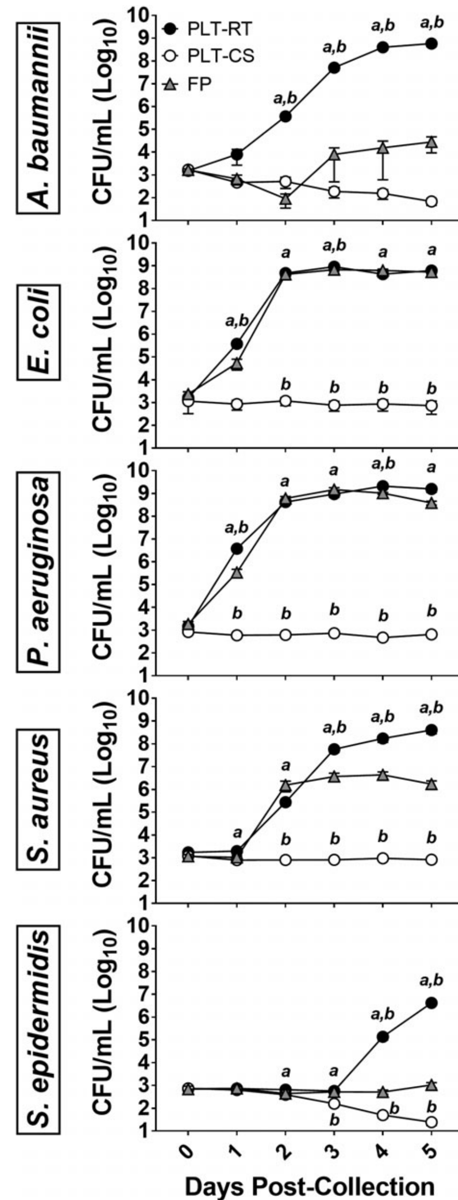

Fig. 1. Bacterial growth is enhanced in the presence of PLTs at room temperature. PLTs or FP were challenged with *A. baumannii, E. coli, P. aeruginosa, S. aureus,* or *S. epidermidis.* PLTs challenged with indicated bacterial isolates were stored at room temperature (PLT-RT) or refrigerated (PLT-CS). Error bars = SEM. Significance between groups determined by t test at each time interval (*a* = significantly different than CS; *b* = significantly different than FP).

RESULTS

Bacterial growth

We initially evaluated bacterial growth in PLTs stored at RT or CS for up to 5 days after collection as per current FDA guidelines. Significant differences in bacterial growth ($p < 0.05$) were detected as early as Day 1 after collection for all gram-negative isolates and Day 2 after collection for both gram-positive isolates (Fig. 1). As expected, growth at RT was significantly greater than that observed in CS samples, with growth in CS samples largely static throughout. Interestingly, despite reports of antimicrobial activity associated with PLT-rich plasma,[19–22] no obvious antimicrobial activity was observed in either RT or CS samples. Because metabolic activity is reduced with refrigeration, we sought to determine if metabolic by-products may contribute to bacterial growth as well. Thus, we repeated the experiment in FP lacking PLTs (Fig. 1). All gram-negative isolates exhibited a significant defect in bacterial growth 24 hours after collection in the absence of metabolically active platelets ($p < 0.05$). Although growth recovered in samples challenged with *E. coli* and *P. aeruginosa* by Day 2 after collection, growth remained largely static throughout in FP samples

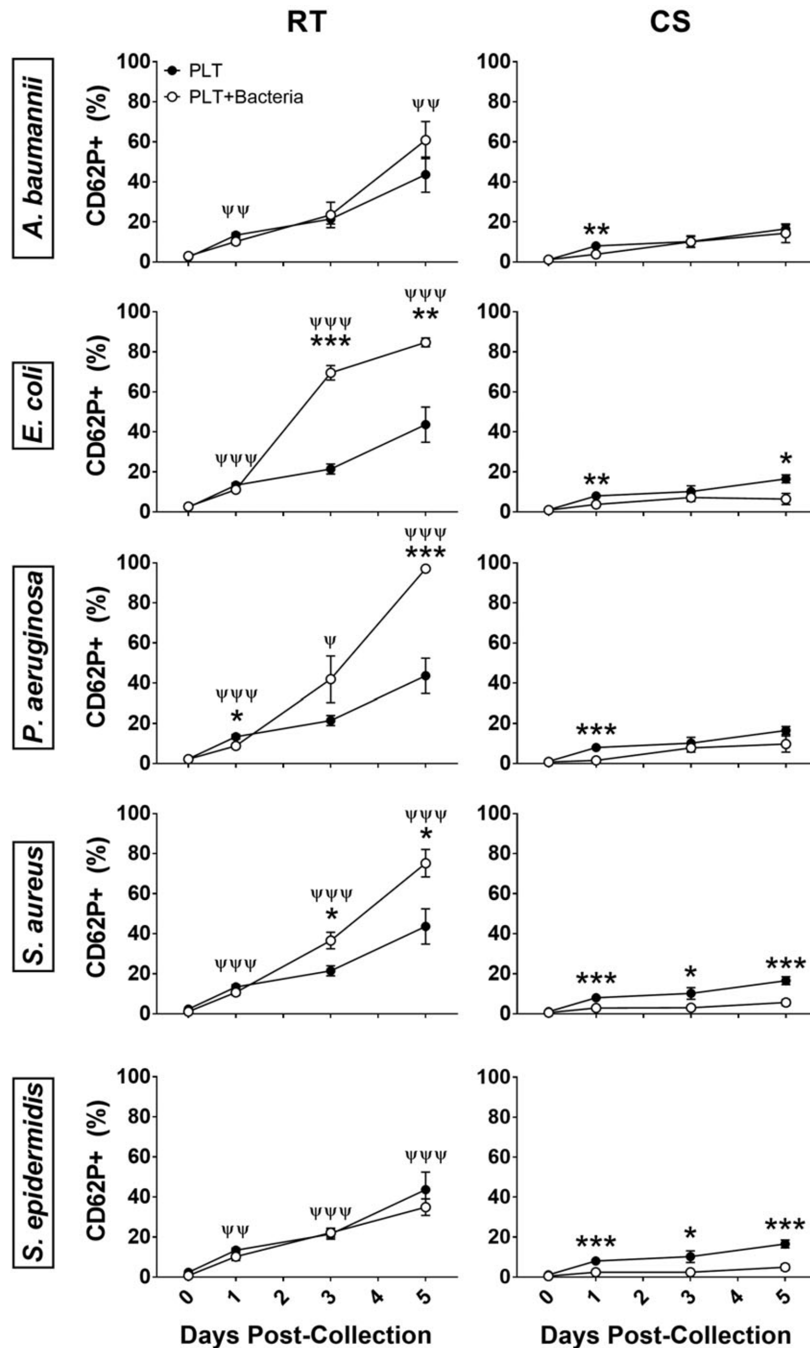

Fig. 2. Cold storage reduces PLT activation in contaminated PLT units. PLTs were challenged with the indicated bacteria and stored at RT or CS. Platelet activation was assessed by flow cytometry detection of CD62P expression. For each graph: uninfected control platelets = PLT; platelets challenged with bacteria = PLT + Bacteria. Error bars = SEM. Significance between groups determined by t test at each time interval. (Differences between PLT and PLT + Bacteria: *p < 0.05; **p < 0.005; ***p < 0.0005. Differences between RT and CS PLT + Bacteria: ψ p < 0.05; ψψ p < 0.005; ψψψ p < 0.0005.)

challenged with *A. baumannii.* Similarly, *S. epidermidis* exhibited a similar trend in the absence of PLTs. Bacterial growth proceeded largely unhindered in FP challenged with *S. aureus* through Day 2 after collection. However, bacterial growth was reduced over 10-fold in FP samples on Days 3 through 5 after collection.

PLT activation

Our laboratory has previously shown that PLTs are activated over time in CS relative to RT.[13–15,17] However, we observed that CS significantly mitigated PLT activation following bacterial challenge with all samples as early as day 1 post-collection with respect to their RT counterparts ($p < 0.05$; Fig. 2).

Furthermore, PLT activation is significantly increased in PLTs challenged with *E. coli*, *P. aeruginosa*, and *S. aureus* by Day 5 after collection relative to uninfected controls at RT ($p < 0.05$). However, despite a detectible increase in bacterial growth, neither *A. baumannii* nor *S. epidermidis* appeared to increase PLT activation at RT relative to uninfected controls to a significant degree.

PLT function

Although PLT aggregation following TRAP-6 stimulus was significantly reduced in CS controls relative to those stored at RT on days 3 and 5 post-collection ($p < 0.05$), CS appeared to preserve PLT function overall following bacterial challenge. PLT aggregation was significantly reduced in RT samples challenged with *E. coli*, *P. aeruginosa*, and *S. aureus* relative to their CS counterparts on Days 3 and 5 after challenge ($p < 0.05$; Fig. 3). Similarly, PLT aggregation was significantly reduced in *E. coli*, *P. aeruginosa*, and *S. aureus* challenged PLT samples relative to uninfected controls at RT on Days 3 and 5 after challenge as well ($p < 0.0005$). While both *A. baumannii* and *S. epidermidis* exhibited significantly reduced platelet aggregation in CS samples relative to their RT counterparts, no significant differences were detected with respect to PLT aggregation in PLT samples challenged with either bacteria relative to uninfected controls at either RT or CS.

PLT glucose consumption

As we observed previously, the presence of PLTs in plasma stored at RT appeared to contribute to bacterial growth. Thus, we assessed PLT metabolism first through glucose consumption. Not surprisingly, glucose consumption was undetectable with CS irrespective of challenge status, while uninfected PLT samples exhibited a gradual decline in plasma glucose indicative of glucose metabolism at RT (Fig. 4). In PLTs challenged with bacteria at RT, significant declines in plasma glucose levels were observed by Day 2 after collection in PLTs challenged with either *E. coli* or *P. aeruginosa* ($p < 0.05$), and Day 3 in PLTs challenged with either *S. aureus* or *A. baumannii* relative to uninfected controls. Glucose consumption appeared to be largely unaffected in PLTs challenged with *S. epidermidis.*

PLT lactate production

In addition to glucose consumption, we also assessed lactate production as a measure of PLT metabolism. While measured lactate levels did rise slightly over the 5-day observation period in CS, levels never exceeded 10 mM in any of the samples, and no significant differences were observed between PLTs challenged with bacteria and uninfected controls (Fig. 5). However, measured lactate levels were significantly ($p < 0.05$) higher in all PLT samples at RT. This rise in lactate had the inverse effect of lowering plasma pH and pO_2 in all challenge groups over the course of the study (Fig. SS1, available as supporting information in the online version of this paper). No significant differences in lactic acid/lactate production were observed between *A. baumannii*, *S. aureus*, or *S. epidermidis* and uninfected controls at RT. However, measured lactate levels in PLTs challenged with *E. coli* at RT increased through Day 2 after collection before rapidly dropping, with significant reductions in plasma lactate detected on Days 4 and 5 after collection relative to uninfected controls ($p < 0.05$). Curiously, despite reported lactic acid/lactate utilization mechanisms,[23–25] lactate levels were significantly increased in PLTs challenged with *P. aeruginosa* beginning on Day 3 after collection and continuing through Day 5 relative to uninfected controls at RT ($p < 0.05$). Furthermore, while most RT-stored PLT samples exhibited a maximum lactate level of around 20 mM, lactate levels in PLTs challenged with *P. aeruginosa* consistently exceeded 30 mM by Day 5 after collection.

In all challenge groups, lactate production significantly correlated with bacterial growth: *A. baumannii* ($r = 0.6424$; $p < 0.0001$); *E. coli* ($r = 0.6415$; $p < 0.0001$); *P. aeruginosa* ($r = 0.7128$; $p < 0.0001$); *S. aureus* ($r = 0.3676$; $p < 0.001$); and *S. epidermidis* ($r = 0.5235$; $p < 0.0001$).

Bacterial growth following addition of lactic acid

Because lactic acid/lactate is the primary metabolic by-product produced by platelets during RT storage, we next sought to determine if it may contribute to the growth of the bacteria. In all cases but one, any defect originally observed in bacterial growth associated with FP was corrected through addition of lactic acid (Fig. 6). Growth of *A. baumannii* in FP supplemented with lactic acid proceeded in a dose-dependent fashion, as did growth of *S. epidermidis.* Growth of *E. coli* was recovered in FP on Day 1 after challenge following addition of lactic acid in excess of 15 mM, while any concentration from 5 to 20 mM lactic acid appeared to restore growth of *P. aeruginosa.* Growth of *S. aureus* in FP supplemented with lactic acid at concentrations of 10 mM or greater significantly increased bacterial growth relative to both FP alone and in PLTs on Day 1 after collection ($p < 0.05$). However, deficits in bacterial growth similar to that observed in FP alone relative to PLTs at RT remained on Days 3 and 5 after collection.

DISCUSSION

STRs are the most common, severe risks associated with PLT transfusion occurring at a rate of approximately 1 of every 2000 units transfused.[1,5,6] The risk of infection arising from PLT transfusion can be directly linked to storage of platelets at RT.[10] However, while it has been assumed that CS would mitigate this risk, to date few, if any, studies have addressed this key point.

Importantly, the bacterial challenge dosage used in this study was grossly exaggerated. As most blood product contamination arises from skin contamination due to inadequate sterilization of the puncture site, the expected bacterial inocula would be significantly less than the 1000 CFU/mL

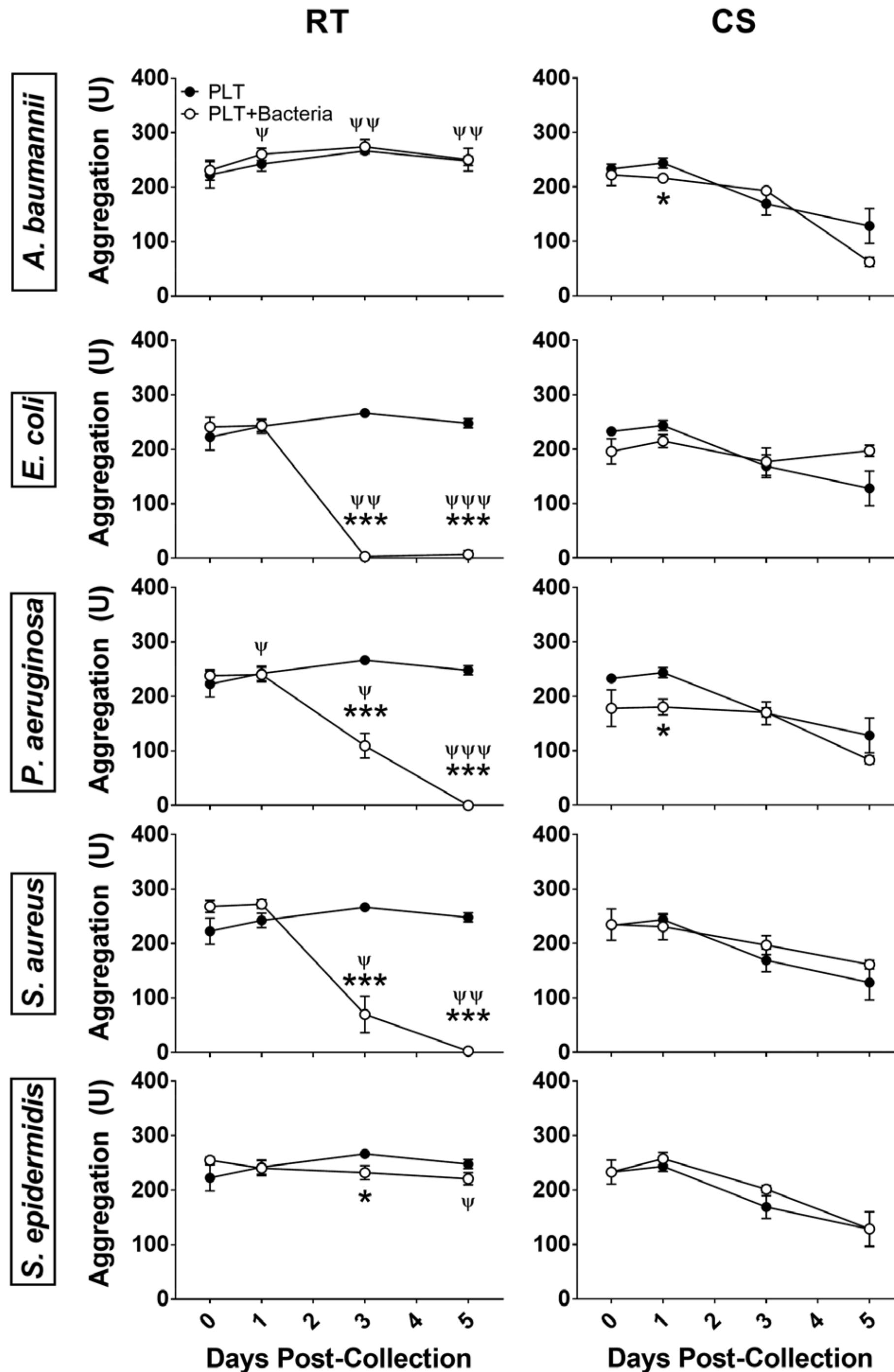

Fig. 3. CS preserves platelet function despite bacterial contamination. PLTs were challenged with the indicated bacteria and stored at RT or CS. Platelet aggregation measured by multiplate following TRAP stimulus. For each graph: uninfected control platelets = PLT; platelets challenged with bacteria = PLT + Bacteria. Error bars = SEM. Significance between groups determined by t test at each time interval. (Differences between PLT and PLT + Bacteria: *p < 0.05; **p < 0.005; ***p < 0.0005. Differences between RT and CS PLT + Bacteria: ψ p < 0.05; ψψ p < 0.005; ψψψ p < 0.0005.)

utilized in this study. However, we have observed that decreased inoculum size only delayed the growth plateau, and not the overall rate of growth (Fig. S2, available as supporting information in the online version of this paper). While each strain studied exhibited unique characteristics with regard to growth and their effects on stored PLTs, we consistently observed that CS lessened the storage lesion resulting from bacterial contamination. Not only was bacterial

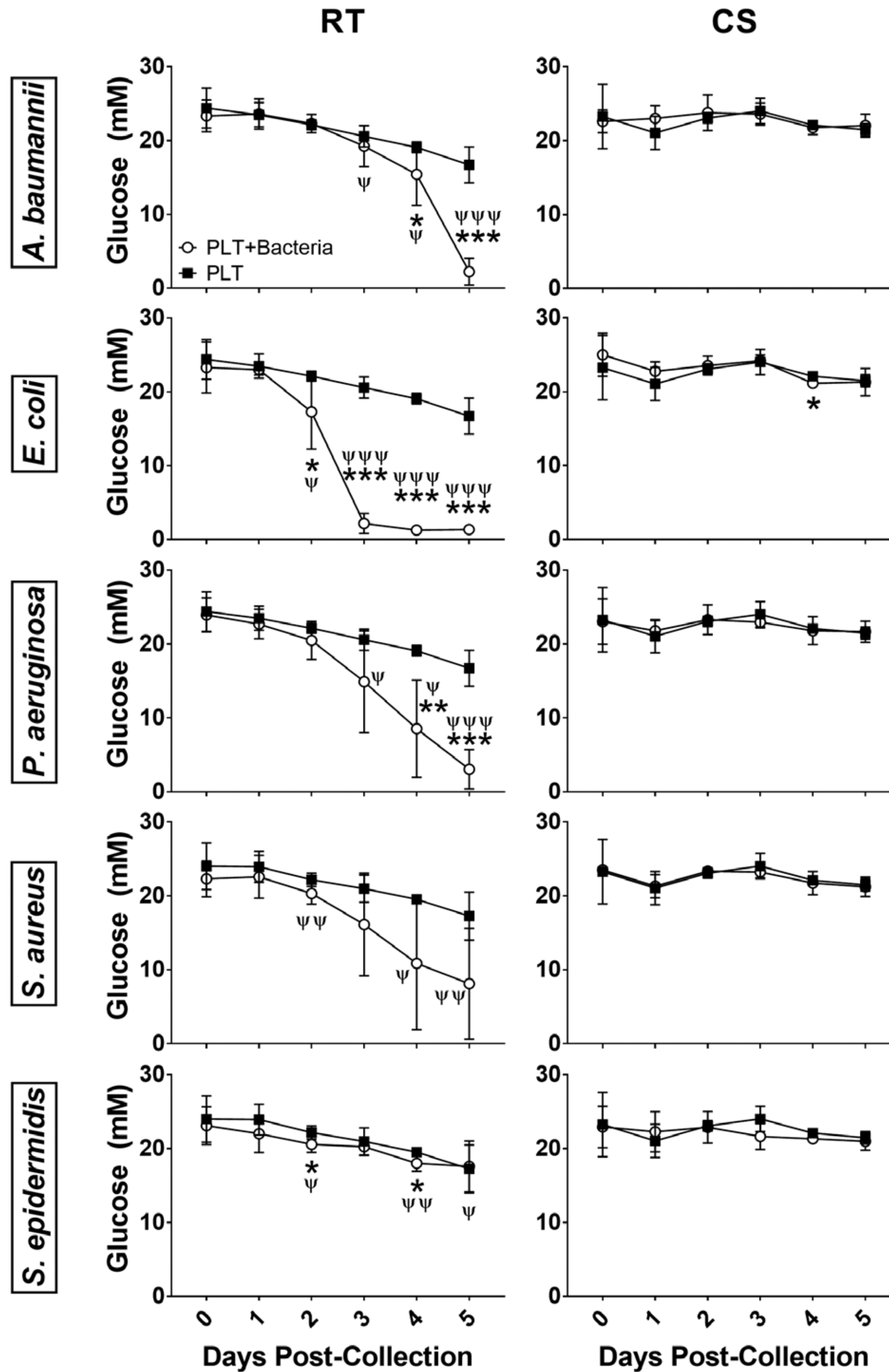

Fig. 4. CS limits glucose consumption. PLTs were challenged with the indicated bacteria and stored at RT or CS. Plasma glucose was monitored daily. For each graph: uninfected control platelets = PLT; platelets challenged with bacteria = PLT + Bacteria. Error bars = SEM. Significance between groups determined by t test at each time interval. (Differences between PLT and PLT + Bacteria: *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. Differences between RT and CS PLT + Bacteria: ψ $p < 0.05$; ψψ $p < 0.005$; ψψψ $p < 0.0005$.)

growth effectively inhibited in every case (Fig. 1), but we also observed a significant reduction in PLT activation over time (Fig. 2). As a result, PLT function, as measured through aggregation following TRAP-6 stimulation, was largely preserved in CS samples over time relative to their RT counterparts (Fig. 3).

When we then examined the effect of PLT metabolism on bacterial growth, we found that bacteria grown in FP lacking PLTs exhibited significant growth defects (Fig. 1). While the 10-fold reduction in bacterial growth observed with *S. aureus* could be directly attributed to declining plasma glucose concentrations, the same could not be said for

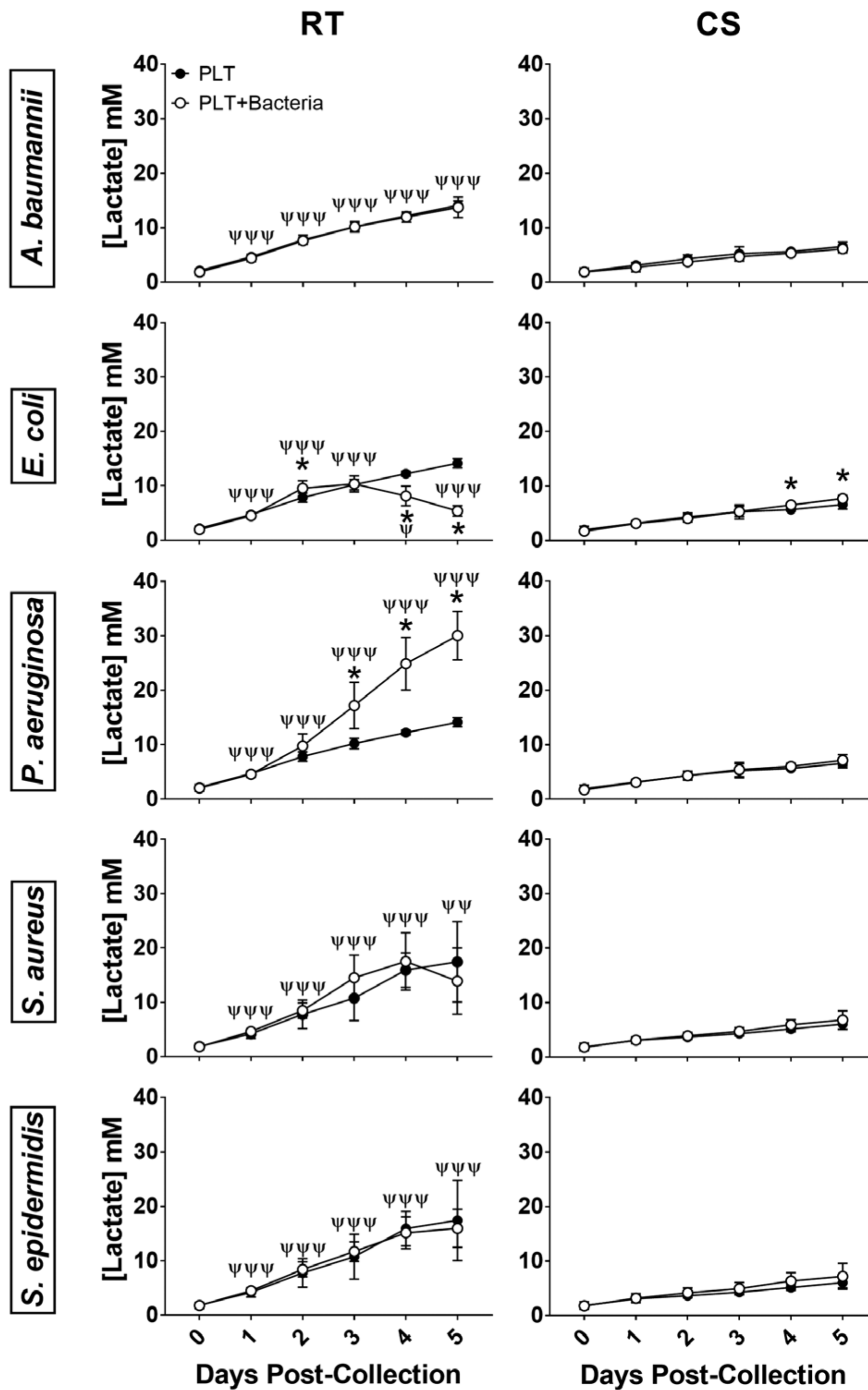

Fig. 5. CS limits lactate availability for bacterial growth. PLTs were challenged with the indicated bacteria and stored at RT or CS, and lactate levels were assessed daily. For each graph: uninfected control platelets = PLT; platelets challenged with bacteria = PLT + Bacteria. Error bars = SEM. Significance between groups determined by t test at each time interval. (Differences between PLT and PLT + Bacteria: *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. Differences between RT and CS PLT + Bacteria: ψ $p < 0.05$; ψψ $p < 0.005$; ψψψ $p < 0.0005$.)

deficiencies observed with any of the other strains used in this study (Fig. 4). When we next examined lactate levels in PLTs stored at RT, we found that measured lactate levels directly correlated with bacterial growth observed in all PLT samples. Curiously, although all samples exhibited correlations between growth and measured lactate levels, only

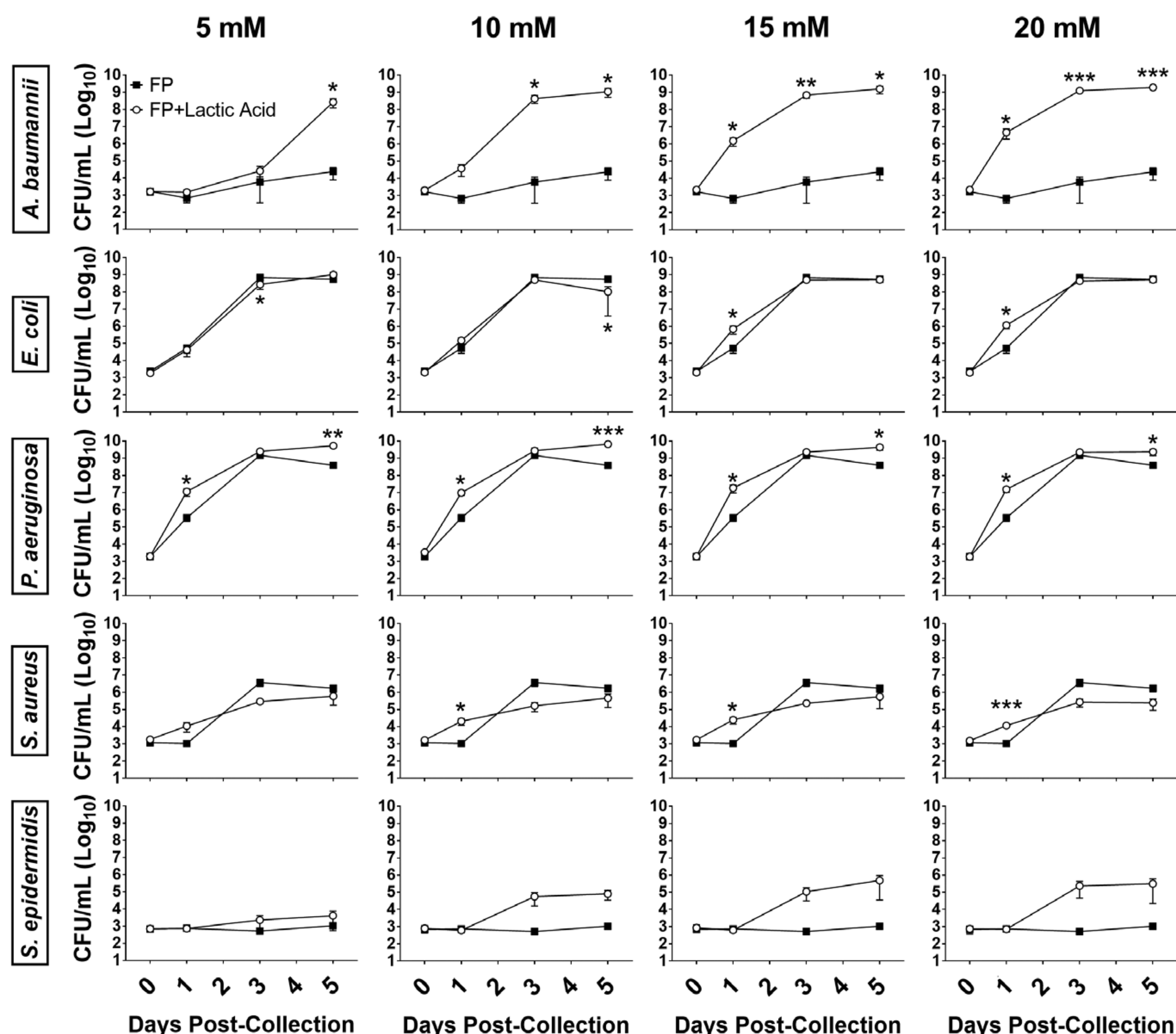

Fig. 6. Bacterial growth in FP following addition of lactic acid. FP was supplemented with 5 mM, 10 mM, 15 mM, or 20 mM lactic acid and challenged with the indicated bacteria. All samples were incubated at RT and bacterial growth was assessed daily. For each graph: bacterial growth in FP without lactic acid (FP), bacterial growth in FP with lactic acid (FP + Lactic Acid). Corresponding lactic acid concentrations listed at top each column. Error bars = SEM. Significance between groups determined by t test at each time interval (*p < 0.05; **p < 0.005; ***p < 0.0005).

E. coli exhibited clear signs of lactic acid/lactate utilization as levels dropped precipitously between Days 3 through 5 after collection (Fig. 5, RT *E. coli*). This drop was immediately preceded by a significant reduction in plasma glucose (Fig. 4, RT *E. coli*), suggesting a conversion from glucose to lactic acid/lactate utilization. A similar, though nonsignificant, trend can be seen with measured lactate levels in PLTs challenged with *S. aureus* on Day 5 after collection (Fig. 5, RT *S. aureus*), which was again preceded by a downward trend in plasma glucose (Fig. 5, RT *S. aureus*). Interestingly, although we observed a significant reduction in plasma glucose on Days 4 and 5 relative to uninfected control at RT (Fig. 4, RT *A. baumannii*), measured lactate levels in RT *A. baumannii* samples overlap with no significant differences detected (Fig. 5, RT *A. baumannii*). However, the fact that we measured no increased lactate levels on Days 4 and 5, despite the significant decline in plasma glucose, would suggest that *A. baumannii* may consume the excess lactic acid/lactate to facilitate bacterial growth. Neither *P. aeruginosa* nor *S. epidermidis* exhibited similar trends.

Given that *E. coli, S. aureus,* and *A. baumannii* exhibited signs of lactic acid/lactate utilization, we next sought to determine if addition of lactic acid to FP could restore bacterial growth (Fig. 6). As expected, lactic acid restored bacterial

growth in FP to levels comparable to that observed with PLTs with samples challenged with *E. coli* and *A. baumannii*. Surprisingly, while addition of lactic acid to FP did result in an increase in bacterial growth at Day 1 after collection in *S. aureus* challenged samples, the 10-fold reduction in growth observed in FP alone persisted. Additionally, addition of lactic acid to FP challenged with *P. aeruginosa* significantly increased bacterial growth at Day 1 after collection, similar to that observed with *E. coli*, despite no obvious signs of lactic acid/lactate utilization otherwise. Similarly, while PLTs challenged with *S. epidermidis* never exhibited significant changes in either glucose or measured lactate levels, growth of *S. epidermidis* progressed in a dose-dependent fashion relative to lactic acid concentration similar to that observed with *A. baumannii*.

Although it has been long assumed that CS would mitigate bacterial contamination and reduce the risk of STR through inhibition of bacterial growth, to our knowledge, this is the first study to directly demonstrate this key advantage. Furthermore, despite changes observed at RT, we consistently observed that PLTs kept at CS exhibit not only significantly reduced bacterial growth but also reduced storage-related PLT activation, glucose consumption, and lactic acid/lactate production while maintaining aggregation function. Meanwhile, PLTs stored at RT exhibited increased metabolism, resulting in much higher levels of lactic acid/lactate production. This ultimately appeared to contribute directly to the enhanced growth of the bacteria we observed in PLTs relative to FP samples under RT storage conditions. Thus, CS would greatly improve the safety of the product. Additionally, the ability to store PLTs and whole blood under the same conditions would eliminate the need for separate PLT incubators and shakers. This, in combination with the extended shelf life possible with CS, would mitigate waste and significantly reduce the costs associated with the use of PLTs, making CS an attractive alternative to the currently mandated RT storage standard.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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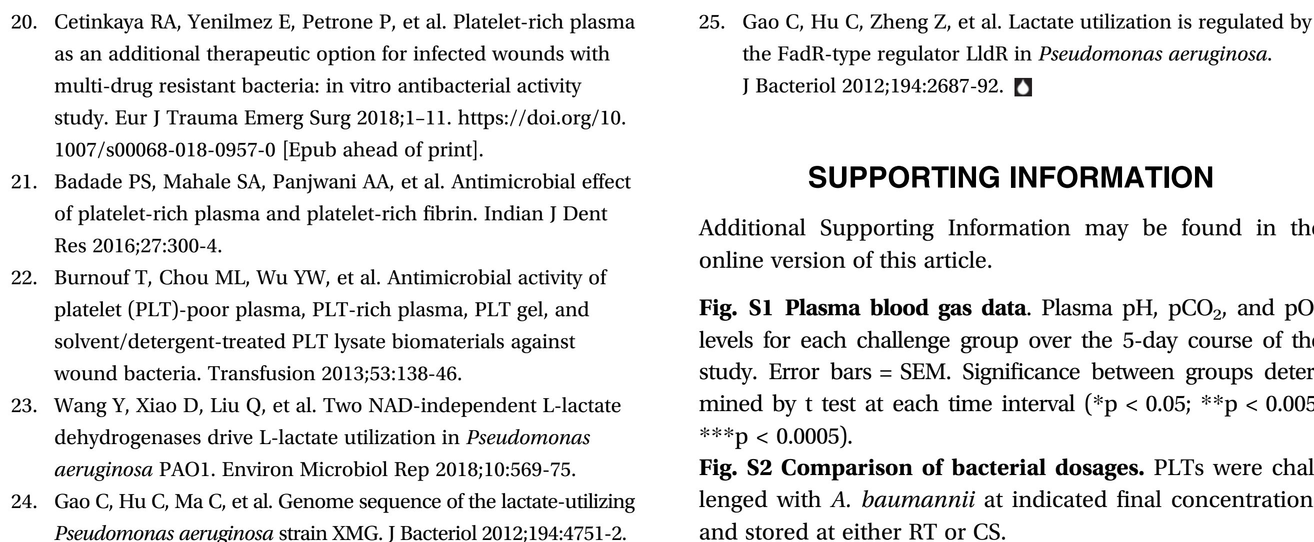

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Plasma blood gas data. Plasma pH, pCO_2, and pO_2 levels for each challenge group over the 5-day course of the study. Error bars = SEM. Significance between groups determined by t test at each time interval (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$).

Fig. S2 Comparison of bacterial dosages. PLTs were challenged with *A. baumannii* at indicated final concentrations and stored at either RT or CS.