PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
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DETECÇÃO DIRETA DE MICRO-ORGANISMOS POR CULTURA, MALDI-TOF E qPCR EM MATERIAL ORTOPÉDICO SONICADO, APÓS DIFERENTES CONDIÇÕES DE PROCESSAMENTO

CURITIBA
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JULIETTE DE MORAES CIESLINSKI

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Dissertação apresentada ao Programa de Pós Graduação em Ciências da Saúde da Pontifícia Universidade Católica do Paraná, para obtenção do título de Mestre em Ciências da Saúde.

Orientação: Prof. Dr. Felipe Francisco Tuon

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CURITIBA
2019

"*One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away.*"

Stephen Hawking

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RESUMO

Introdução: Infecções de próteses ortopédicas são complicações associadas à alta mortalidade e custos. O padrão ouro para detecção microbiana nessas infecções é a cultura de múltiplos tecidos peri-prótese. Entretanto, este método pode ser pouco sensível. A cultura do fluido sonicado da prótese pode melhorar a recuperação dos micro-organismos devido à ruptura do biofilme microbiano. **Métodos:** Foram produzidos biofilmes de *Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Candida albicans* em parafusos ortopédicos, que foram armazenados em diferentes recipientes e temperaturas antes da sonicação. Após a sonicação, o fluido obtido foi cultivado e analisado por espectrometria de massas (MALDI-TOF) e PCR em tempo real (qPCR), com objetivo de detectar os micro-organismos presentes no biofilme aderido aos parafusos. **Resultados:** Houve redução da contagem microbiana em aproximadamente um *log* após a refrigeração dos parafusos que continham biofilme de *P. aeruginosa* e *S. aureus*. Todos os micro-organismos envolvidos nos biofilmes dos parafusos foram detectados por cultura, MALDI-TOF e qPCR. **Conclusão:** As reduções significativas nas contagens de UFC ocorreram apenas nos grupos armazenados em saco plástico, pois as alterações nas condições de temperatura e umidade, devido à fraca vedação do recipiente, podem ter favorecido a morte celular. Entretanto, esta redução não foi importante para este modelo estudado, visto que não afetou a detecção microbiana. É possível identificar os micro-organismos diretamente no fluido sonicado por MALDI-TOF, desde que a contagem microbiana seja suficiente. Não houve diferenças na detecção por qPCR entre os parafusos processados imediatamente ou após refrigeração. Porém, é necessário avaliar se a refrigeração poderia afetar a recuperação microbiana em amostras clínicas de próteses removidas em procedimento cirúrgico, onde a contagem microbiana pode ser menor. Um estudo clínico para avaliar o *bioburden* obtido após a sonicação de próteses poderia esclarecer esta questão.

Palavras-chave: biofilme; sonicação; parafuso ortopédico; infecção; prótese.

ABSTRACT

Background: Prosthetic joint infections are complications associated with high morbidity and cost. The gold standard for microbial detection in joint infections is the multiple culture of peri-prosthetic tissue. However, this method may be less sensitive. The culture of sonicated fluid can improve the microorganisms recovery by rupturing the microbial biofilm. **Methods:** We produced biofilms of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* on orthopedic screws, which were stored under different conditions and temperatures before sonication. After sonication, the protocols of culture, mass spectrometry by MALDI-TOF and qPCR were performed using sonicated fluid, aiming to detect the microorganisms involved in biofilm. **Results:** Bacterial bioburden decreased approximately one log after the refrigeration period in *P. aeruginosa* and *S. aureus* biofilm screws. All the microorganisms involved in screws biofilm were detected with MALDI-TOF and qPCR. **Conclusion:** Significant reductions in CFU counts occurred only in groups stored in the plastic bag, because changes in temperature and humidity may favor cell death. However, this variation is not important for this model, since it did not affect the detection due to the high counts obtained. Microbial identification by MALDI-TOF in sonicated fluid is feasible, provided that the sample has enough bioburden. With qPCR, there were no differences between detection in screws processed immediately or after refrigeration. It is necessary to think if the refrigeration period would affect microbial recovery in an explanted prosthesis. A clinical study to evaluate the bioburden obtained after prosthesis sonication could further elucidate this issue.

Keywords: biofilm; sonication; orthopaedic screw; infection; prosthesis.

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LISTA DE ABREVIATURAS

ATCC	*American Typing Culture Collection*
BHI	*Brain Heart Infusion*
BHQ-1	*Black Hole Quencher*-1
BHQ-2	*Black Hole Quencher*-2
CFU	*Colony Forming Unit*
CY5	*Cyanine dye* 5
Ct	*Cicle Treshold*
DNA	*Deoxyribonucleic Acid*
FAM	5(6)-*carboxyfluorescein*
gDNA	*Genomic Deoxyribonucleic Acid*
HCCA	4-*cyanohydroxycinnamic acid*
HMDS	Hexametildisilazano
IADO	Infecções Associadas a Dispositivos Ortopédicos
ITS-2	*Internal Transcribed Spacer* 2
LOD	*Limit of Detection*
MALDI-TOF	*Matrix Assisted Laser Desorption Ionization – Time of Flight*
MEV	Microscopia Eletrônica de Varredura
MS	*Mass Spectrometry*
NTC	*No Template Control*
PB	*Plastic bag*
qPCR	*Real Time - Polymerase Chain Reaction*
RC	*Rigid container*
RR	Recipiente rígido
rRNA	*Ribosomal ribonucleic acid*
SEM	*Scanning electron microscopy*
SP	Saco plástico
TSA	*Triptic soy agar*
UFC	Unidades formadoras de colônia

SUMÁRIO

1. INTRODUÇÃO

Com os avanços tecnológicos constantes na área médica, o uso de dispositivos implantáveis tem se tornado cada vez mais frequente, e tem sido um fator importante de melhoria nos cuidados de saúde e qualidade de vida dos pacientes (DAROUICHE, 2001; DONLAN, 2001; ZIMMERLI, 2004; CALIFANO, 2012; CRNICH, 2012; GANDHI, 2012). A área que mais se beneficiou com esse avanço foi a ortopedia, cujos procedimentos de implantes para restaurar a função das articulações afetadas, segmentos ósseos fraturados e membros comprometidos estão aumentando progressivamente (MONTANARO, 2011).

Próteses articulares totais e dispositivos de fixação de fraturas são essenciais para o tratamento de pacientes com enfermidades ortopédicas. No entanto, estes dispositivos médicos tornam-se vulneráveis à contaminação, tanto pela microbiota colonizante, quanto por fontes ambientais (HIRSEMANN, 2005), favorecendo assim a ocorrência de infecções ósseas associadas ao implante (MONTANARO, 2011; OSMON, 2013; ZIMMERLI, 2004). A colonização bacteriana do dispositivo médico não só pode evoluir para a infecção clínica, como também pode afetar negativamente sua função (DAROUICHE, 2001). Para alguns pacientes, as infecções de próteses articulares frequentemente requerem múltiplas cirurgias, terapia antimicrobiana em longo prazo e períodos prolongados de reabilitação (STREET, 2017).

Infecções associadas a próteses articulares são as menos frequentes, mas representam a complicação com maior morbidade e custo (TRAMPUZ, 2006). As taxas de infecção após o implante de próteses de quadril e joelho são inferiores a 2%. De modo geral, cerca de 5% dos dispositivos de fixação interna são infectados (DAROUICHE, 2004), enquanto que a incidência após fixação de fraturas expostas pode exceder 30% (TRAMPUZ, 2006; TRIBBLE, 2018).

A maioria das infecções associadas a dispositivos ortopédicos (IADO) está associada aos serviços de assistência à saúde (DUDECK, 2015). Não há dados regionais publicados sobre a incidência das IADO, mas no Hospital Universitário Cajuru, referência em atendimentos de trauma no sul do Brasil, são realizadas anualmente

5000 cirurgias ortopédicas. Destas, em média 50 evoluem com quadro infeccioso, normalmente causado por bacilos gram negativos e estafilococos, muitas vezes resistentes a várias classes de antibióticos (TUON, F. Comunicação pessoal). O diagnóstico rápido, preciso e confiável da infecção é necessário para auxiliar na escolha correta de tratamento, particularmente para micro-organismos resistentes a antibióticos (STREET, 2017).

Os micro-organismos que colonizam os dispositivos médicos podem frequentemente se agregar e formar biofilmes, causando danos aos tecidos adjacentes (ZHAO, 2015). Biofilmes são formados quando os micro-organismos se aderem de forma irreversível a uma superfície e produzem polímeros extracelulares, formando uma matriz estrutural. Esta superfície pode ser inerte ou composta por organismos vivos, como bactérias ou fungos (MARQUES, 2007).

A estrutura do biofilme desempenha um papel importante na patogênese das IADOs, pois protege os micro-organismos do sistema imune devido à função de barreira física e fisiológica da membrana do biofilme. Internamente a esta membrana, as células que estão aderidas à estrutura do biofilme são as chamadas sésseis, enquanto aquelas livres e dispersas são denominadas planctônicas (Figura 1).

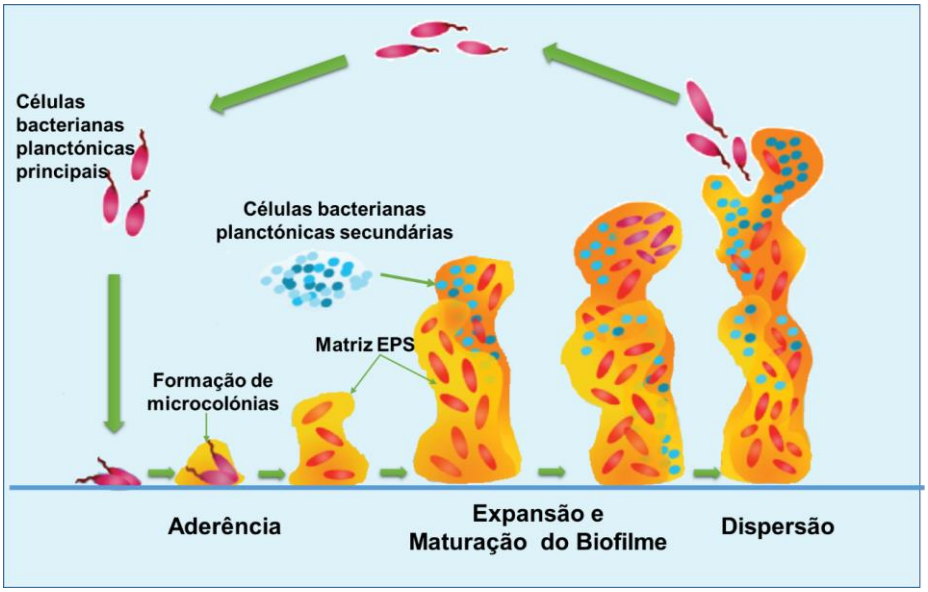

Figura 1: Fases da formação do biofilme. (Fonte: Tremblay, 2014)

As bactérias incorporadas em biofilmes podem se tornar resistentes à antibióticos e desinfetantes, à fagocitose e aos sistemas de defesa inflamatória do hospedeiro (MONTANARO, 2011; THURLOW, 2011). Dessa forma, os micro-organismos estão protegidos do sistema imunológico do hospedeiro e têm maior resistência aos antibióticos do que as bactérias planctônicas, dificultando o tratamento médico e requerendo remoção do implante, debridamento ósseo extenso, excisão do osso e tecidos moles infectados e subsequente tratamento antimicrobiano prolongado (CALIFANO, 2012; ZHAO, 2015). Além disso, a formação do biofilme pode prejudicar a detecção dos micro-organismos aderidos, levando à falha no diagnóstico da infecção (STREET, 2017).

A patogênese dessas infecções depende da interação entre o microrganismo, o dispositivo e o hospedeiro. Fatores bacterianos são provavelmente os mais importantes, enquanto os fatores do dispositivo são os mais passíveis de modificação com o objetivo de prevenir infecção (DAROUICHE, 2001). A superfície dos dispositivos ortopédicos, normalmente compostas por aço inoxidável, titânio ou polipropileno, pode ser contaminada por micro-organismos que se aderem, iniciando o crescimento celular e, consequentemente, levando à formação de biofilme (MARQUES, 2007).

Vários micro-organismos são capazes de participar em maior ou menor intensidade dos processos de adesão e formação do biofilme. As bactérias mais comumente isoladas de articulações infectadas são do gênero *Staphylococcus*. Alguns estudos reportam a associação deste microrganismo com a infecção ortopédica em 75% dos casos, sendo que nos casos em que houve uso de algum dispositivo médico, o número aumenta para 82,3% (BOLES, 2011; MONTANARO, 2011; TUON, 2019)

O padrão ouro para detecção microbiana em infecções articulares é a cultura múltipla de tecido ósseo peri protético (ATKINS, 1998; OSMON, 2013). No entanto, este método pode ser pouco sensível e, em média, apenas 62% das bactérias causadoras da infecção são detectadas (ATKINS, 1998; DUDAREVA, 2018). Nesse contexto, a técnica de sonicação têm se destacado por aumentar a sensibilidade das culturas de dispositivos médicos onde há formação de biofilme (TRAMPUZ, 2007). A cultura do fluido de sonicação de próteses explantadas pode melhorar a recuperação do

microrganismo pela ruptura do biofilme bacteriano (STREET, 2017). Isso porque o material que será cultivado é proveniente do implante, e não do tecido ósseo adjacente.

A sonicação de dispositivos como auxílio diagnóstico tem sido descrita desde 1998 e, desde então, vários autores descrevem-na como melhor opção para diagnóstico de infecções articulares quando comparada à cultura de múltiplos tecidos peri-prótese (TRAMPUZ, 2007; BJERKAN, 2009; PIPER, 2009; BOGUT, 2014; TANI, 2018; YAN, 2018; UEDA, 2019). A sonicação é uma técnica que utiliza o ultrassom (som acima da faixa de frequência audível) para remover partículas aderidas a uma superfície. Em meios líquidos, o ultrassom se propaga com baixa atenuação e forma bolhas de vapor de alta pressão (cavitação) que, ao colidir com a superfície, remove o que está aderido, inclusive o biofilme. Para o descolamento suave do biofilme, o implante é sonicado em meio líquido e com ultrassom de baixa frequência (40 kHz). O biofilme da superfície do implante é removido por meio de bolhas de cavitação de baixa energia, de modo que não há destruição significativa dos micro-organismos (RENZ, 2015).

Além disso, várias técnicas de investigação molecular têm sido descritas para melhorar a sensibilidade do diagnóstico, seja o teste molecular diretamente do tecido ou do material sonicado (TUNNEY, 1999; ACHERMANN, 2010; GOMEZ, 2012; PORTILLO, 2012; CAZANAVE, 2012; RYU, 2014). Mais recentemente, a espectrometria de massa por MALDI-TOF *(Matrix Assisted Laser Desorption Ionization – Time of Flight)* vem sendo utilizada de várias formas para detecção direta de micro-organismos em amostras biológicas com intuito de obter identificação precoce como uma alternativa à cultura (CHEN, 2013; BAZZI, 2016; BARBERINO, 2017). Apesar de todos esses avanços no diagnóstico das IADOs, a cultura tecidual ainda continua sendo o exame padrão ouro.

2. JUSTIFICATIVA

O uso de próteses ortopédicas é de grande importância para o tratamento de pacientes que necessitam de fixação nas articulações. No entanto, estes dispositivos estão propensos à contaminação, tanto pela microbiota colonizante, quanto por fontes ambientais (HIRSEMANN, 2005). Esta contaminação favorece a ocorrência de infecções ósseas associadas ao implante (MONTANARO, 2011; OSMON, 2013; ZIMMERLI, 2004) e afeta negativamente sua função (DAROUICHE, 2001). Os micro-organismos presentes nas próteses podem formar biofilmes, o que os torna resistentes à antibióticos e desinfetantes, à fagocitose e ao sistema imune do hospedeiro. Além disso, a formação do biofilme pode prejudicar a detecção dos micro-organismos aderidos, levando à falha no diagnóstico da infecção (STREET, 2017).

A técnica de sonicação têm se destacado por aumentar a sensibilidade das culturas de dispositivos médicos onde há formação de biofilme (TRAMPUZ, 2007; BJERKAN, 2009; BOGUT, 2014; TANI, 2018). Porém, apesar de se tratar de uma técnica simples e que não requer profissional especializado, esta não se encontra disponível em muitos serviços, principalmente devido à falta de padronização e estudos aplicados na área. Nos casos em que é desejável a realização da cultura por este método, a opção que muitos hospitais têm é enviar a prótese para processamento em um laboratório que possua esta técnica em sua rotina. Para que este envio ocorra de forma a preservar a integridade do material coletado e a viabilidade dos possíveis micro-organismos colonizantes, condições ideais de transporte são necessárias. Entretanto, não há relatos em literatura que descrevam qual o recipiente de transporte mais adequado e também qual o tempo e temperatura ideais para transporte até o processamento inicial, para que as propriedades do biofilme sejam preservadas, a fim de manter a viabilidade microbiana para a detecção em cultura.

Diante disso, evidencia-se a necessidade de técnicas práticas e padronizadas que facilitem o processo como um todo. Ainda, a possibilidade de identificar o micro-organismo diretamente do fluido obtido da sonicação, seja por métodos moleculares ou proteômicos, pode tornar o diagnóstico mais rápido e efetivo. Além disso, não há

estudos sobre a interferência dos processos pré-analíticos sobre técnicas de biologia molecular ou de espectrometria de massa. Até o presente momento, não existem dados sobre o uso da técnica de espectrometria de massa pelo MALDI-TOF como metodologia de identificação direta de micro-organismos utilizando o fluido sonicado. Este método poderia acelerar a identificação microbiológica para uma a duas horas, ao passo que para a cultura convencional são necessárias 24 a 72 horas.

3. OBJETIVO

Avaliar a detecção de micro-organismos presentes em biofilmes formados *in vitro* em parafuso ortopédico, por meio de sonicação, após diferentes condições de processamento.

3.1. OBJETIVOS ESPECÍFICOS

3.1.1. Desenvolver modelos de biofilmes com cepas de *Pseudomonas aeruginosa*, *Staphylococcus aureus* e *Candida albicans,* produzidos *in vitro* em parafuso ortopédico de titânio;

3.1.2. Verificar a estrutura dos biofilmes produzidos por meio de microscopia eletrônica de varredura (MEV);

3.1.3. Comparar a contagem de unidades formadoras de colônia (UFC) obtidas após a sonicação dos parafusos ortopédicos: (i) utilizando saco plástico e recipiente rígido; (ii) imediatamente após a produção do biofilme e após sete dias de refrigeração (2 à 8°C);

3.1.4. Detectar micro-organismos utilizando espectrometria de massa por MALDI-TOF, diretamente do fluido sonicado;

3.1.5. Detectar DNA microbiano por qPCR diretamente do fluido sonicado.

4. ARTIGO

A metodologia detalhada, bem como os resultados e a discussão, constam no texto do artigo completo resultante desta pesquisa, descrito a seguir.

Para esta pesquisa não foi necessária avaliação pelo comitê de ética

A empresa NEOORTHO® Produtos Ortopédicos participou deste estudo fornecendo os parafusos de interferência de titânio.

4.1. *MANUSCRIPT* PARA PUBLICAÇÃO

Title:

Direct detection of microorganisms in sonicated orthopedic devices after *in vitro* biofilm production and different processing conditions

Running title:

Biofilm Sonication

Authors:

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Keywords: biofilm; sonication; screw; infection; model

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ABSTRACT

Background: Prosthetic joint infections are complications associated with high morbidity and cost. The gold standard for microbial detection in joint infections is the multiple culture of the peri-prosthetic tissue. However, this method may have low sensitivity. The fluid cultures after sonication can improve the recovery of the microorganisms, by rupturing the microbial biofilm. **Methods:** We produced biofilms of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* on orthopedic screws, which were stored under different conditions and temperatures before sonication. After sonication, the mass spectrometry by MALDI-TOF, qPCR and culture protocols were performed using the sonicated fluid, for detecting the microorganisms involved in the biofilm. **Results:** The bacterial bioburden decreased by approximately one *log* after the refrigeration period, in the screws containing *P. aeruginosa* and *S. aureus* biofilms. All the microorganisms involved in the screw biofilms were detected with MALDI-TOF and qPCR. **Conclusion:** Significant reductions in CFU counts occurred only in groups stored in the plastic bag, indicating that changes in temperature and humidity may favor cell death. However, this variation is not important for this model as it did not affect the detection owing to the high counts obtained. Microbial identification by MALDI-TOF in sonicated fluid is feasible, provided that the sample has enough bioburden. With qPCR, there were no differences between the detection in the screws processed immediately or after refrigeration. It is necessary to consider whether or not the refrigeration period would affect microbial recovery in an explanted prosthesis. A clinical study to evaluate the bioburden obtained after sonication of prosthesis could further clarify this issue.

Keywords: biofilm; sonication; screw; infection; model

INTRODUCTION

Prosthetic joint infections are complications associated with high morbidity and cost (1). Biofilm-associated microorganisms are the main etiological agents (2, 3). Biofilms are formed when the microorganisms adhere irreversibly to a surface and produce extracellular polymers, forming a structural matrix (4). The biofilm structure plays an important role in the pathogenesis of prosthetic joint infections. It protects the microorganisms of the immune system and can also impair the detection of adhered microorganisms. This leads to a failure to diagnose the infection (5, 6).

The gold standard for microbial detection in joint infections is multiple cultures of the peri-prosthetic tissue (7, 8). However, this method may have low sensitivity; on an average, only 62% of the bacteria that cause infections are detected (7, 9). In this context, the use of the sonication technique has been highlighted as it increases the sensitivity of the medical device cultures (1). The sonicated fluid cultures from the explanted prostheses can improve the recovery of microorganisms by rupturing the bacterial biofilm. This is because the material that is used in the culture is derived from the implant, and not from the adjacent bone tissue (5).

Several techniques of molecular investigation of sonicated fluid have been described, with the aim of improving the sensitivity of the diagnosis or detection of peri-prosthetic infection (10-14). More recently, MALDI-TOF (Matrix Assisted Laser Desorption Ionization) mass spectrometry has been applied in several conditions for the direct detection of biological samples to obtain early identifications of the microorganisms, as an alternative to culture (15-17). Despite

253 the progress in the diagnosis of infections associated to orthopedic implants, tissue culture
254 remains the gold standard tool.

255 Although sonication is a simple technique, it is not available in many settings, mainly due
256 to the lack of standardization and applied studies in the field. There are no reports in the literature
257 that compare the transport containers or the ideal time and temperature for transportation for
258 maintaining microbial viability in the biofilm.

259 The aim of this study was to evaluate the sonication technique with a plastic bag and the
260 effect of refrigeration on microorganism detection with conventional culturing, MALDI-TOF MS
261 and qPCR assay on an orthopedic screw model.

METHODS

Pilot study of in vitro biofilm production and sonication

The quality of the biofilm formation (biofilms of *Staphylococcus aureus* (ATCC® 25923™), *Pseudomonas aeruginosa* (ATCC® 27853™) and *Candida albicans* (ATCC® 14053™), which were produced on the screws) was evaluated using a titanium screw model (Neoortho®, Curitiba, Brazil). Two biofilm screw sets were produced for each microorganism. After the biofilm production, the first set was sent directly for visualization by Scanning Electron Microscopy (SEM) to observe the biofilm structure. The second set was submitted for the sonication protocol described below, following which it was observed using SEM.

For biofilm production, we used a protocol previously described (18). Briefly, strains of *P. aeruginosa* and *S. aureus* were reactivated in nutrient agar and incubated for 24 hours at 37°C; the *C. albicans* strain was reactivated in the same way and incubated for 48 hours at 37°C. An isolated colony of these microorganisms was dissolved in BHI broth (Brain Heart Infusion) and incubated for 24 hours at 37°C. The treated broth was centrifuged for 5 minutes at 207×g. The supernatant was discarded and the obtained pellet was washed three times with 0.9% NaCl. In the last wash step, the pellet was dissolved in a new BHI broth to obtain a bacterial suspension prepared to match the turbidity of the 0.5 tube of McFarland turbidity standard, using a nephelometer (Alfakit, Florianópolis, Brazil). From this suspension, a 1:10 dilution was made in BHI broth until concentrations of 10^7 CFU/mL for bacteria and 10^5 CFU/mL for yeast were achieved. This broth was spread into sterile 6-well plates until it covered the titanium screws (one screw per well) for 90 minutes so that cells could adequately adhere to the titanium screw. The screws were transferred to a new sterile 6-well plate containing 0.9% NaCl in order to remove

planktonic cells from the material. After this, screws were transferred to another sterile 6-well plate and submerged in 3 mL of BHI at 37°C for 24 hours for bacteria and 48 hours for yeast. During this step, the cells adhered to the device surface formed the biofilm. After this step, the culture medium was aspirated and the screws were submerged into 0.9% NaCl in order to remove the residues and unfixed cells. After this washing step, the devices were stored in 15 mL conical tubes (Alfa Hospitalar®, São Paulo, Brazil) for further processing.

The first set of biofilm screws of each of the three microorganisms was preserved for direct visualization under SEM, with the aim of characterizing the structure of the different biofilms formed. The second set of biofilm screws was transferred to a sterile conical tube with 10 mL of 0.9% NaCl, and sonicated for 5 minutes in an ultrasonic bath using a Soniclean 15 (Sanders Medical, Santa Rita da Sapucaí, Brazil) at a frequency of approximately 40 kHz and temperature of 35°C (1). After the sonication step, the liquid was inoculated in TSA agar for growth evaluation after the detachment of the biofilm. The remaining liquid was discarded, and the screws were stored in conical tubes for further SEM analysis.

For SEM visualization, the samples were transferred into sterile glass petri dishes filled with the primary fixative agent (0.68g sucrose, 0.42 g sodium cacodylate, 0.6 mL 30% glutaraldehyde) (Merck, Darmstadt, Germany) and 19.4 mL of deionized water in sufficient quantity to cover the screw for 45 minutes. After that, the screws were transferred to a buffer (composed of sucrose and sodium cacodylate at the above concentrations) for 10 minutes. Then, the samples were dehydrated in alcohol in a series of increasing concentrations: ethanol 35%, ethanol 50%, ethanol 70%, ethanol 100%, and HMDS PA (hexamethyldisilazane) (Merck, Darmstadt, Germany) for 10 minutes in each. After the fixation step, the models were metallized with gold particles in a metallizing equipment with a Q150R ES rotary pump (Quorum

Technologies, Lewes, UK), and later fixed in a metal base for observation under SEM, which was a PentaFET Precision (Oxford Instruments, Abingdon, UK) at 5.0 kV. Observations were made with magnifications between 2,000 and 30,000×.

Biofilm production for evaluation of different processing conditions and microbial detection methods

The evaluation of sonication on titanium screws was made with *in vitro* biofilm models of *Staphylococcus aureus* (ATCC® 25923™), *Pseudomonas aeruginosa* (ATCC® 27853™) and *Candida albicans* (ATCC® 14053™). The biofilms were produced following the previously described methodology (18). Twenty-four biofilm screws were produced for each microorganism, which were stored under different conditions before sonication. These 24 screws were divided into 2 groups: those stored in a plastic bag (PB) and those stored in a rigid container (RC), with 12 screws for each group. For the screws stored in a PB, 6 of them were sonicated immediately after the biofilm formation and the other 6 were refrigerated for 7 days prior to sonication. The same practice was followed for the group stored in RC, as shown in flowchart (figure 2). We used sterile 50 mL traditional plug seal capped polypropylene tubes (Alfa Hospitalar®, São Paulo, Brazil) for sonication with RC. For plastic bags, we used sterile polyethylene sampling bags with a removable seal and a wire closure system (Labplas®, Sainte-Julie, Canada).

The sonication protocol was previously described (1). Briefly, 20 mL of 0.9% NaCl was added to cover the screw collected in the transport containers (PB or RC). These containers were

vortexed for 30 seconds, placed inside the ultrasonic bath, sonicated for 5 minutes at a frequency of 40 kHz at 35°C, and vortexed again for 30 seconds.

After the sonication step, the resulting fluid was serially diluted to 1:1,000,000 in order to obtain isolated colonies for counting. From this dilution, 100 µL were inoculated in TSA agar and subsequently incubated for 48 hours at 35ºC. Colony growth was evaluated in CFU/mL. One milliliter aliquots of the sonicated fluid were stored for further studies (for qPCR and MALDI-TOF methodologies).

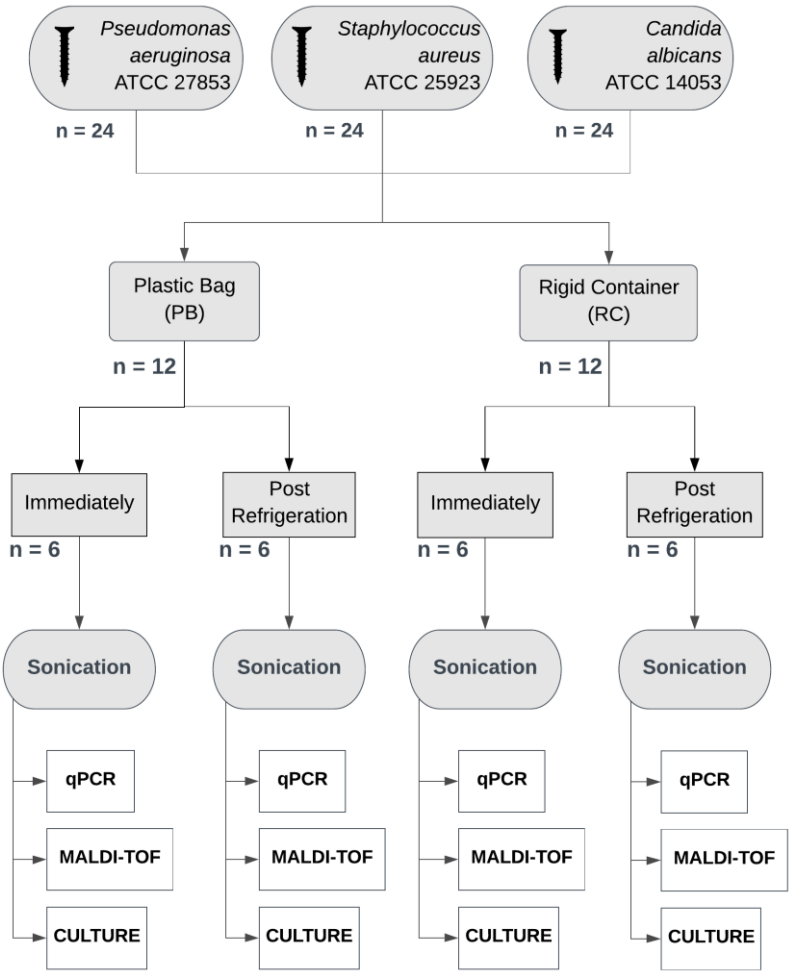

Figure 2: Sample processing sequence. (Legend - qPCR: real-time polymerase chain reaction; MALDI-TOF: Matrix Assisted Laser Desorption Ionization - Time of Fight)

Direct detection protocol using MALDI-TOF mass spectrometry

Direct microorganism detection using MALDI-TOF mass spectrometry was performed on Vitek® MS equipment (BioMérièux, Durham, NC). Sample extraction for processing was adapted from the protocol previously described (19). Briefly, 4 mL of the sonication fluid was centrifuged at 367×g for 5 minutes and the obtained pellet was washed with deionized water. The pellet was re-suspended in 50 µL of deionized water and 900 µL of absolute alcohol was added. After vortexing, the tube was centrifuged at 18,000×g for 2 minutes and the supernatant was discarded. Fifty microliter of formic acid (70% v/v) and 50 µL of acetonitrile were added. After vortexing, the tube was centrifuged at 18,000×g for 2 minutes. Then, 1 µL of the supernatant was spotted directly onto the target plate. After drying, each inoculum was covered with 1 µL of the HCCA matrix solution (4-cyanohydroxycinnamic acid) (BioMérièux, Durham, NC). Again, after drying, samples were analyzed using the VITEK® MS System. Quality control was performed using reference strain of *Escherichia coli* (ATCC® 8739™). All this procedure was performed in triplicate.

Direct detection using real-time PCR (qPCR)

Direct molecular detection of microbial gDNA was performed using real-time PCR for 16S rDNA gene screening (for bacteria) and ITS-2 region (for yeast). Microbial DNA was extracted using a PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, using 1 mL of the sonication fluid to obtain 50 µL of extracted DNA.

For the molecular detection of 16S rDNA, a TaqMan Universal PCR Master Mix (Applied Biosystems, Inc., Foster City, CA) was used and the detection of 16S rDNA was adapted from the protocol previously described (20), using forward and reverse primers and a probe with the following sequences: 5'-TGGAGCATGTGGTTTAATTCGA-3', 5'-TGCGGGACTTAACCCAACA-3' and (CY5)-5'-CACGAGCTGACGACARCCATGCA-3'-(BHQ2) (21). The detection of the ITS-2 region was adapted from the protocol previously described (22), using forward and reverse primers and a probe with the following sequences: 5'-GGAGGGCATGCCTGTTTG-3', 5'-CAAGTCGTATTGCTCAACACCAA-3' and (FAM)-5'-TCGTTTCTCCCTCAAACCGCTGGG-3'-(BHQ1).

The 16S protocol was performed in triplicate for each sample, using 12.5 µL of TaqMan Universal PCR Master Mix, 8.7 µL of ultrapure water, 0.6 µL of each primer (forward and reverse; 20 mM), 0.6 µL of probe (10 mM), and 2 µL of DNA giving a total volume of 25 µL per well. Furthermore, no template control (NTC) (the same protocol, but with water rather than DNA) and positive control, using 0.1 ng/µL of *E. coli* gDNA (Applied Biosystems, Inc., Foster City, CA), were performed. The reactions were subjected to amplification in an ABI-7500 Fast real-time PCR instrument (Applied Biosystems, Inc., Foster City, CA), using the following parameters: 50ºC for 2 min, 95ºC for 10 min, 40 cycles of 95ºC for 15 s, and 60 ºC for 1 min. A standard curve for 16S rDNA was created to determine the efficiency and analytical sensitivity of the assay.

The ITS-2 protocol was performed in triplicate for each sample, using 10 µL of TaqMan Universal PCR Master Mix, 3.8 µL of ultrapure water, 0.4 µL of each primer (forward and reverse; 20 mM), 0.4 µL of probe (20 mM) and 5 µL of DNA for a total volume of 20 µL per well. Furthermore, no template control (NTC) (the same protocol, but with water rather than

DNA) and positive control, using *C. albicans* (ATCC® 14053™) gDNA were performed. The reactions were subjected to amplification in an ABI-7500 Fast Real-Time PCR instrument (Applied Biosystems, Inc., Foster City, CA), using the following parameters: 50ºC for 2 min, 95ºC for 10 min, 45 cycles of 95ºC for 15 s, and 60ºC for 1 min. A standard curve for the ITS-2 region was created to determine the efficiency and analytical sensitivity of the assay.

Saline solution was inoculated with *S. aureus* (ATCC® 25923™), *P. aeruginosa* (ATCC® 27853™) in progressive concentrations from 10^8 to 10^2 CFU/mL and *C. albicans* (ATCC® 14053™) in progressive concentrations from 10^6 to 10^0 CFU/mL (performed in triplicate) to determine the standard curve. The Ct (cycler threshold) values were used to calculate the performance of 16S rDNA and ITS-2 region qPCR. The last three concentrations detected in the standard curve ($10^2 \pm 1$) were amplified with 30 repetitions, to define the limit of detection (LOD), which must have 100% of molecular targets amplified to assure minimal detection with 95% CI.

Statistical analysis

The SEM data are qualitative and descriptive. For the comparison of the different sonication methods and screw storage, the median CFU/mL was analyzed by non-parametric Mann Whitney test. The difference in CFU/mL was significant when $p < 0,05$.

RESULTS

After *in vitro* biofilm production, the screws were submitted to SEM. There, the bacterial cells of *P. aeruginosa*, *S. aureus* and yeast cells of *C. albicans* were observed, as well as the formation of an extracellular matrix layer involving the cells, which can be observed in Figure 3. A group of screws was sonicated and submitted to SEM. The presence of biofilm was also observed, but with a lower number of cells and extracellular matrix in some regions (figure 3). This result was expected because sonication is the only method not able to completely remove the biofilm adhered to a surface (23).

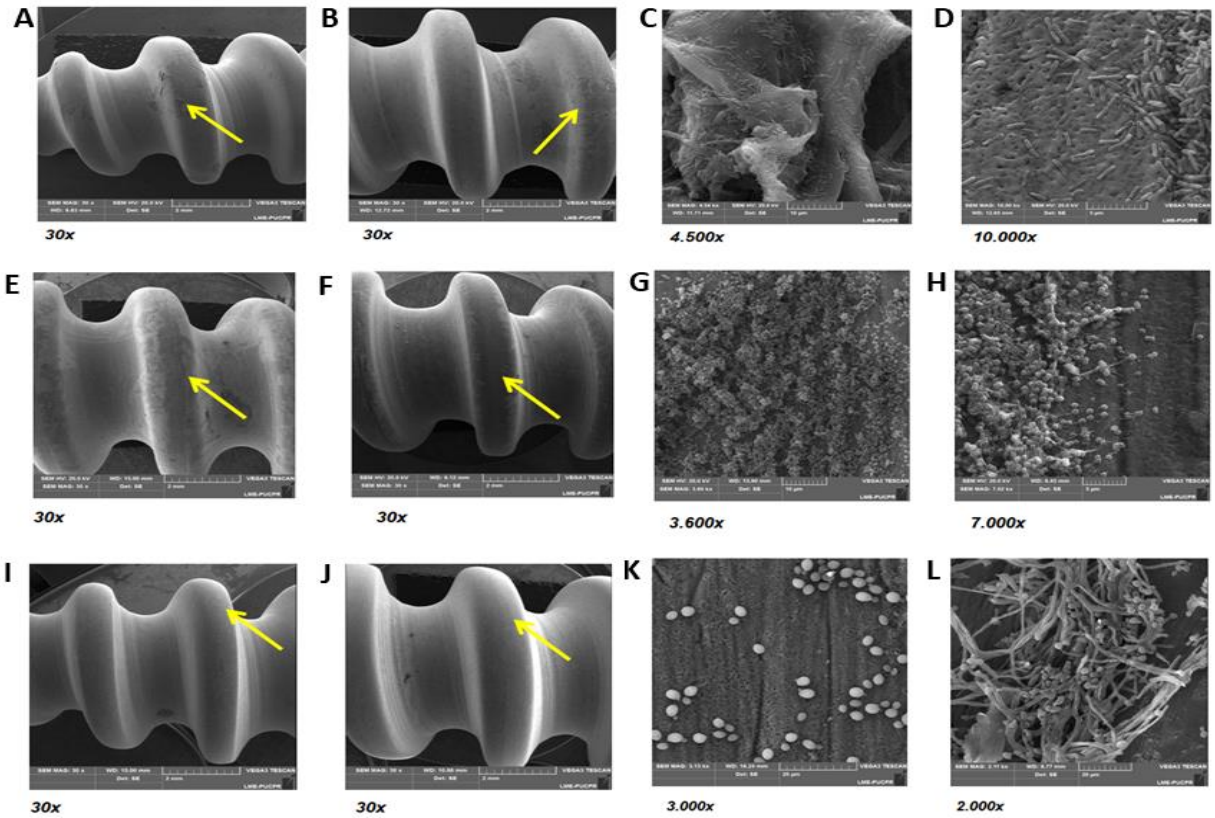

Figure 3: Scanning electron microscopy showing biofilm adhesion on the surface of the titanium screw. (A and C) *P. aeruginosa* biofilm before sonication. (B and D) *P. aeruginosa* biofilm after sonication. (E and G) *S. aureus* biofilm before sonication. (F and H) *S. aureus* biofilm after sonication. (I and K) *C. albicans* biofilm before sonication. (J and L) *C. albicans* biofilm after sonication. Yellow arrows indicate biofilm attached to the screw surface.

After processing the screws, the sonicated fluid was cultured for colony count and bioburden estimation in the post sonication sample. The bacterial counts in CFU/mL of sonicated fluid are shown in Table 1. As expected, the count obtained in the group that was immediately processed was high, around 10^7 CFU/mL for bacteria and 10^5 CFU/mL for yeast. Bacterial bioburden decreased after the refrigeration period for seven days. This reduction was significant in the screws treated with *P. aeruginosa* and *S. aureus* and stored in plastic bag (figure 3).

After sonication, sonicated fluid was used for direct microbial detection using MALDI-TOF mass spectrometry. All microorganisms involved in screw biofilms were detected with 99.9% reliability.

	***S. aureus* (CFU/mL)**		***P. aeruginosa* (CFU/mL)**		***C .albicans* (CFU/mL)**	
	Immediately	**Post refrigeration**	**Immediately**	**Post refrigeration**	**Immediately**	**Post refrigeration**
RC 1	1.2×10^8	5×10^6	9×10^7	4.3×10^7	1.2×10^5	1.3×10^5
RC 2	1.3×10^8	1.2×10^7	9.3×10^7	3×10^6	1.6×10^5	1×10^5
RC 3	1.6×10^8	3.9×10^8	7.1×10^7	9×10^6	1.4×10^5	1×10^5
RC 4	1.6×10^8	2.1×10^8	1.2×10^8	2.5×10^7	9.1×10^4	1×10^5
RC 5	3.5×10^8	2.4×10^8	7.9×10^7	4×10^6	1.1×10^5	1×10^5
RC 6	3.6×10^8	2.6×10^8	1.7×10^7	2.9×10^8	1.1×10^5	1.4×10^5
Median	1.4×10^8	2.3×10^8	8.4×10^7	1.7×10^7	1.2×10^5	1×10^5
PB 1	3.1×10^8	4.1×10^7	1.8×10^7	1×10^6	5.9×10^4	9.1×10^4
PB 2	4.5×10^8	5.1×10^7	1.8×10^8	2.1×10^7	9.5×10^4	7.7×10^4
PB 3	5.3×10^8	1.3×10^8	2.3×10^8	3×10^6	1.2×10^5	8.7×10^4
PB 4	5.4×10^8	4.1×10^8	1.6×10^8	4×10^6	1.5×10^5	1×10^5
PB 5	4.9×10^8	1×10^8	8×10^6	1×10^6	6.8×10^4	8.2×10^4
PB 6	4.1×10^8	1.8×10^7	7×10^6	1×10^6	1×10^5	6.7×10^4
Median	4.7×10^8	7.9×10^7	9×10^7	2×10^6	9.9×10^4	8.4×10^4

Table 1: Bioburden in CFU/mL of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* in sonicated fluid samples, in immediate and post-refrigeration groups, with six replicates each (RC: Rigid Container; PB: Plastic Bag).

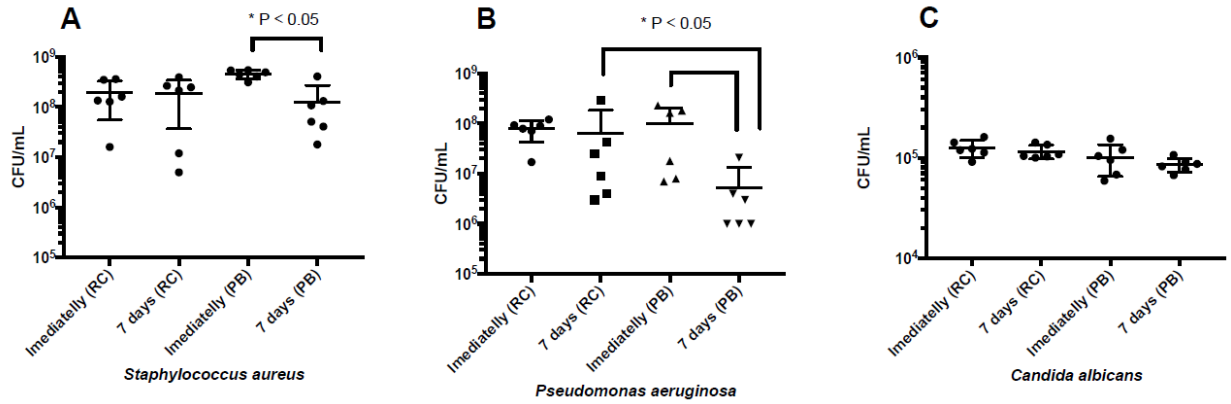

Figure 4: (A) Distribution of *S. aureus* samples after different processing conditions; (B) Distribution of *P. aeruginosa* samples after different processing conditions; (C) Distribution of *C. albicans* samples after different processing conditions.

For direct molecular detection of microbial gDNA, standard curves of 16S rDNA and ITS-2 region were previously created. The Ct_{curve} values decreased progressively with bacterial load. For *P. aeruginosa*, the values varied between 14.76±0.08 for 10^8 CFU/mL and 33.94±0.31 for 10^2 CFU/mL, with an efficiency of 100.93%, slope value of -3.3, and R^2 value of 0.997. For *S. aureus*, they varied between 17.05±0.35 for 10^8 CFU/mL and 36.32±0.35 for 10^2 CFU/mL, with an efficiency of 100.05%, slope value of -3.321, and R^2 value of 0.996. The results of *C. albicans* varied between 17.72±0 for 10^6 CFU/mL and 34.95±0.19 for 10^1 CFU/mL, with an efficiency of 95.72%, slope value of -3.429, and R^2 value of 0.999. The LOD obtained was 10^3 UFC/mL for both *P. aeruginosa* and *S. aureus*, and 10^1 UFC/mL for *C. albicans*. After the LOD definition, the sonicated fluid samples were analyzed. The molecular targets (16S rDNA and ITS-2 region) were detected in all the samples. The mean Ct_{sample} of each group is shown in Table 2.

		Immediately	Post Refrigeration
S. aureus	**RC**	20.484	20.071
	PB	20.465	18.650
P. aeruginosa	**RC**	18.532	18.474
	PB	17.838	19.679
C. albicans	**RC**	22.021	22.832
	PB	22.541	22.625

Table 2: Mean Ct_{sample} obtained in qPCR of sonicated fluids, processed immediately and after refrigeration, in RC and PB groups (RC: Rigid Container; PB: Plastic Bag).

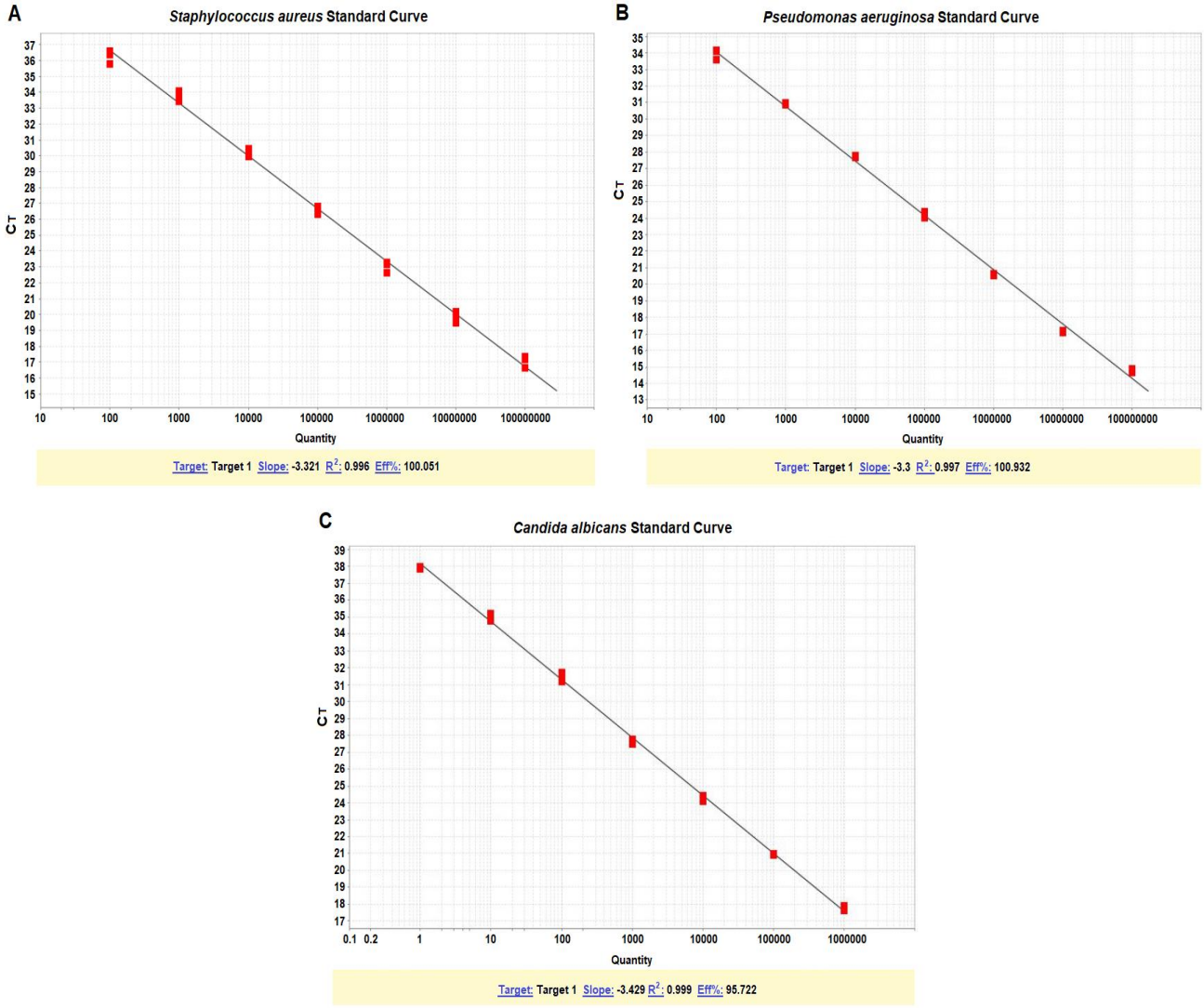

Figure 5: (A and B) Standard curves of 16S rDNA (*S. aureus* and *P. aeruginosa*); (C) Standard curve of ITS-2 region (*C. albicans*).

DISCUSSION

In this model, a bacterial suspension of 10^7 CFU/mL and a yeast suspension of 10^5 CFU/mL was used to create an in vitro biofilm. The colony count obtained after sonication was high, around 10^7 CFU/mL for bacteria and 10^5 CFU/mL for yeast. Sonication uses ultrasound to remove particles adhered to a surface. In liquid media, ultrasound propagates with low attenuation and forms bubbles of high-pressure vapor that, when colliding with the surface, remove what is adhered, including the biofilm. For the smooth detachment of the biofilm, the implant is sonicated in a liquid medium with low frequency ultrasound. During sonication, the biofilm is removed by means of low energy cavitation bubbles, so that there is no significant destruction of the microorganisms, as we observed (1).

When comparing the bioburden obtained in screws stored in RC or PB, if processed immediately, there was no significant difference in colony counts. The screws were refrigerated for seven days to analyze if this condition could maintain the bioburden without change, thus allowing for further detection of microbial growth in the culture. Bacterial bioburden decreased in most groups after the refrigeration period of seven days. This reduction was significant in the groups of screws with *P. aeruginosa* stored in plastic bags, where the count in the immediate group was 9×10^7 CFU/mL and the count in the post-refrigeration group was 2×10^6 CFU/mL ($p < 0.05$). This was also observed with *S. aureus* colony counts, which reduced from 4×10^8 CFU/mL in the immediate SP to 1×10^7 CFU/mL in post-refrigeration SP ($p < 0.05$).

Some factors are determinants of microbial viability, especially that of prokaryote microorganisms, such as temperature and humidity. Low temperatures favor the loss of water on a solid surface, and studies have shown that the consequences of desiccation of the

microorganisms include shrinkage of capsular layers, an increase in intracellular salt concentrations, macromolecule agglomeration, changes in cell compartment volumes, changes in biophysical properties (e.g., surface tension), reduced fluidity (viscosity increase), damage to the external layers such as pili and membranes, electric charge acquisition, and changes in physiological processes (e.g. growth stoppage) (24, 25). We observed that significant reductions in CFU counts occurred only in groups stored in the plastic bag. The rigid containers, which have a thicker surface and are completely sealed, can act as a protective factor. They avoid loss of water and contribute to the maintenance of microbial viability. The plastic bag in this model did not form a complete seal, so changes in temperature and humidity may have affected the microorganisms in a more important way, favoring cell death. For *C. albicans*, no significant reduction in counts was observed when collecting containers and processing times.

Although the bioburden of *S. aureus* and *P. aeruginosa* decreased by approximately one *log* after the refrigeration period, this variation is not important for this model as it did not affect the detection in culture owing to the high counts obtained (higher than 10^4). However, in this model, a bacterial suspension of 10^7 CFU/mL was used to create an *in vitro* biofilm. Therefore, the counts obtained after sonication were expected to be higher. It is necessary to consider whether the refrigeration period would affect microbial recovery in an explanted prosthesis during a surgical procedure, where although biofilm is involved, the bioburden is usually smaller. In these samples, the bioburden can range from 10^1 to 10^5 (1, 11, 26, 27). Recent studies have evaluated sonicated fluids for cultures and have reported counts of over 10^2 CFU/mL for most samples (5, 28, 29). A clinical study to evaluate the bioburden obtained after prosthesis sonication could further throw light upon this issue.

In general, using the extraction protocol chosen for this study, it was possible to detect microorganisms directly from the sonicated fluid using mass spectrometry. However, the ability of this method to identify microorganisms depends on microbial concentrations. The sensitivity of MALDI-TOF MS for microbial identification is between 10^4 and 10^5 CFU (30). Previous studies that evaluated the use of MALDI-TOF for microbial identification in urine samples showed that concentrations between 10^4 and 10^5 CFU/mL were necessary for correct detection (19, 31). Therefore, microbial identification in sonicated fluid is feasible, provided that the sample has enough bioburden. This may advance diagnostic techniques of prosthetic and orthopedic device infections, since this direct detection precludes the necessary microbial growth period in culture for its subsequent identification (at least 24 hours). This means that just 2 hours after the prosthesis has been removed, we can tell the doctor which microorganism is adhering to the device and is likely causing the infection.

Prior to sample analysis by qPCR, it was necessary to determine the assay efficiency and analytical sensitivity. For that, standard curves of 16S rDNA and ITS-2 region were obtained. These curves showed the detection of molecular targets even at the lowest concentrations and the coefficient of determination (R^2) obtained was close to 1, which was satisfactory for both curves (32). The LOD obtained was 10^3 for both *P. aeruginosa* and *S. aureus*, and 10^1 for *C. albicans*, since at this concentration, all of the 30 repetition curves were positive/amplified. The Ct is the cycle number at which the fluorescence generated by real-time PCR crosses the fluorescence threshold. The more amplified the product (in this case, 16S rRNA and ITS-2 region) is in the sample, the lower is the Ct value, as fewer amplification cycles will be required to detect it. The Ct_{curve} values were used to evaluate the performance of 16S rDNA qPCR. In this study, at the

lowest detectable concentration (10^3), the Ct_{curve} for *P. aeruginosa* was 30.91 and for *S. aureus*, it was 33.74. For *C. albicans*, at concentration 10^1, the Ct_{curve} was 34.95.

After defining the method sensitivity, sonicated fluid samples were analyzed. In all samples 16S rDNA and the ITS-2 region were detected. According to Ct_{sample} values, there were no differences between detection in screws processed immediately or after refrigeration, in RC and PB groups. Although this method does not have excellent clinical applicability, as it only shows the presence of bacterial DNA in the samples, it is the basis for future studies using multiplex PCR with probes directed to specific microorganisms, as previously described in studies using clinical samples (33-35).

We obtained high CFU counts because we started from a high inoculum to *in vitro* biofilm formation, which does not correspond to true bioburden in orthopedic devices, where biofilm is present. In a clinical study, counts less than 10^3 CFU/mL may not be detected by mass spectrometry or molecular methods. Despite that, it is possible to sonicate at a certain volume and concentrate all suspended microorganisms. This may increase the total amount of microbial cells and achieve LOD. Moreover in this model, the sonication fluid is basically composed of physiological solution. In a clinical model, detachment of cell debris and other substances that have adhered to the prosthesis may be interfering. Nevertheless, these methodologies have been previously described in blood culture samples. A clinical study is necessary to evaluate this issue.

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CONFLICTS OF INTEREST

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5. CONSIDERAÇÕES FINAIS

Foram desenvolvidos modelos *in vitro* de biofilmes de cepas de *Pseudomonas aeruginosa*, *Staphylococcus aureus* e *Candida albicans,* produzidos nos parafusos ortopédicos de titânio, que foram posteriormente visualizados por microscopia eletrônica de varredura (MEV). Observou-se a presença de células bacterianas e leveduriformes, envoltas em uma camada de matriz extracelular. Após a sonicação destes parafusos, ainda foi possível observar a presença dos micro-organismos aderidos à superfície dos parafusos.

Posteriormente, foram avaliadas as diferentes condições de processamento. Um conjunto de parafuso foi processado logo após a obtenção do biofilme maduro, simulando o envio imediato de uma prótese/dispositivo retirado em centro cirúrgico para processamento no laboratório de análises clínicas. Outro conjunto foi mantido em refrigeração, simulando o processamento de uma prótese/dispositivo que não pôde ser enviado imediatamente para o laboratório. Ainda, foram analisados dois tipos de recipientes para coleta: saco plástico e recipiente rígido. Finalmente, foi possível comparar as contagens de UFC obtidas após a sonicação dos parafusos. Concluiu-se que, no grupo em que a coleta foi feita em saco plástico e refrigerado antes do processamento, houve redução na recuperação bacteriana, mas que este modelo precisa ser estudado em amostras clínicas para avaliar se esta redução é clinicamente relevante e se pode interferir na detecção microbiana.

Foi possível detectar os micro-organismos diretamente no fluído sonicado por MALDI-TOF e qPCR em todos os grupos de parafusos analisados. Estes métodos podem trazer agilidade no diagnóstico das infecções associadas a dispositivos ortopédicos e auxiliar na conduta antimicrobiana, visto que a identificação microbiológica pode ser realizada e informada ao clínico em uma ou duas horas, ao passo que para a identificação por cultura convencional seriam necessárias 24 a 72 horas.

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