Effects of different pretreatment methods on microbial recovery of infected tissues

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Abstract

This study aimed to evaluate the effects of different pretreatment methods on the degree of microbial recovery in infected tissues. Standard strains of *Staphylococcus aureus* (SA), *Escherichia coli* (EC) and *Candida albicans* (CA) were used to construct single-surface, full-surface, and internal infection models in sterile pork tissue. Manual milling (MM), mechanical homogenization (MH), ultrasonic lysis (UL), dithiothreitol (DTT), and direct culture (DC) were used to pretreat infection tissues, the ability of the different pretreatment methods to achieve pathogen recovery in the different bacterial infection models was compared. At the same time, periprosthetic tissues collected from periprosthetic joint infection (PJI) were pretreated with the same methods. We showed that regardless of whether the single-surface or full-surface infection model was used in SA, EC, and CA infection, the microbial acquisition in the MH group was significantly higher than that in the MM (P <0.01) and UL groups (P <0.01). In the internal infection model, the microbial acquisition of the MH group was significantly higher than that of the MM (P <0.01), UL (P <0.01), DTT (P <0.01), and DC groups (P <0.01). In the PJI cases, the number of bacterial colonies obtained by MH was significantly higher than that obtained by other pretreatment methods (P = 0.004). The effects of MH and DTT in microbial recovery were significantly better than that of DC, UL and MM, and these methods can be used to process multiple tissue samples at the same time, which can further improve the efficiency of clinical microbial diagnosis.

Background

Periprosthetic joint infection (PJI) is a serious complication after joint arthroplasty, which brings a heavy economic burden to patients and society [1]. Microbial culture is essential for the diagnosis and treatment of PJI [2,3]. At present, the traditional laboratory diagnostic methods include synovial fluid, sonication fluid and periprosthetic tissues, but in many cases, synovial/sonication are insufficient, resulting in poor sensitivity of microbial culture. But it is relatively easy to obtain tissue samples intraoperatively, but the positive rate is not high if tissues are not pretreated properly [4]. Due to different various levels toughness of tissues and different distribution of bacteria on the tissue surface, if proper pretreatment is not performed, it will affect the release and recovery of pathogenic bacteria. Therefore, optimizing the pretreatment method of tissue specimens to make full use of PJI tissue samples is of great significance for improving the sensitivity of microbial culture as a diagnostic tool.

The current mainstream tissue pretreatment methods reported in clinical microbiology laboratories are direct tissue culture (DC) or manual milling (MM) [5]. However, MM is a tedious process in which contamination can easily be introduced. Studies have shown that mechanical homogenization (MH), dithiothreitol (DTT), and ultrasonic lysis (UL) can increase the release of bacteria from tissue [678]. However, these methods are still controversial, and currently there are no researches that compares different specimen pretreatment methods in vitro.

Therefore, in this study, the abovementioned pretreatment methods were used to treat infection tissues in vitro and PJI tissue specimens. The recovery colony-forming units (CFU) were compared to evaluate the

efficiency of different pretreatment methods.

Methods

1. Establishment of infection tissues

Fresh pork was frozen and cut into cubes with a size of 0.5 × 0.5 × 0.5 cm^3, each weighing approximately 40 mg. After immersion in diluted penicillin G (500 U/ml) and streptomycin (600 μg/ml) for 1 hour, the samples were washed three times with PBS.

The strain of *Staphylococcus aureus* (*S. aureus*) ATCC 25923 (American Type Culture Collection, USA) the strain of *Escherichia Coli* (*E.coli*) ATCC 25922 (American Type Culture Collection, USA), and the strain of *Candida albicans* (*C. albicans*)ATCC 90029 (American Type Culture Collection, USA) were used to establish infection model in vitro. In order to simulate PJI infection as much as possible, in which pathogen may be distributed on a single surface or multiple surfaces even inside tissues [6], therefore, three tissues infection models were established (*Fig* 1): ▯ Single surface infection: pipette aspirate 10 μL (200 CFU) bacterial dilution (*S. aureus /E. coli/Candida albicans*) to colonized a single surface of the pork cube. ▯ Full surface infection model: Microorganisms were colonized on the entire surface of pork cubes, with 10 μL (200 CFU) for each surface. ▯ Internal infection model: Ten microliters (200 CFU) of bacterial dilution was injected into the center of the pork cube with a syringe for colonization. Fifty pieces of each pathogen model were made, and the control group included pork cubes injected with the same amount of saline.

2. Various pretreatment methods for tissue infection models in vitro

Fifty samples of each infection model were randomly assigned to MH group, MM group, DTT group, UL group, and DC group. For pretreatment: ▯ MH group: tissues were transferred to 2 mL Eppendorf (EP) tubes with 1 mL LB culture medium (Haibo Biotechnology Co., Ltd., Qingdao, Chian), vortexed for 15 min, and put into a fully automatic rapid grinder (Jingxin Industrial Development Co., Ltd., Shanghai, China) at 40 Hz for 60-90 s until the tissue specimen was homogenized. ▯ MM group: tissues were transferred to 2 mL EP tubes with 1 mL LB culture medium and manually ground with a disposable sterile grinding rod until a homogenate appeared. ▯ DTT group: the shock time and DTT concentration established in previous in vitro studies was used [9]. After soaking in 1 g/L DTT (Haibo Biotechnology Co., Ltd., Qingdao, China) for 15 min at room temperature, specimens were transferred to a 2 mL EP tube and 1 mL of LB liquid medium was added to seal the tubes. ▯ UL group: tissues were transferred to 2 mL EP tubes with 1 mL LB culture medium, vortexed and shaken for 30 s and placed in an ultrasonic cleaner (Wuxi Woxin Instrument Co., Ltd., Jiangsu, China), sonicated at 40 Hz for 5 min, vortexed for 30 s, centrifuged at 4 000 r/min for 15 min, the supernatant was discarded, and the pellet was resuspended in sterile saline. ▯ DC group: tissues were transferred to 2 mL EP tubes with 1 mL LB culture medium and vortexed for 15 min.

After pretreatment, the inoculated specimens were centrifuged at 5500 rpm/min after the above four tissue processing methods for 20 s to spin down impurities. After centrifugation, 100 μL of each solution was inoculated onto Columbia blood agar plates (Thermo Fisher Scientific, USA) and cultured overnight at 37 °C in a biochemical incubator (Shanghai Qixin Scientific Instrument Co., Ltd, China). And CFUs were obtained and calculated.

3. Various tissues pretreatment methods performed on periprosthetic tissues from PJI

A total of 30 original specimens were collected from 8 PJI patients according to the American Society for Musculoskeletal Infection (MSIS) criteria[10]. Each original specimen was divided into 5 equal portions (each = 50 mg) and treated with the above methods (MH, MM, DTT, UL, DC) to obtain inoculated specimens.

One hundred microliters of each of the inoculated samples was inoculated onto Columbia blood agar plates (Qingdao Haibo Biotechnology Co., Ltd., HBPM0153) and CDC anaerobic blood agar plates (Qingdao Haibo Biotechnology Co., Ltd., HBPM16) under aerobic and anaerobic conditions, respectively. Samples were incubated in a biochemical incubator (Shanghai Qixin Scientific Instrument Co., Ltd., China) at37 ℃ for 14 days, and colony growth was observed 2, 4, 7, and 14 days after incubation. If there was colony growth, the CFU per ml were calculated.

This study was approved by institutional review board, and all patients signed informed consent forms.

4. Statistical analysis

The continuity variables were expressed as mean±standard deviation. One- way ANOVA was used to compared the differences between groups. All statistical analysis was performed on GraphPad Prism 8 .0. P <0.05 was considered statistically significant.

Results

1. **Microbial biomass recovered from single-surface infection models by various methods**

In the SA infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 611 ± 101 CFU/ml (95% CI: 538-683), 999 ± 141 CFU/ml (95% CI: 898- 1100), 609 ± 96 CFU/ml (95% CI: 540-679), 938 ± 136 CFU/ml (95% CI: 840-1035), and 533 ± 108 CFU/ml (95% CI: 455-611), respectively. The amount of microorganisms obtained in MH group was significantly higher than that in the MM (P <0.01), SC (P <0.01), and UL groups (P <0.01), but there was no difference with that of the DTT group (P = 0.26). (*Fig* 2A).

In the EC infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 451 ± 192 CFU/ml (95% CI: 313-588), 1373 ± 132 CFU/ml (95% CI: 1278-1468), 480 ± 135 CFU/ml (95% CI: 383-576), 1347 ± 162 CFU/ml (95% CI: 1231-1463), and 393 ± 178 CFU/ml (95% CI: 265-520), respectively. The amount of microorganisms obtained in the MH group was significantly higher than that in the MM (P <0.01), UL (P <0.01), and DC groups (P <0.01), but there was no difference from that of the DTT group (P = 0.71). (*Fig* 2A).

In the CA infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 440 ± 70 CFU/ml (95% CI: 391-488), 818 ± 122 CFU/ml (95% CI: 730-905), 435 ± 68 CFU/ml (95% CI: 386-484), 771 ± 108 CFU/ml (95% CI: 694-849), and 360 ± 85 CFU/ml (95% CI: 298-421), respectively. The amount of microorganisms obtained in the MH group was significantly higher than that in the MM (P <0.01), UL (P <0.01) and DC groups (P <0.01), but there was no difference with that of the DTT group (P = 0.945). (*Fig* 2A).

2. **Microbial biomass recovered from full-surface infection models by various methods**

In the SA infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 4014 ± 888 CFU/ml (95% CI: 3378-4650), 6268 ± 1019 CFU/ml (95% CI: 5539-6997), 3960 ± 875 (95% CI: 3334-4568) CFU/ml, 6227 ± 1000 CFU/ml (95% CI: 5512-6942), and 3255 ± 1048 CFU/ml (95% CI: 2505-4005), respectively. The amount of microorganisms obtained in the MH group was significantly higher than that in the MM (P <0.01), UL (P <0.01) and DC groups (P <0.01), but there was no difference compared with that of the DTT group (P = 0.924). (*Fig* 2B).

In the EC infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 3079 ± 934 CFU/ml (95% CI: 2410-3747), 7058 ± 920 CFU/ml (95% CI: 6400-7717), 3060 ± 902 CFU/ml (95% CI: 2415-3706), 6853 ± 858 CFU/ml (95% CI: 6239-7466), and 2533 ± 637 CFU/ml (95% CI: 2077-2989), respectively. The amount of microorganisms obtained in the MH group was significantly higher than that in the MM (P <0.01), the UL (P <0.01), and DC groups (P <0.01), but there was no difference compared with that of the DTT group (P = 0.964). (*Fig* 2B).

In the CA infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 3080 ± 842 CFU/ml (95% CI: 2477-3682), 5274 ± 966 CFU/ml (95% CI: 4583-5966), 2971 ± 890 CFU/ml (95% CI: 2334-3608), 5269 ± 853 CFU/ml (95% CI: 4658-5879), and 2287 ± 851 CFU/ml (95% CI: 1678-2896), respectively. The amount of microorganisms obtained in the MH group was significantly higher than that in the MM (P <0.01), UL (P <0.01), and DC groups (P <0.01), but there was no difference compared with that of the DTT group (P = 0.989). (*Fig* 2B).

3. **Microbial biomass recovered from internal infection models by various methods**

In the SA infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 267 ± 68 CFU/ml (95% CI: 218-316), 636 ± 90 CFU/ml (95% CI: 571-701), 300 ± 73 CFU/ml (95% CI: 247-353), 270 ± 63 CFU/ml (95% CI: 224-315), and 208 ± 95 CFU/ml (95% CI: 140-277), respectively. The amount of

microorganisms obtained in the MH group was significantly higher than that in the MM (P <0.01), SC (P <0.01), DTT (P <0.01), and UL groups (P <0.01). (*Fig* 2C).

In the EC infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 313 ± 78 CFU/ml (95% CI: 257-369), 698 ± 134 CFU/ml (95% CI: 601-794), 298 ± 72 CFU/ml (95% CI: 246-351), 306 ± 93 CFU/ml (95% CI: 239-373), and 234 ± 82 CFU/ml (95% CI: 175-293), respectively. The amount of microorganisms obtained in the MH group was significantly higher than that in the MM group (P <0.01), UL group (P <0.01), DTT group (P <0.01), and DC group (P <0.01). (*Fig* 2C).

In the CA infection groups, the average CFU of the MM, MH, UL, DTT, and DC groups were 169 ± 60 CFU/ml (95% CI: 125-212), 359 ± 55 CFU/ml (95% CI: 319-399), 164 ± 58 CFU/ml (95% CI: 122-206), 172 ± 58 CFU/ml (95% CI: 130-213), and 119 ± 47 CFU/ml (95% CI: 85-154), respectively. The amount of microorganisms obtained in the MH group was significantly higher than that in the MM group (P <0.01), UL group (P <0.01), DTT group (P <0.01), and DC group (P <0.01). (*Fig* 2C).

4. **Culture results incontrol samples**

Negative (sterile) control samples consisted of 30 cubes of pork tissue (10 for each single-surface/full-surface/internal model). In the MM group, growth of *Streptococcus viridans* and *Staphylococcus epidermidis* was found in the negative control of the single-surface and full-surface models, respectively. In the negative control of the UL group, a pork cube was found to have growth of the bacterial contaminant *Corynebacterium* (Table 1).

5. **Tissue culture results of periprosthetic joint infection specimens**

The numbers of colonies obtained from PJI patient specimens subjected to various pretreatment methods are listed in Table 2. Out of a total of 30 original samples from 8 patients, 10 original samples received at least one positive result, distributed in 7 cases. Only one patient's 4 original specimens were negative with the various pretreatment methods. The total number of pathogenic bacterial colonies obtained by MH was significantly higher than that obtained by the other pretreatment methods (*Fig* 2D, P = 0.004).

Discussion

This study differs from other studies in that it provides a more comprehensive assessment of all currently reported pretreatment methods, with recovery capabilities for different representative pathogens and different distribution models. This study used fresh pork as a specimen for in vitro experiments since multiple infection methods were analyzed, and a large amount of sample was required. It was not possible to obtain enough soft tissue from a single human case for the experiment to ensure sample uniformity [11]. To reduce the possibility of contamination, the pork cubes were immersed in PBS containing double antibodies (mixture of penicillin and streptomycin) for 1 hour and then washed for

later use. Thereafter, we used tissue samples from clinically confirmed infection cases to further verify the ability of the above methods to recover bacteria.

To study the effects of different pretreatment methods on different microbial species, we selected *S. aureus*, *E. coli*, and *C. albicans* as representatives of gram-positive bacteria, gram-negative bacteria, and fungi to construct infection models. The single-surface inoculation of 2×10^2 bacteria avoided massive bacterial growth and facilitated counting [12]. The inoculation volume of 10 μL was based on our experimental experience. This amount of inoculum was stable enough for colonization but the amount of fluid was not excessive enough for it to slip off the surface of the tissue specimen.

A contradiction exists in using pretreatment methods, because insufficient pretreatment can result in not enough bacteria being released, and excessive pretreatment may reduce bacterial viability. Mohamed Askar reported that the use of mechanized steel ball grinding and homogenization may reduce the recovery of bacteria from tissue samples [6]. The results of this study showed that the various pretreatment methods had no significant effect on the viability of various bacteria. This may have been related to our use of precooled working fluid for various pretreatment operations to reduce heat generation from processing and thereby avoid loss of bacterial vitality.

This study found that the average CFU acquired with MH in every model was more than that with DC and MM, which is basically consistent with the results of Sylvio Redanz *et al*. [11]. The pretreatment methods of DTT and SF were added in this study because the literature reports that the mucolytic agent DTT can homogenize tissues and release the microorganisms in tissue samples [7]. The application of SF to break the biofilm on the surface of a prosthesis and to thus release bacteria has been recognized by many researchers [13–16]. The results of this study show that DTT's ability to separate bacteria on the surface is similar to that of MH, while SF's ability to elute bacteria from tissue surfaces is not as good as that of MH and DTT. In the internal infection model, only MH facilitated high levels of bacterial isolation, and DTT's bacterial recovery ability dropped to the same level as that of MM and UL. The above results confirm that MH has a good ability to recover pathogens under various complex conditions.

Finally, we collected tissue specimens from PJI patients diagnosed according to MSIS standards and processed the specimens with MH, MM, DTT, and UL. The results were consistent with the animal experiments. The number of colonies obtained by MH was significantly greater than that obtained by other treatment methods, which further confirms the superiority of MH compared with other methods under clinical conditions. This is also consistent with the reports of Mohamed Askar, Sylvio Redanz and others [611].

Because tissue sample processing takes longer than synovial fluid processing, false positives caused by contamination during processing are also a concern. This study found that the MH and DTT methods were not associated with false positives in the negative control samples. Two samples were contaminated with the MM method, and one sample was contaminated with the SF method. The reason may be that, with MH and DTT, the samples are basically sealed during processing, and MM exposes the

samples to air during grinding, while the use of a water bath in the SF method increases the chance of contamination.

This study has the following limitations: This study only selected common pathogens, such as *S. aureus*, *E. coli*, and *C. albicans*, and did not study rare pathogens associated with PJI, such as *Mycobacterium tuberculosis* and non-tuberculous mycobacteria, mycoplasma, etc. ; 2. The purpose of this study was to evaluate the recovery rate of bacteria by different tissue treatment methods, and it was not possible to clearly determine their actual diagnostic efficiency in clinical applications.

Conclusion

Mechanical homogenization and dithiothreitol released the bacteria in tissues significantly better than ultrasonic lysis, manual milling, and direct culture methods. In internal infection models, the effect of mechanical homogenization was better than that of dithiothreitol. The mechanical homogenization and dithiothreitol methods were not conducive to contamination, and multiple tissue samples could be processed at the same time with these methods, which may improve the efficiency of clinical microbial diagnosis.

Abbreviations

SA: *Staphylococcus aureus*; and

EC: *Escherichia coli*;

CA: *Candida albicans*;

MM: Manual milling;

MH: mechanical homogenization;

UL: ultrasonic lysis);

DTT: dithiothreitol;

DC: direct culture;

ATCC: American Type Culture Collection;

PJI: Periprosthetic joint infection;

CFU: colony-forming units.

Declarations

Ethics approval and consent to participate: This study was approved by the Ethics Committee and Institutional Review Board of our institution. Informed consent was obtained from each patient before the data was collected.

Consent for publication: Participants gave informed consent for publication.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no conflicts of interest.

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Authors' contributions: X Fang, Y Cai, L Zhang: analyzed data, wrote the manuscript; X Yang, L Nie : literature review, collected data;, W Li, B Yang: revised manuscript; Z Huang, C Zhang: English edited; W Zhang, Z Guan: created the concept, designed the study, analyzed data, wrote the manuscript.

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Tables

Table 1 Detection and quantification of microorganisms in sterile pork cubes of various infection models

	MH	MM	DTT	UL	DC
Single surface infection model	0/10	1/10 *Streptococcus* green (10 CFU)	0/10	0/10	0/10
Full surface infection model	0/10	1/10 *Staphylococcus epidermidis* (20 CFU)	0/10	0/10	0/10
Internal infection model	0/10	0/10	0/10	1/10 Corynebacterium (20 CFU)	0/10

Table 2 Number of colony forming units (CFU) obtained from PJI patient specimens by various pretreatment methods

Original specimen	Pathogenic bacteria	MH	MM	DTT	UL	DC
1 (case 1)	*Staphylococcus epidermidis*	10	0	0	1	0
2 (case 2)	*Staphylococcus aureus*	360	50	179	60	34
3 (case 3)	*E.coli*	187	23	45	8	10
4 (case 3)	*E.coli*	68	40	20	23	30
5 (case 5)	*Staphylococcus epidermidis*	96	22	36	28	16
6 (case 6)	*Enterococcus faecalis*	46	0	33	0	0
7 (case 6)	*Enterococcus faecalis*	89	17	46	34	0
8 (case 7)	*Staphylococcus epidermidis*	147	0	36	28	0
9 (case 7)	*Staphylococcus epidermidis*	15	0	0	0	0
10 (case 8)	*Staphylococcus aureus*	283	12	16	26	10
Total		1041	164	329	208	100

Figures

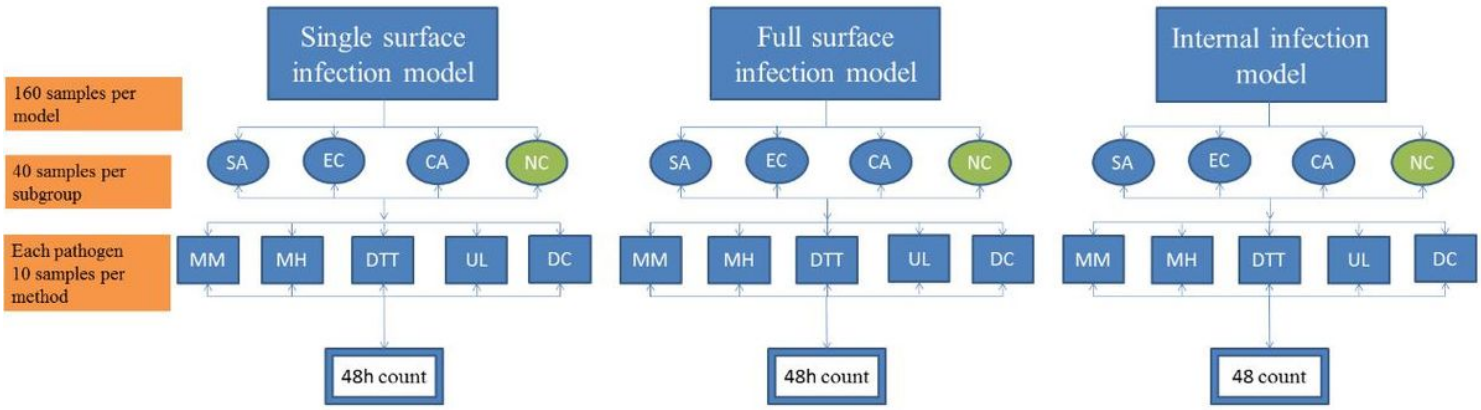

Figure 1

Establishment of infection tissues in vitro.

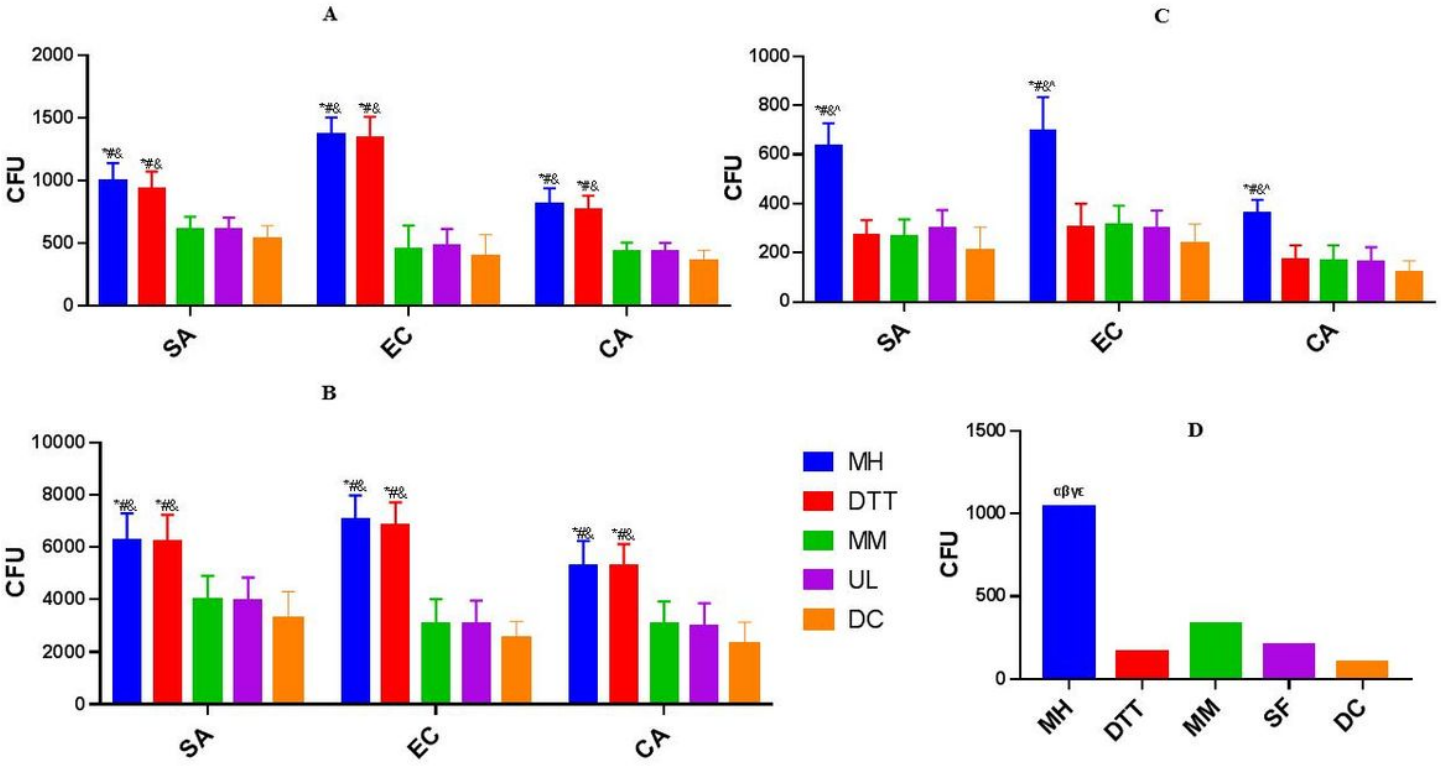

Figure 2

Quantitative recovery of microorganisms from inoculated tissues. Tissue cubes were separately inoculated on 1 surface (A), on six surfaces (B), or on the inside of the samples (C) with 2 × 102 CFU of S. aureus, E. coli, or C. albicans, respectively. Number of colony forming units (CFU) obtained from PJI patient specimens by various pretreatment methods(D).CFU = colony forming units; *P < 0.01 compared to the DTT group, #P< 0.01 compared to the MM group, &P< 0.01 compared to the SF group, ^P< 0.01 compared to the DC group. αP < 0.05 compared to the DTT group, β P< 0.05 compared to the MM group, γP < 0.05 compared to the SF group, εP < 0.05 compared to the DC group