

RESEARCH ARTICLE

Synovial fluid NMR-based metabolomics in septic and aseptic revision total knee arthroplasty: Implications on diagnosis and treatment

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Abstract

Periprosthetic joint infection (PJI) is one of the most challenging complications following total knee arthroplasty. Despite its importance, there is a paucity of reports in the literature regarding its pathogenesis. Recently, cellular metabolic reprogramming has been shown to play an important role in the progression and outcome of infectious diseases. Therefore, the aim of this study was to evaluate the metabolites composition of the synovial fluid from patients with PJI or aseptic failure of total knee arthroplasties. The synovial fluids from 21 patients scheduled for revision total knee arthroplasty (11 with the diagnosis of PJI and 10 with aseptic failures) were analyzed using 1D ¹H NMR spectroscopy. Univariate and multivariate statistical analyzes were used to identify metabolites that were differentially abundant between those groups. A total of 28 metabolites were identified and five of them found to be differentially abundant between infected and non-infected synovial fluids. Lactate, acetate and 3-hydroxybutyrate were found to be in a higher concentration, and glucose and creatine were found reduced in the synovial fluid from PJI patients. Synovial fluid from patients with PJI exhibit a distinct metabolic profile, possibly reflecting metabolic adaptation that occurs in the infected periprosthetic microenvironment. Further research and studies are warranted to gain a broader insight into the metabolic pathways engaged by both pathogen and immune cells in the context of a PJI.

KEYWORDS

metabolomics, periprosthetic joint infection, synovial fluid, total knee arthroplasty

1 | INTRODUCTION

Periprosthetic joint infection (PJI) is a challenging complication after total knee arthroplasty. Although its incidence remains stable, the high demand for knee arthroplasty leads to an increase in the absolute number of PJIs.¹ Nowadays, PJI is referred to as the leading cause of knee arthroplasty revision surgery, and is associated with

morbidity and mortality, resulting in a significant degree of physical impairment and increased healthcare costs.²

In PJI microenvironment, a complex interaction between the infecting pathogen and components of the host immune system takes place, resulting in cellular and molecular changes that reflect the state of infection and may be used for PJI diagnosis as well as for monitoring treatment response.^{3,4} However, despite its importance

and the growing body of knowledge focusing on its treatment, microbiological profile and diagnosis, there is a paucity of information about its pathogenesis.

Most data on the pathophysiology of PJIs derive from reductionist approaches that seek to investigate the role of a single isolated component in a larger biological system.⁵ However, infectious diseases are particularly complex as they involve the consideration of two main components, the host and the pathogen, thus requiring a thorough understanding of the connection between the parties to have a comprehensive knowledge of them.⁶

Metabolomics, which refers to the determination and quantification of metabolites within a biological system, is a high-throughput methodology that can aid in the discovery of disease mechanisms and biomarkers.⁷ Such an approach may reveal several aspects of the intricate connection among pathogen, host immune response and energy metabolism. Metabolomics is a central omics in the flux of information since metabolites pools reflect the instantaneous status of a biological system in conditions either with health or with disease.⁸ However, this has not been explored in relation to PJI. Therefore, the aim of this study is to use NMR spectroscopy to compare the metabolic profile of synovial fluids from patients with PJI with those from aseptic failures.

2 | METHODS

This study was conducted in a tertiary healthcare center specialized in high-complexity orthopedic surgery and was approved by the Human Ethics Committee of the *Instituto Nacional de Traumatologia e Ortopedia Jamil Haddad* (INTO), Rio de Janeiro, Brazil. All patients provided an informed consent for the purpose of this study. Synovial fluid was collected from patients undergoing knee arthroplasty revision surgery. Exclusion criteria included systemic inflammatory disease, insufficient synovial fluid volume, single positive microbiological culture and lack of appropriate clinical and laboratorial data to confirm or exclude a PJI.

All participants were evaluated according to the International Consensus Meeting on Musculoskeletal Infection 2013 (ICM) criteria.⁹ Synovial fluid was obtained during the revision procedure, before the arthrotomy. Three to five millilitres were drawn into vials for aerobic and anaerobic cultures (Becton, Dickinson and Company). Culture dishes were held for 14 days and discarded after turning into positive results according to a laboratory protocol. In addition to that, five to seven periprosthetic tissue samples were also harvested and sent for microbiological analyzes. Histopathological analyzes were also performed on periprosthetic membranes.

The cohort of participants for the study was divided into two groups. The infection group was composed of patients with two or more positive cultures of periprosthetic tissue with growth of the same pathogen. Patients in the non-infection group displayed 3 or more of the minor criteria, except for the single positive culture, that was an exclusion criteria.

During the study period, from January 2020 to April 2021, 36 patients were considered potentially eligible for the study. Of these,

15 patients were excluded due to acute PJI (3), chronic inflammatory disease (2), lack of adequate clinical and laboratory data (4) and insufficient synovial fluid volume (4). Therefore, the study population consisted of 21 patients: 11 with PJI and 10 with aseptic failure. Patient demographics is summarized Table 1. Infected and non-infected groups did not differ regarding age, body mass index, and comorbidities. The identified pathogens and their respective frequencies are displayed in Table 2. Two patients had polymicrobial infections (*Staphylococcus aureus* and *Enterococcus faecalis*; and *S. aureus*, *Klebsiella aerogenes* and *S. epidermidis*). Within the infected cohort, 4 acute and 7 chronic PJIs were identified. No patients in the infected group presented with fistula.

Synovial fluid was collected before the arthrotomy. An aliquot of the collected synovial fluid was transferred to Ethylene Diamine Tetra Acetic acid containing tubes (Becton Dickinson), centrifuged at 4800g for 10 min, and the cell-free supernatant was aliquoted in sterile tubes and stored at -80°C until further analysis. The samples were kept frozen for a maximum of 6 months until the preparation and performance of the analysis by Proton NMR spectroscopy.

The sample preparation was done as described previously.¹⁰ Briefly, a sample of synovial fluid was thawed at room temperature and immediately centrifuged at 6000g for 40 min at 4°C for additional removal of debris and protein aggregates. Then, 500 μL of the supernatant were mixed with 1 mL of sodium phosphate buffer solution (50 mM) with pH 7.4, containing 100% deuterated water (Cambridge Isotope Laboratories, Inc.). To obtain a macromolecule-depleted fluid, this solution was filtered through an Amicom filter (3-kDa cutoff) (Merck), according to the manufacturer's instructions, and then centrifuged at 3000g, for 40 min at 4°C . The eluted material was collected and 600 μL were transferred for 5 mm diameter NMR tubes.

One and two-dimensional ^1H spectra were acquired in a Bruker Avance III HD spectrometer, operating at 500 MHz, in TopSpin 3.2 software (Bruker) and IconNMR 4.6.7 software. One-dimensional ^1H spectra were acquired with excitation sculpting for water suppression (ZGESGP pulse sequence), 1k scans, 64K TD in the acquisition time of 3.27 s, relaxation delay of 1.74 s and sweep width 20 ppm at 25°C . For assignments the two-dimensional ^1H - ^1H total correlation spectroscopy (TOCSY) spectrum was acquired. The assignments were carried out through the HMDB 5.0, BMRB databases,^{11,12} and COLMAR-TOCSY platform,¹³ with ^1H chemical shift cutoff (ppm) of 0.03 ppm, matching ratio cutoff of 0.6, direct matching method for aqueous database, as shown by the Table S1.

After acquisition, spectra were processed in different steps on MestReNova version 14.2.1-276840.02 ppm binning by average sum method phase and baseline correction and spectra calibration. To eliminate artifacts compounds we removed the buckets referring to, water, regions with negative signs, regions referring to ethylenediaminetetraacetic acid, glycerol and ethanol (present in filter and in asepsis, respectively). Finally, binning table was normalized by the sum of intensities.

MetaboAnalyst 5.0 was used for multivariate statistical analyzes.¹² We used the unsupervised principal component analysis (PCA) to create a statistical classificatory model. Multiple *t*-test was

TABLE 1 Patients characteristics.

Variável	Non-infection (n = 10)	Infection (n = 11)	p
Age (median [IQR]; years)	75 [68–78]	71 [66–78]	0.587 ^a
Sex (n)			0.182 ^b
Male	2	6	
Female	8	5	
BMI (kg/m ²) (mean ± SD)	31.1 ± 4.9	31.6 ± 6.2	0.833 ^a
Comorbidities			
High pressure	9	9	–
Diabetes	2	3	
Coronary disease	1	2	
Kidney disease	1	3	
Hypothyroidism	0	2	
COPD	1	1	
Vascular insufficiency	1	1	
Hepatitis	0	1	
CRP (mg/L)	0.72 ± 0.72	9.4 ± 11.06	0.03
ESR (mm/h)	41 ± 31	64 ± 33	0.13
% SF-PMN (%)	26.4 ± 17.7	59.1 ± 37.9	0.03
SF-leukocyte count (cells/mm ³)	15177 ± 43338	133719 ± 239733	0.01

Abbreviations: BMI, body mass index; IQR, interquartile range; SF, synovial fluid.

^at-test.

^bChi square.

TABLE 2 Causative microorganisms.

Microorganism	Frequency [n (%)]
<i>Staphylococcus aureus</i>	4 (36)
<i>Staphylococcus cohnii</i>	2 (18)
<i>Staphylococcus epidermidis</i>	2 (18)
<i>Enterococcus faecalis</i>	2 (18)
<i>Staphylococcus lugdunensis</i>	1 (9)
<i>Staphylococcus hominis</i>	1 (9)
<i>Acinetobacter baumannii</i>	1 (9)
<i>Klebsiella aerogenes</i>	1 (9)

used for univariate analysis, and a $p < 0.05$ was considered statistically significant following correction for multiple testing using the Benjamini-Hochberg false discovery rate method.

PCA is a widely used method for multivariate analysis of metabolomics data, which takes a large dataset with several variables, takes into account the relationships between them, and reduce them to a smaller set of summary indices. Such approach allows the identification of major trend and features, as it order the variables

evaluated according to their variation. Therefore, PCA reduces the complexity of large datasets as it captures the essence of the data into a few key components, which convey the most variation in the dataset. Principal components are a few linear combinations of the original variables that maximally explain the variance of all the variables. Therefore, PCA score plots show cluster of samples based on their similarity. The loading plot shows how strongly each variable influences a component. The closer a variable is to 1 or –1 the greater its influence in the component.

3 | RESULTS

A representative ¹H NMR spectrum of infected and non-infected synovial fluid is detailed in Figure 1. Twenty-five metabolites were identified and are listed in Table 3. The unsupervised PCA score plot, in which each point represents one patient, showed a partial distinction between infected and non-infected groups. The first two principal components of this model explain 74.4% of the variance between groups, with lactate being the major discriminating metabolite. Notably it was possible to identify two distinct metabolic patterns within the infected group with one of them clearly overlapping the non-infected group (Figure 2A).

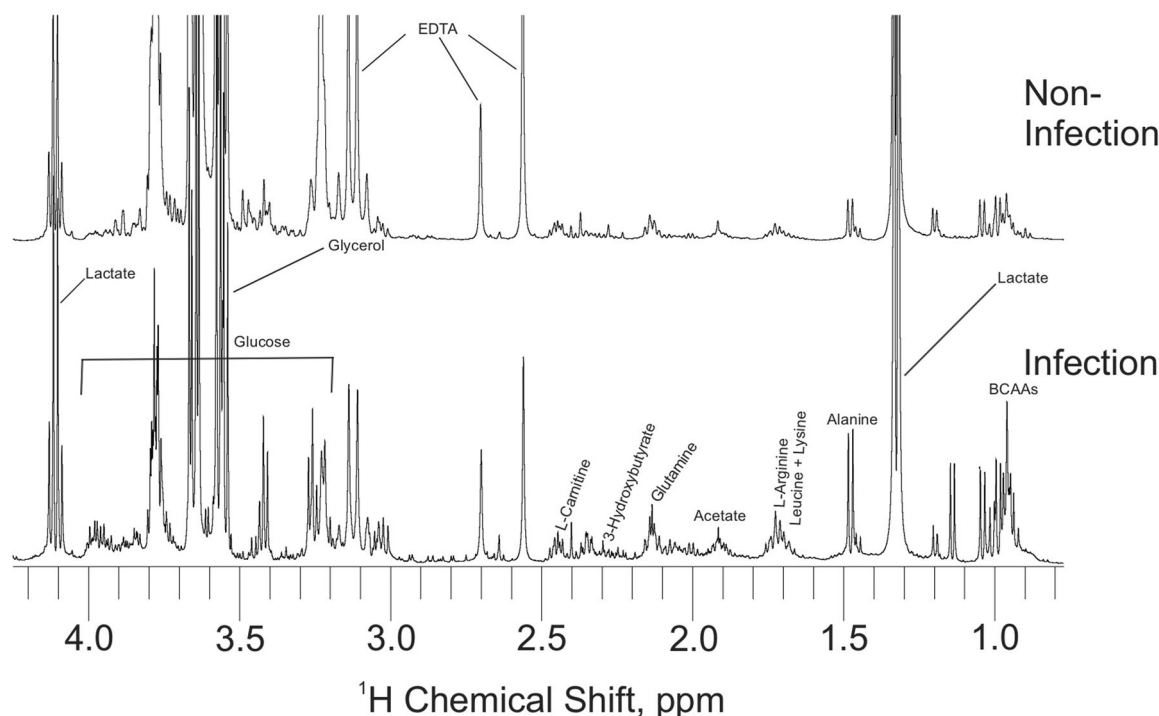


FIGURE 1 Representative spectra of synovial fluid from aseptic failure (top) and periprosthetic joint infection (bottom). Lines indicate the peaks of some of the metabolites identified.

Analysis of the PCA loading plot indicated that PJI samples tend to have a higher amount of lactate, and that in the non-infected ones a greater concentration of glucose can be found (Figure 2B).

Multiple *t*-test was performed to identify metabolites that were differently abundant between the groups. The five metabolites that showed different concentrations between both groups were: lactate, acetate and 3-hydroxybutyrate, that were elevated in infected synovial fluid, and glucose and creatine, that were found in higher concentration in non-infected group. When we considered the false discovery rate, a correction for multiple *t*-tests, lactate and glucose still showed significantly different between both groups (Table 4).

Once we identified two distinct profiles among patients with PJI, we specifically analyzed these patients to disclose the possible causes of such variation. When infected samples were categorized according to a timeline (acute or chronic), we could not obtain a clear separation between groups (Figure S1). However, when this set of samples were categorized considering the identification of *S. aureus* as the (or one of the) causative pathogens, we observed a clear separation between both groups. Loading plot analyzes revealed that synovial fluid from *S. aureus* infected patients tend to have higher amounts of lactate and acetate (Figure 3).

4 | DISCUSSION

In infectious diseases, new evidence has shown that pathogen and host cells engage in bidirectional modulation of their respective metabolism, thus regulating cellular function, which dictates disease

evolution and outcomes.^{14–16} Here we have performed a comprehensive analysis of the metabolites in the synovial fluid of patients with PJI and showed that the infected periprosthetic micro-environment has a distinct metabolic profile characterized by increased levels of lactate, acetate and 3-hydroxybutyrate and reduced levels of glucose and creatine. Besides, our results reveal that synovial fluid from PJIs caused by *S. aureus* display increased amounts of lactate compared to synovial fluid from infections by other pathogens. This study differs from previous ones as it is the first that evaluates exclusively patients with knee prostheses in both the PJI and the control groups.¹⁷

Recent evidence has shown that a critical step in the orchestration of immune cells is the reprogramming of their cellular metabolism,^{18,19} which means that cellular energy metabolism pathways are altered during the activation and function of immune cells and that intermediates and metabolic by-products of these pathways modulate immune cell function.²⁰ On the other hand, pathogens also regulate their metabolism during the course of the infection and such an alteration directly impacts their virulence.¹⁶

The presence of neutrophils is the hallmark in PJIs. Upon pathogen sensing, those cells primarily utilize glucose and glycolysis to fuel their respiratory burst, that refers to the rapid generation of the reactive oxygen species (ROS) through the catalytic conversion of molecular O₂ to ROS.²¹ Activated neutrophils display an increased surface expression of glucose transporters which is accompanied by an increased uptake of glucose that is converted into pyruvate through the glycolytic pathway.²² Under hypoxic conditions, when cellular oxygen demand exceeds supply, pyruvate is oxidized to

TABLE 3 Metabolites annotated in synovial fluid.

Identified metabolites	Function of metabolite
Butanol	Produced in small amounts by gut microbial fermentation through the butanoate metabolic pathway
Leucine	Branched chain amino acid (BCAA), involved in stress, energy and muscle metabolism
L_Valine	BCAA involved in stress, energy and muscle metabolism
3_Hydroxybutyrate	Ketone body produto of partial-degradation of BCAA
L_Threonine	Acts an immunostimulant and also can probably promote cell immune defense function
Lactate	Plays a role in several biochemical processes and is produced from pyruvate via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise
Alanine	Is an important participant as well as regulator in glucose metabolism. Is also an important amino acid for lymphocyte reproduction and immunity
Acetate	This short-chain fatty acids is a major end product of bacterial fermentation of fiber. Is also used in lipid synthesis and energy derivation, and act as a key regulatory agent through which cells respond to changing physiological conditions, nutrient and oxygen deprivation and other signals and stressors
Lysine	Lysine plays several roles in humans, most importantly proteinogenesis, but also in the crosslinking of collagen polypeptides, uptake of essential mineral nutrients, and in the production of carnitine, which is key in fatty acid metabolism
L_Glutamic_acid	Plays an important role in the body's disposal of excess or waste nitrogen. In many respects glutamate is a key molecule in cellular metabolism
L_Glutamine	Inovlved in cell survival and proliferation
3_Hydroxypropionic_acid	It is an intermediate in the breakdown of BCAA
L_Carnitine	Plays a key role in lipid metabolism and beta-oxidation
Citrate	Citrate is an intermediate in the TCA cycle that is a central metabolic pathway for all animals, plants, and bacteria
Creatine	Is a naturally occurring non-protein compound that facilitates recycling of adenosine triphosphate (ATP)
L_Phenylalanine	Is the precursor of the amino acid tyrosine
L_Histidine	Is a precursor for histamine and carnosine biosynthesis
Ethanolamine	Is an initial precursor for the biosynthesis of two primary phospholipid classes, phosphatidylcholine and phosphatidylethanolamine
L_Cystine	Oxidized dimeric form of cysteine involved in glutathione synthesis in cells from immune system
L_Tyrosine	Important amino acid in many proteins, peptides and even enkephalins, the body's natural pain reliever
D_Glucose	This monosaccharide is the primary source of energy for all living organisms
Betaine	Widely regarded as an anti-oxidant
Taurine	Cell-membrane stabilizer, and a facilitator in the transport of ions such as sodium, potassium, calcium, and magnesium
Choline	Essential vitamin in various metabolic processes, and in lipid metabolism
Glycerol	Forms the backbone of fatty acids in fats. When the body uses stored fat as a source of energy, glycerol and fatty acids are released into the bloodstream

lactate and nicotinamide adenine dinucleotide (NAD⁺).²³ Although the establishment of a hypoxic environment has not yet been specifically addressed in PJI, previous studies have shown that in inflammatory conditions like rheumatoid arthritis or osteoarthritis, synovial fluid pO₂ levels are reduced.^{24,25} Hypoxia in the synovial fluid of rheumatoid arthritis affected patients was characterized by a low synovial pH and increased lactate, 3-D-hydroxybutyrate and acetate levels.^{26,27} Remarkably, the establishment of a hypoxic environment is a common feature during inflammation associated with bacterial infection,²⁸ and a previous study has shown that

neutrophils at the site of biofilm infection consume high amounts of oxygen, thus creating an anaerobic environment.^{29,30}

In addition to hypoxia, it has been shown that activated neutrophils do not fully oxidize glucose even under normoxic conditions, on the contrary, pyruvate is preferentially oxidized to lactate through the engagement of Warburg metabolism, which is characterized by an increase in the rate of glucose uptake and preferential production of lactate to synthesize nicotine adenine dinucleotide phosphate (NADPH) to support its respiratory burst.^{18,31,32}

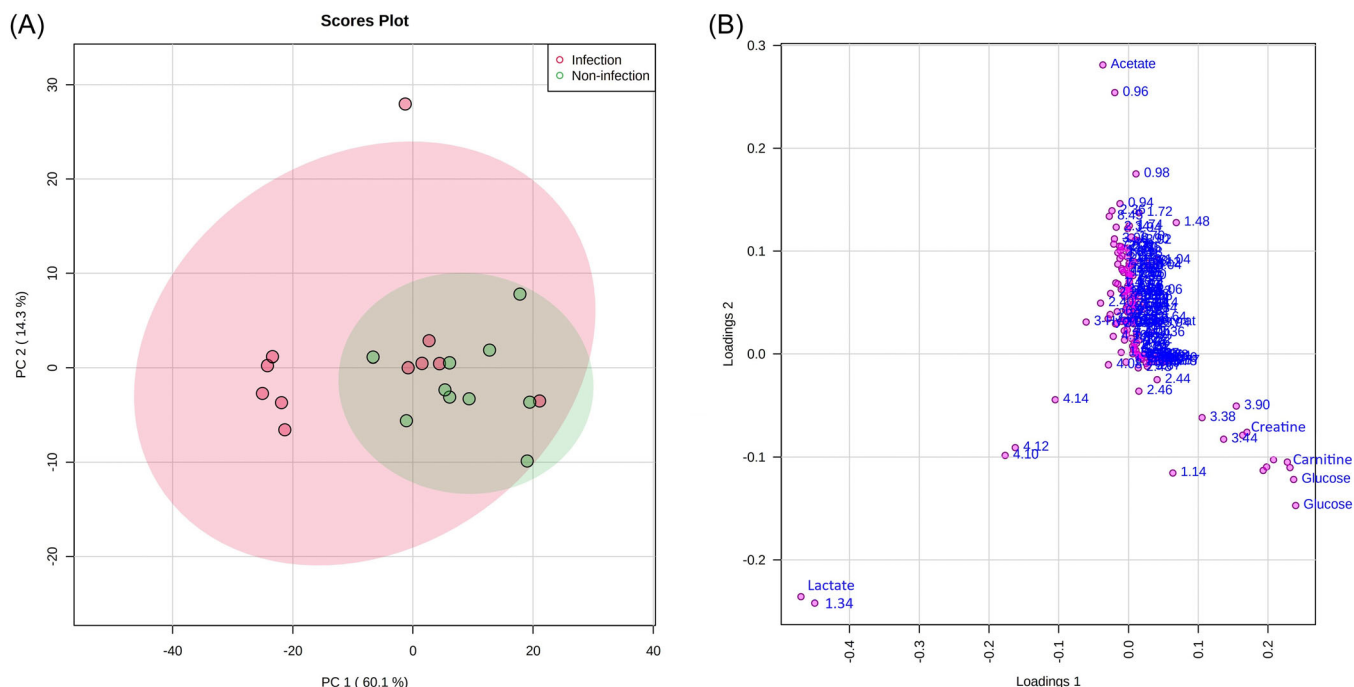


FIGURE 2 (A) Principal component analysis (PCA) score plot of synovial fluid from patients with (red) and without (green) periprosthetic joint infection. Two distinct groups of patients can be seen in the group of patients with PJI. (B) Loading plot where the metabolites that mostly contributed for the variation between PJI and non-PJI groups can be identified. PJI, periprosthetic joint infection.

TABLE 4 Differently abundant metabolites.

Metabolite	^1H chemical shift (ppm)	Non-infection	Infection	Status ^a	p value	Q value*
Lactate	1.32	4785 ± 976	7052 ± 2454	↑	<0.0001	<0.0000
Glucose	3.46–3.50	938 ± 398	448 ± 308.1	↓	<0.00001	0.0002
Creatine	3.92	585.6 ± 198.9	310.7 ± 201	↓	0.007	0.1
Acetate	1.92	235.1 ± 52.1	447 ± 540.5	↑	0.03	0.445
3-Hydroxybutyrate	1.20	242.2 ± 183	448 ± 260	↑	0.045	0.481

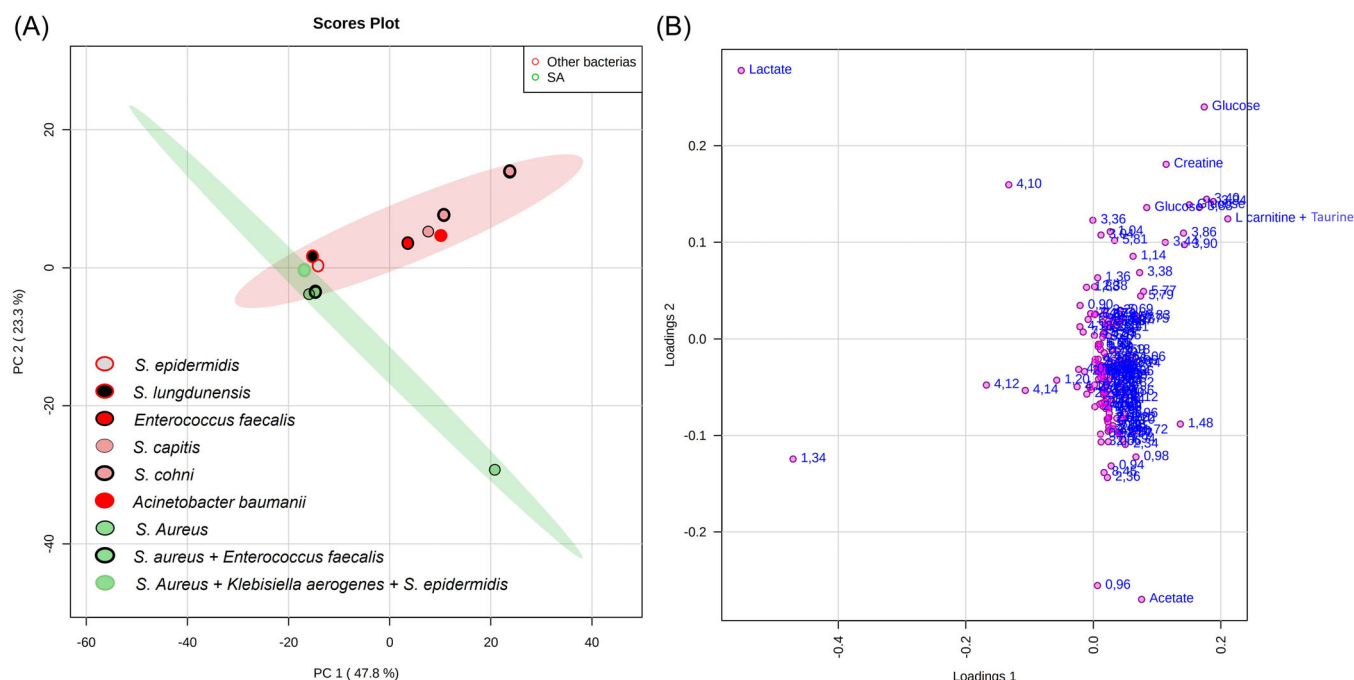
^aInfected cohort related to non-infected. Data are presents as mean relative intensity ± standard deviation. Multiple t-test.

Considering the above, the reduced levels of glucose coupled with increased levels of lactate observed in our study may suggest both the establishment of a hypoxic environment or the engagement of Warburg metabolism by synovial fluid infiltrating neutrophil. Further studies are needed to specifically address the processes taking place within periprosthetic infected microenvironment.

From a pathogen perspective, during infection, several aspects of host immune response limits bacterial respiration, (e.g., massive oxygen consumption by innate immune cells, iron chelation, nitric oxide production).^{33,34} Additionally, it has been shown that a variety of microbes enter glycolysis, an energetically less efficient pathway, in a mechanism known as overflow metabolism, that is the equivalent to the Warburg effect.³⁵ Through this process, substrates like glucose, are not fully oxidized through the respiratory pathway, and as a result metabolites like acetate, lactate and methanol are excreted. To succeed in this metabolic scenario of competition for

glucose, it has been shown that pathogens like *S. aureus*, one of the most prevalent pathogens in PJIs, display increased capacity for carbohydrate import, with a preferential absorption of glucose as a metabolic adaptation that allows a more efficient uptake of carbohydrates.³⁶

In bacteria, overflow metabolism is thought to be a mechanism to facilitate the biosynthesis of molecules required for invasion and growth.¹⁶ This mechanism has been associated with a fast pathogen growth and it has been proposed that the synthesis of the enzymes required for respiration is more costly than for those required for partial oxidation of glucose, what would be energetically inefficient in supporting fast growth in comparison to the incomplete oxidative metabolism.³⁵ Previous studies with *E. coli* have shown that the overflow metabolism pathway is associated with increased production of acetate, also pointing out this pathway as a potential source of increased acetate within synovial fluid from PJI patients.³⁷



Periprosthetic joint infection microenvironment

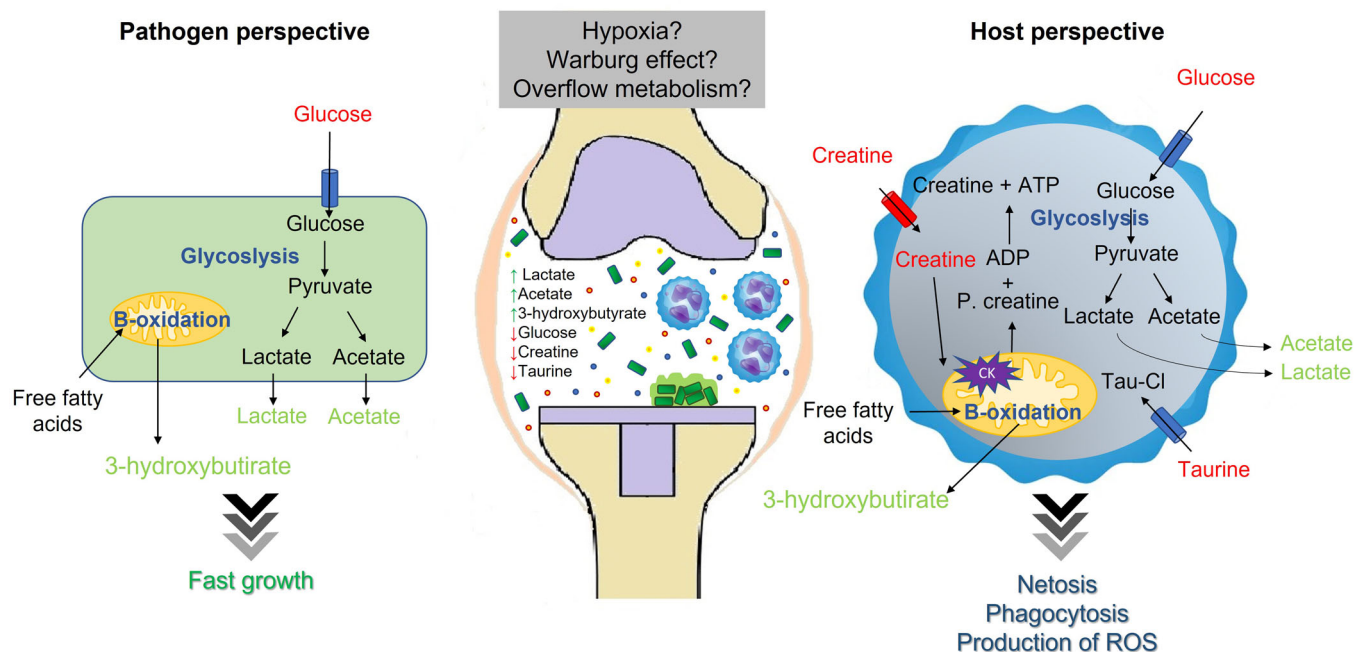


FIGURE 4 Proposed metabolic adaptations underlying metabolic alterations identified within synovial fluid from patients with periprosthetic joint infection. The left side of the image represents the putative metabolic pathways engaged in bacterial cells. Engagement in overflow metabolism would result in increased glucose uptake reducing extracellular levels of such metabolite. This would be accompanied by increased production of lactate and acetate. From a host perspective (right side), the establishment of a hypoxic environment or the engagement of Warburg metabolism by synovial fluid infiltrating neutrophil would shift metabolic pathway to glycolysis, resulting in reduced levels of glucose and the production of acetate and lactate.

Taken together our results show that the synovial fluid from patients with PJI display a distinct metabolic profile, suggesting the establishment of metabolic adaptations in host cells and bacteria within periprosthetic infected microenvironment.

AUTHOR CONTRIBUTIONS

Alan Mozella: design, data acquisition, analysis, and interpretation of data. **Hugo Cobra:** design, analysis, and interpretation of data. **Idemar Palma:** analysis and interpretation of data. **João Antonio Matheus Guimarães:** analysis and interpretation of data. **Rodrigo Salim:** analysis and interpretation of data. **Gilson Costa:** data acquisition, analysis and interpretation of data. **Ana Carolina Leal:** design, data acquisition, analysis and interpretation of data.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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