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Evaluation of Mouse Wound Models for Probiotics-Based Wound Infection Prevention Study



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14. ABSTRACT Probiotics are live microorganisms that have shown a health benefit when taken in adequate amounts. Typically, these are given as food supplements and have been shown to play a role in disease remediation, particularly in gastrointestinal conditions such as inflammatory bowel disease. In the case of a diarrheal infection, probiotics have been thought to assist in reduction of “bad” bacteria through competition by “good” bacteria. There has been some interest in using these organisms in early wound care to aid in prevention of infection in both traumatic and surgical settings. We used established methods to produce typical wounds in a reproducible manner using a murine model. The wound models were challenged with known infection-causing bacteria and then treated with either probiotic bacteria or a control. However, the primary goal was not to develop a probiotic treatment regime but rather to develop a murine model that is appropriate for rapid screening of any experimental wound treatment and is also suitable for determination of parameters of that treatment. We attempted to create four wounding models: (1) simple full thickness skin incision, (2) stitched skin flap, (3) burn (with liquid nitrogen), and (4) skin incision with circulatory disruption. Of the four wound models attempted, only two were completely reproducible (models 1 and 2). Model 4, producing the circulatory disruption by tourniquet, is easily reproducible, but creating a punch on the extremity of the mouse was not reproducible. This model was not pursued, as it could not be consistently reproduced using mice. It is advised that if this model is of interest, a larger species, such as rats, should be used. The punch and skin flap models are reproducible murine models for wounding and infection. The use of probiotic (<i>Lactobacillus reuteri</i>) had limited efficacy in reducing <i>Pseudomonas aeruginosa</i> . Probiotic reduced <i>Pseudomonas aeruginosa</i> infection in model 1 but not model 2. Probiotic had no beneficial effect in limiting <i>Streptococcus pyogenes</i> infection.					
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1.0 SUMMARY

Despite the wide use of antibiotics in trauma care, infection remains a common complication and represents a serious source of morbidity and mortality. Infection rates for field injuries can vary widely due to environmental conditions at the time of injury and the type of injury. Often, extremity injuries have impaired vascularization that can further reduce the natural defenses against infection. Poorly vascularized injuries, under suboptimal environmental conditions, can be further impacted by delays in treatment due to transit time to a point of primary care. New treatments that can improve patient care in these situations would be highly desirable. The objectives of this study were to (1) establish reproducible, small animal models of infectious wounds and (2) assess if probiotics represent an effective therapy for infected wounds. Four wound models—simple full thickness injury (“punch”) model, stitched skin flap model, cryo burn model, and extremity injury model—were inoculated with *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Lactobacillus reuteri*, or a combination. After 48 hours the mice were sacrificed and biopsies were collected for analysis. All wound biopsies were evaluated for bacterial load using a manual counting method and reported as colony-forming units per gram tissue. Both the punch and skin flap models are viable, reproducible mouse models of infection. The cryo burn and skin incision with circulatory disruption models were not reproducible. Application of probiotic (*L. reuteri*) had some limited efficacy in reducing *P. aeruginosa*. Probiotic significantly reduced *P. aeruginosa* infection in the punch model but not the skin flap model. Probiotic had no beneficial effect in limiting *S. pyogenes* infection in either model.

2.0 INTRODUCTION

Probiotics are live microorganisms that literature has shown can confer a health benefit when taken in adequate amounts [1-3]. Typically, these are given as food supplements and have been shown to play a role in disease remediation, particularly in gastrointestinal conditions such as inflammatory bowel disease. In the case of a diarrheal infection, probiotics have been thought to assist in reduction of “bad” bacteria through competition by “good” bacteria. Recently, there has been some interest in using these organisms in early wound care to aid in prevention of infection in both traumatic and surgical settings. Such a product could be lyophilized and incorporated into a field trauma kit.

However, to properly assess if probiotics can be used in this manner, it is critical to evaluate them in an appropriate animal model. The model should be low cost, yet closely approximate the early wound as it may be experienced by the warfighter. To this end, the objective of this study was to evaluate the ability of probiotics to limit and/or reduce bacterial infection in different models of wounds, from simple dermal wounds to more complex wounds [4-7].

The University of Cincinnati was contracted to conduct this study with two specific objectives: (1) to establish reproducible, small animal models of infectious wounds and (2) to assess if probiotics represent an effective therapy for infected wounds.

3.0 MATERIALS AND METHODS

3.1 Animals

This study was approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. All research was conducted in compliance with applicable federal laws and regulations relating to animals used in experimentation. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

CF1 (6-week; male) mice were acquired through The Jackson Laboratory (Bar Harbor, ME). The CF1 mouse, an albino outbred mouse strain, serves as a general multipurpose model for research. The mice were received from the same supplier, acclimated, randomly separated into their respective groups, and then placed into individual cages 1 day before the study for further acclimation.

3.2 Bacterial Growth

Pseudomonas aeruginosa SBI-N (gift from Shriner's Hospital, Cincinnati, OH) was grown in trypticase soy broth (BD, Franklin Lakes, NJ) for 15-18 hours at 37°C in an incubator with constant shaking at 200 RPM. The bacteria were spun down at 3000 g and resuspended in 2.5 mL Dulbecco's phosphate-buffered saline (DPBS), and a 20% transmittance sample was made in DPBS using a Spectronic 20D+ (Milton Roy). Seven 1:10 dilution samples were made, and 100 µL of each was plated on trypticase soy agar plates (BD) and allowed to grow overnight in a 37°C incubator with 5% carbon dioxide (CO₂). Colonies were counted (in colony-forming units, CFU) the next day and concentrations calculated.

Streptococcus pyogenes (ATCC 19615, American Type Culture Collection, Manassas, VA) was grown in trypticase soy broth (BD) for 15-18 hours at 37°C in an incubator with 5% CO₂ and no shaking. The bacteria were spun down at 3000 g and resuspended in 2.5 mL DPBS, and a 20% transmittance sample was made in DPBS using a Spectronic 20D (Milton Roy). Seven 1:10 dilution samples were made, and 100 µL of each was plated on trypticase soy agar plates (BD) and allowed to grow overnight in a 37°C incubator with 5% CO₂. Colonies were counted (in CFU) the next day and concentrations calculated.

Lactobacillus reuteri MM4-1A (ATCC PTA-6475) was grown in lactobacilli MRS broth (BD) for 15-18 hours at 37°C in an incubator with 5% CO₂ and no shaking. The bacteria were spun down at 3000 g and resuspended in 2.5 mL DPBS, and a 20% transmittance sample was made in DPBS using a Spectronic 20D (Milton Roy). Seven 1:10 dilution samples were made, and 100 µL of each was plated on lactobacilli MRS agar plates (BD) and allowed to grow overnight in a 37°C incubator with 5% CO₂. Colonies were counted (in CFU) the next day and concentrations calculated.

3.3 Wound Models

3.3.1 Simple Full Thickness Injury ("Punch") Model. The day before surgery, mice were placed under isoflurane and the hair from the dorsal side of the mouse was shaved with a clipper and completely removed using a depilatory cream for 3 minutes. The mice were placed in individual cages and allowed to acclimate overnight. On the day of surgery, mice were placed

under anesthesia using an intraperitoneal ketamine/xylazine mixture. The dorsal skin was disinfected using a betadine scrub followed by a 70% ethanol rinse, two times, and allowed to air dry. The mouse was placed on its side on a sterile sheet. The dorsal skin of the chest from the midline was pulled up with fingers, and a 6-mm-diameter sterile biopsy punch was used through the folded skin (both layers) to create two symmetrical full thickness excisional wounds beside the midline. The wounds were inoculated with 1×10^6 CFU *P. aeruginosa*, 5×10^5 CFU *S. pyogenes*, 5×10^6 CFU *L. reuteri*, or a combination of bacteria with the probiotic in a 25- μ L sample. Control mice underwent the same wounding procedure, but sterile DPBS was used instead of bacteria. The wounds were covered with a sterile N-Terface membrane and the dorsal skin was protected using a Tegaderm dressing. The mice were allowed to recover and sacrificed after 48 hours, and samples were obtained to determine bacterial load.

3.3.2 Stitched Skin Flap Model. The mice were prepped as in the simple full thickness model, but after the punch the skin was lifted on the sides of the punch to produce raised flaps. The wounds were inoculated relaxed but not with the flap open using the same conditions as before. Before the wounds were dressed, two retaining stitches were placed on the sides of the punch to maintain flap closure. Mice were sacrificed after 48 hours and samples were obtained to determine bacterial load.

3.3.3 Cryo Burn Model. The mice were prepped as in the previous two models and a mild freezing injury was produced using a Wartner cryogenic wart removal pen (Medtech Products, Irvington, NY). The dorsal skin was exposed to the pen two times for 15 seconds on each side of the midline. The wounds were allowed to return to room temperature and inoculated using the same conditions as above. The wounds were dressed as before, the mice were sacrificed after 48 hours, and samples were obtained to determine bacterial load. This injury did not produce a sufficient enough wound to make it a viable model.

3.3.4 Extremity Injury Model. The day before surgery, mice were placed under isofluorane and the hair from the hind quarters of the mouse was shaved with a clipper and completely removed using a depilatory cream for 3 minutes. The mice were placed in individual cages and allowed to acclimate overnight. On the day of surgery, mice were placed under anesthesia using an intraperitoneal ketamine/xylazine mixture.

A tourniquet (small braces rubber band) was placed around the upper portion of the hind quarter and a 3-mm sterile biopsy punch was used to create a wound below the tourniquet. The wound was inoculated as before and dressed while the tourniquet remained on the hind quarter. After 2 hours the dressing was removed and the rubber band was cut. New sterile dressings were applied, the mice were sacrificed after 48 hours, and samples were obtained to determine bacterial load. This model was not ideal, as the punch consistently went through the muscle layer, producing a more severe wound.

3.4 Bacterial Growth

After 48 hours, the mice were sacrificed using CO₂ gas and the dressings were removed. Photographs were taken of the wounds and biopsies were collected for analysis. Entire wounds were excised from the mouse, including 2 mm of surrounding tissue, weighed, and placed in either sterile DPBS for bacterial counts or in 10% neutral buffered formalin for histological

analysis (R = histology, L = counts). After all the samples were collected, the wounds were homogenized (Kinematica Polytron, Bohemia, NY) in a 50-mL conical tube containing 2 mL sterile DPBS for 1 minute. The homogenate was filtered through a 70- μ m cell strainer (BD) that was rinsed with 5 mL DPBS. The samples were centrifuged at 3000 g for 10 minutes and room temperature and resuspended in 2 mL DPBS. A 1:10 dilution was made, and 100 μ L was plated on the appropriate plate for culture per bacterial strain. A MacConkey agar plate was inoculated in addition to the bacterial strain plates to measure naturally occurring bacteria in the wounds. The plates were placed in a 37°C incubator with 5% CO₂ overnight, and the CFU colonies were manually counted and concentrations calculated per gram tissue.

3.5 Data Collection

All wound biopsies were evaluated for bacterial load using a manual counting method and reported as CFU/g tissue. Routine hematoxylin and eosin slides were stained and images were acquired using a 10x objective on a ZEISS Axio Imager M2 microscope and an Axiocam 1Cc5 color camera using ZEISS Zen Pro 2012 software (ZEISS, Jena, Germany). A modified Brown-Hopps stain was also performed to stain for Gram-negative and Gram-positive bacteria and was imaged as stated above.

4.0 RESULTS

4.1 Model Development

These wound models consisted of (1) simple full thickness skin incision, (2) stitched skin flap, (3) burn (with liquid nitrogen), and (4) skin incision with circulatory disruption [2,4,8,9].

We initially focused on the simple skin and stitched flap wounds, as these were the most straightforward and relevant models proposed. An initial challenge was the high level of naturally occurring bacteria on the mouse skin. These bacteria prevented accurate assessment of the growth of seeded bacteria. To overcome this problem, we used skin scrubs, which greatly reduced, but did not eliminate, the level of endogenous bacteria. However, the scrubs did reduce the amount of endogenous bacterial to a level that did not interfere with the experimental design.

The burn model using liquid nitrogen-cooled rod did not produce a full thickness burn. We then tried several alternative approaches, including placing the mouse in a mold modeled after our scald burn and then placing the exposed dorsal skin of the mouse on a liquid nitrogen-cooled metal block. This produced a better burn than the glass rod, but the skin stuck to the metal block, which introduced unacceptable variability to the wound. We next tried placing the exposed dorsal skin of the mouse directly into liquid nitrogen. This produced a severe burn, but the skin stuck to the sides of the plastic mold. Next, we produced a burn using a Wartner cryogenic applicator pen. The cooled applicator tip was held on for 20 seconds times two applications. This method is easier to control and no molds are needed. The burn is not full thickness but could be if applied three to four times in the same area. This could be an expensive route, however, because the Wartner pen kits are expensive and are only usable for a limited number of mice.

The production of the circulatory disruption by tourniquet was easily accomplished; however, creating a punch on the extremity was not reproducible. Applying a punch only on the

skin, and not through the muscle, is not an easy task, as the surface area is very small and there is not a lot of give in the skin itself, like the back provides. This model was not pursued, as it could not be consistently reproduced using mice. It is advised that if this model is of interest, a larger species, such as rats, should be used.

4.2 Bacterial Infection of Wound Models and Effects of Probiotic Application

After developing the model systems, we needed to determine if the probiotic would colonize a wound. To test this, we used the simple full thickness injury model (the “punch” model). As the data in Figure 1 demonstrate, we were able to colonize the wound site with the probiotic.

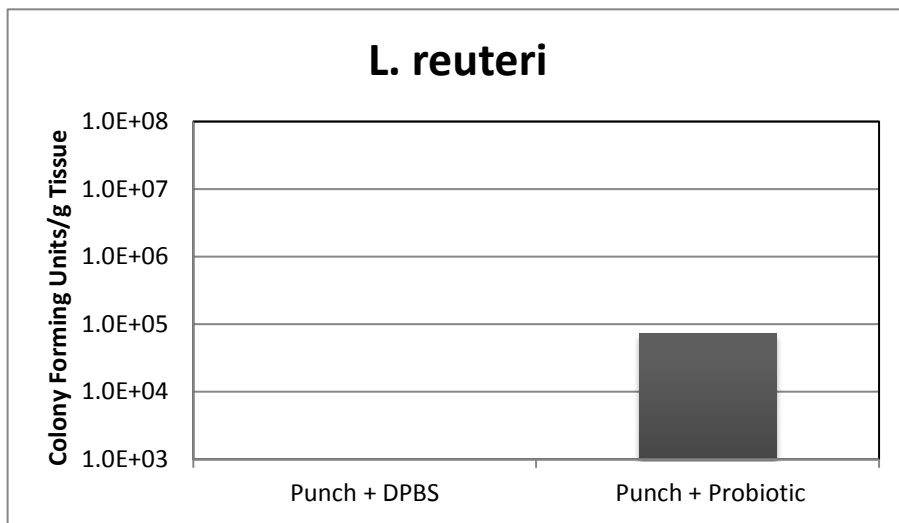


Figure 1. Colonization of the wound site with probiotic.

We next evaluated each model system for its ability to provide an adequate bed for infection of seeded *P. aeruginosa* or *S. pyogenes*. Once we found the appropriate amount of each of these bacteria to seed the wounds, we tested the ability of probiotic (*L. reuteri*) to limit or reduce bacterial infection.

4.3 Simple Full Thickness Injury (“Punch”) Model

In the punch model, we effectively established bacterial infection with both *P. aeruginosa* and *S. pyogenes*. The addition of probiotic significantly reduced *Pseudomonas* infection ($p=0.004$) but not *Streptococcus* infection ($p=0.337$) (Figure 2).

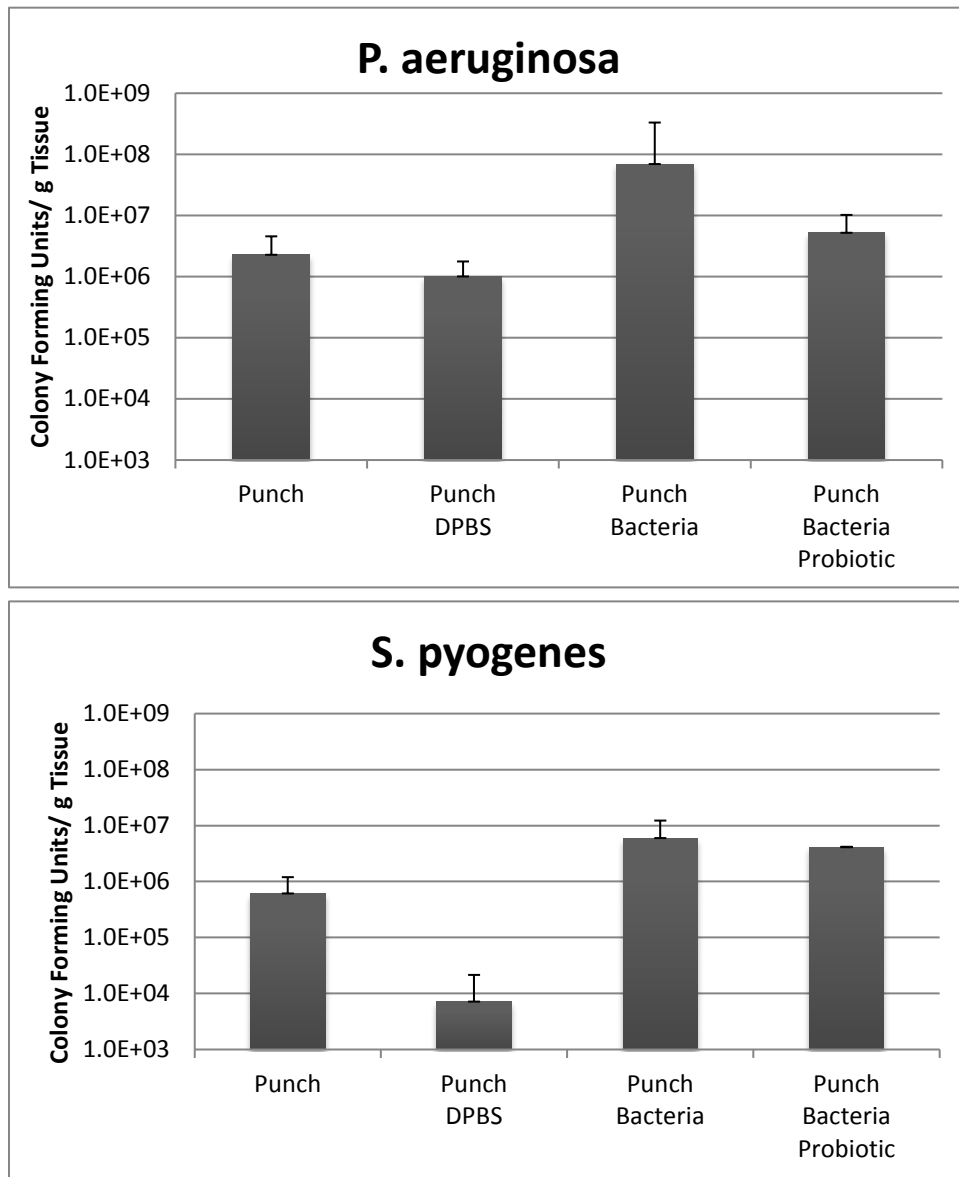


Figure 2. Simple full thickness injury (“punch”) model.

4.4 Stitched Skin Flap Injury (“Skin Flap”) Model

In the skin flap model, we effectively established bacterial infection with both *P. aeruginosa* and *S. pyogenes*. The addition of probiotic had no significant effect on *Pseudomonas* ($p=0.087$) or *Streptococcus* ($p=0.120$) infections (Figure 3).

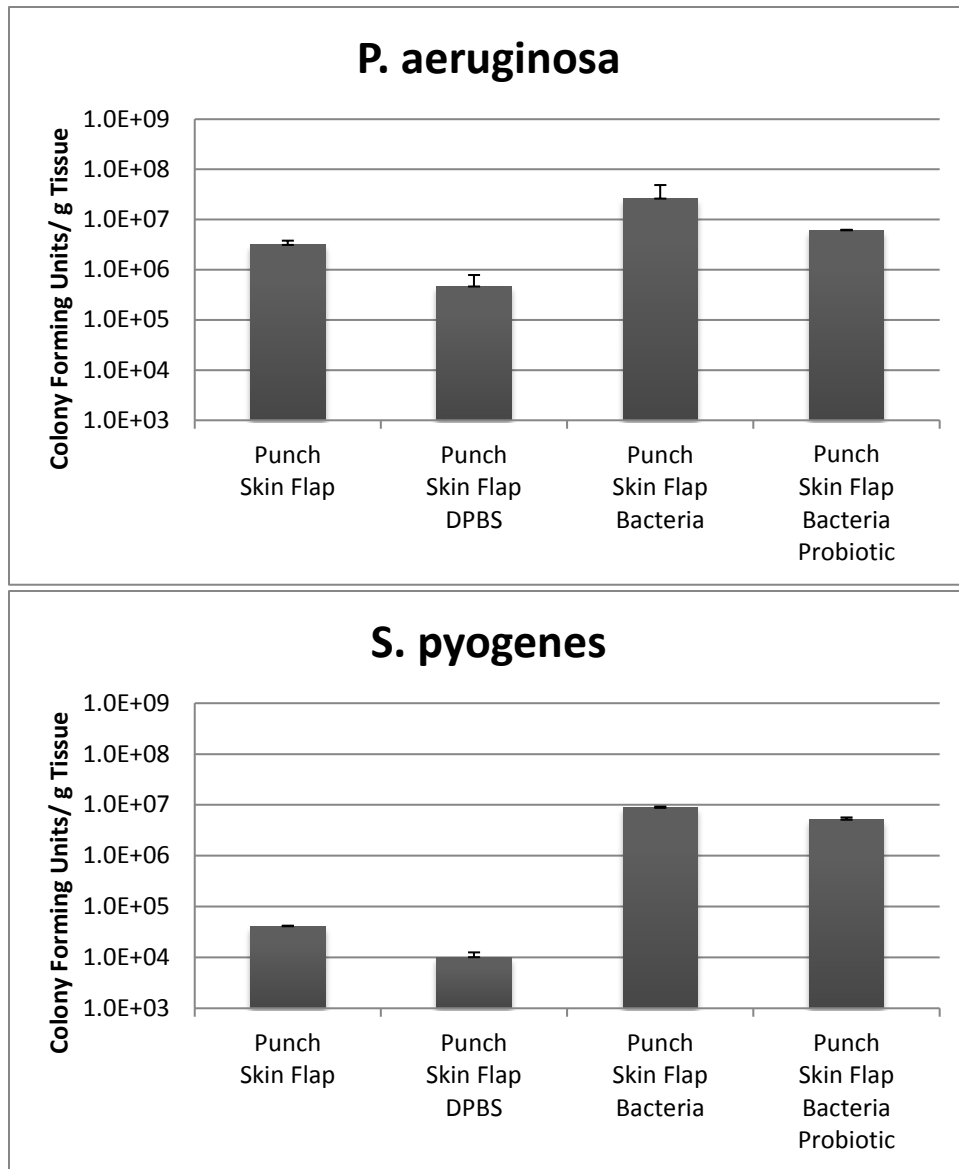


Figure 3. Stitched skin flap injury (“skin flap”) model.

4.5 Cryo Burn Model

As indicated in section 4.1 above, developing a full thickness burn was problematic. Despite this, we assessed the ability of bacteria to seed these injury sites. Unfortunately, we were unable to establish bacterial growth using this model. No data on bacterial growth are provided, as we were not able to measure any bacterial growth in this model.

5.0 CONCLUSIONS

Both the punch and skin flap models are viable, reproducible mouse models of infection. The cryo burn and skin incision with circulatory disruption models were not reproducible. We were able to create a wound induced by cryo burn, but the wound was not full thickness and could not be effectively seeded with bacteria to create an infection. We were unable to reproducibly produce a wound of skin incision with circulatory disruption, primarily because of the size of the animal and the lack of available surface area to create the wounds. Application of probiotic (*Lactobacillus reuteri*) had some limited efficacy in reducing *Pseudomonas aeruginosa*. Probiotic significantly reduced *Pseudomonas aeruginosa* infection in the punch model but not the skin flap model. Probiotic had no beneficial effect in limiting *Streptococcus pyogenes* infection in either model.

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LIST OF ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
CFU	colony-forming unit
CO₂	carbon dioxide
DPBS	Dulbecco's phosphate-buffered saline