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The Effect of Staphylococcus aureus on Stiffness of Cortical Bone

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May 2017

To the Dean of the Graduate School:

We are submitting a thesis written by Ariel N. Kunde entitled The Effect of

Staphylococcus aureus on Stiffness of Cortical Bone. We recommend acceptance in

partial fulfillment of the requirements for the degree of Master of Science in Biology.

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THE EFFECT OF *STAPHYLOCOCCUS AUREUS* ON STIFFNESS OF

CORTICAL BONE

A Thesis

Presented to the Faculty

Of the

College of Arts and Sciences

In Partial Fulfillment

Of the

Requirements for the Degree

Of

Master of Science

In the Department of Biology

Winthrop University

May 2017

by

Ariel N Kunde

Abstract

Osteomyelitis, a term for bone infection, is a common cause of hospitalization in the United States. Infection leading to osteomyelitis is almost always a product of bacterial origin. Although polymicrobial presence is seen at infection sites of osteomyelitis, *Staphylococcus aureus* is most commonly isolated and found to be the cause of more than 95% of bone infection in adults. This organism is a common commensal of humans that is carried by an estimated 60% of the US population. *S. aureus* is transferred by infected asymptomatic individuals, and its ability to proliferate under a variety of environmental conditions contributes to the organism's role as a pathogen in hosts with compromised immunity. This study examines the effect that infection of *Staphylococcus aureus* has on cortical bone stiffness. The data collected may be relevant information for bone-graft banks storing donor tissues. A preliminary experiment showed the ability of *S. aureus* to infect cortical bone by traveling through its porous structure. Frozen, cortical bone samples were thawed, sterilized with alcohol, inoculated with nutrient broth containing *S. aureus* and then disinfected with chlorhexidine gluconate. Stiffness measurements of each sample were recorded before and after bacterial contamination to allow each sample to serve as its own control. One cube out of every testing batch was not inoculated in bacteria to serve as a control. The results demonstrate that *S. aureus* infection of bone does not significantly decrease bone stiffness in the axial orientation ($p>0.05$). In the transverse orientation a significant decrease was observed in the experimental group ($p<0.05$) but not in the control group. A significant decrease was also seen in stiffness of the radial orientation in both the experimental and control groups ($p<0.01$). This project is especially interesting as there is little published literature regarding *in vitro* bacterial infection of bone.

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Introduction

Osteomyelitis is bone inflammation caused by bacterial infection that most often affects children and adults over 50 years of age (especially in patients with risk factors such as diabetes). The end-result of untreated osteomyelitis is bone necrosis and distraction of bone structure, which leads to a decrease in the ability of bone to support physiological loads. While bone tissue can heal and remodel its structure to ameliorate its mechanical properties (such as stiffness and strength), so far no study has tested the mechanical properties of cortical bone tissue affected by osteomyelitis. With increased usage of bone banks as a source of bone graft supply, it is important to screen for any possible pathology that may affect bone quality and the bone graft success to function normally in the receiving patient. This study examines the effect that *Staphylococcus aureus* (the most commonly isolated bacteria from infected bones) has on cortical bone stiffness.

Osteomyelitis

Osteomyelitis, a term for bone infection, is a common cause of hospitalization in the United States (Brady et al. 2006). Individuals with specific risk factors such as diabetes, trauma, surgery, and intravenous drug use are more likely to suffer from infection (Chihara and Segreti 2010). The prevalence of osteomyelitis is increasing primarily due to the increase in worldwide diabetes cases and the rise in the number of insertions of prosthetic devices. It is estimated that a quarter of all diabetes patients will suffer from foot complications that are susceptible to osteomyelitis (Chihara

and Segreti 2010). Prosthetic infection is a cause of contiguous focus osteomyelitis. The process puts an individual at much higher risk of contracting infection for the first two years following the procedure (Lew and Waldvogel 2004). Individuals are highly susceptible to infection after insertion of prosthetic devices even if contamination is with microorganisms that are rarely associated with disease (Lew and Waldvogel 2004).

Osteomyelitis can be classified based on the duration of infection (chronic or acute), etiology (Waldvogel classification system), or based on anatomic and physiologic characteristics (Cierny-Mader staging system). Acute osteomyelitis is typically thought of as progressing over several days or weeks (Lew and Waldvogel 2004) and occurring before osteonecrosis (Chihara and Segreti 2010). Chronic osteomyelitis evolves over months or years, typically recurs in the same area, and is accompanied by destruction of bone (Lew and Waldvogel 2004). According to the Waldvogel classification system, osteomyelitis can be categorized into three types: hematogenous, contiguous focus, and vascular insufficiency (Lew and Waldvogel 2004). According to Waldvogel et al. (1970), hematogenous osteomyelitis contributed to 19% of all cases while 47% were classified as contiguous focus and 34% were associated with vascular insufficiency.

Hematogenous osteomyelitis is caused by bacterial infection of bone through the bloodstream and commonly occurs in long bones or vertebral bones (Brady et al. 2006). Hematogenous osteomyelitis is associated primarily with infection in long bones of children and acute conditions (Gentry 1997). In long bone osteomyelitis of adults, infection begins in the diaphysis and can spread through the medullary canal (Brady et al. 2006). Studies have indicated a decrease in cases of hematogenous osteomyelitis and an

increase in contiguous osteomyelitis (Espersen et al. 1991). Modern vaccination procedures may be influential in the decline in cases of hematogenous osteomyelitis (Brady et al. 2006).

Contiguous focus osteomyelitis is indiscriminate in that it can affect any bone in any individual and occurs due to trauma, bone surgery, or joint replacement (Lew and Waldvogel 2004). Infection from open fractures is another cause of contiguous focus osteomyelitis, and has a reported incidence rate as high as 23% (Carek et al. 2001). Most of those suffering from vascular insufficiency osteomyelitis have diabetes and are typically affected in the small bones of the feet, talus, calcaneus, distal fibula, and tibia (Brady et al. 2006). Medical treatment often fails in these patients (due to the lack of circulation characterized by vascular insufficiency) and is more likely to end in amputation (Gentry 1997). Prevalence of vascular insufficiency osteomyelitis is increasing due to the concurrent rise of diabetes (Chihara and Segreti 2010). According to the Center for Disease Control, there were 21 million individuals diagnosed with diabetes in the United States as of 2014. As many as 25 percent of these individuals with diabetes will present with foot complications, and one third of these patients will show signs of osteomyelitis (Chihara and Segreti 2010). With an estimated rise in worldwide diabetes cases to over 360 million by 2030 (Chihara and Segreti 2010), osteomyelitis occurrence could likely follow suit.

The third classification system, known as the Cierny-Mader staging system, is the most recent tool designed to help classify and diagnose osteomyelitis while providing therapeutic and clinical information (Carek et al. 2001). In this system, osteomyelitis is

characterized depending on the etiology and manifestation of the infection (medullary, superficial, localized, and diffused) combined with the host´s physiological status (normal or immunocompromised) (Cierny et al. 1985). The advantage of using the Cierny-Mader system is that it is dynamic and classifications are alterable based on patient prognosis (Carek et al. 2001). A downfall of this system is that it does not apply to special cases of osteomyelitis including osteomyelitis of smaller bones or prosthesis associated osteomyelitis (Carek et al. 2001).

Although polymicrobial presence is usually seen at infection sites of osteomyelitis, *Staphylococcus aureus* is the most commonly isolated bacteria (Chihara and Segreti 2010). *S. aureus* causes 60-90% of bone infections in children and more than 95% of infections in adults (Rosa et al. 2015). These bacteria adhere to fibronectin and other structural proteins at the infection site and proceed to create a biofilm that has the ability to block vascular tunnels (Kronig et al. 2015). In some cases, necrotized bone can become sequestered and bacteria can continue to proliferate even after antibiotic treatment (Lew and Waldvogel 2004). Antibiotic resistance is due in part to genetic mutations of certain strains and biofilm associated factors including reduced diffusion of the antibiotic into the bioflim (Brady et al. 2008; Foster 1996).

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a gram positive, coccus shaped species of bacteria that is a common commensal of humans (Foster 1996). An estimated 60% of the US population temporarily carries this organism while 20% are permanently inhabited

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(Kluytmans et al. 1997). *S. aureus* is transferred by infected asymptomatic individuals and can cause complications in hosts with compromised immunity (Madigan et al. 2012). *S. aureus* are able to colonize and replicate in any human tissue resulting in the foundation of a diverse repertoire of complications such as bacteremia, osteomyelitis, toxic shock syndrome, food poisoning, and endocarditis (Lowy 1998).

S. aureus* contains a number of virulence factors such as adhesins, toxins, and other protein-degrading enzymes that enhance its invasiveness and pathogenic ability. These virulence factors have been demonstrated to promote colonization and growth in animal tissues (Gould et al. 2012). Ryden et al. (1997) proposed that *S. aureus* have developed cell receptors that recognize and bind to amino acid sequences in extracellular matrix proteins of bones as an evolutionary mechanism to increase virulence. Adhesins, for example, are cell surface receptors that allow the pathogen to gain access to the host by binding to laminin, fibrin, collagen, and other components of bone matrix (Foster 1996; Tong et al. 2015). In prosthetic mediated infections, *S. aureus* interaction with fibrinogen is primarily responsible for adherence (Cunningham et al. 1996). Nilsson et al. (1997) proposed that the presence of capsular polysaccharides, namely the CP5 serotype, protected *S. aureus* from phagocytosis, which increased its survivability in a murine septicemia model. Therefore, capsular polysaccharides are virulence factors that allow *S. aureus* to survive in the blood stream and migrate to susceptible tissue such as bone.

Another characteristic that contributes to the virulence of *S. aureus* is its production of toxins such as leukocidins. Leukocidins are able to inhibit phagocytosis by forming pores in the membranes of polymorphonuclear leukocytes (Foster 1996). A

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specific leukocidin, Panton-Valentine leukocidin (PVL) was found to be associated with increased infection, disease persistence as well as bone deformation in a rabbit osteomyelitis model (Cremieux et al. 2009). Furthermore, small colony variant (SCV) strains of *S. aureus* are also associated with osteomyelitis because they exhibit reduced virulence allowing them to persist intracellularly, and then revert back to wild-type phenotype to infect new cells (Gould et al. 2012). Specifically, the initial inoculum of *S. aureus* can switch phenotype to produce SCV's that exhibit decreased metabolic activity, increased adhesin expression, and produce fewer lytic enzymes to promote survival in host cells (Proctor et al. 2014).

Enzymes such as coagulase are also produced by *S. aureus*. Coagulase can aid in the protection of these bacteria against the host cell immune response by binding to prothrombin (Cunningham et al. 1996). This complex activates the conversion of fibrinogen to fibrin which is thought to inhibit phagocytosis long enough to allow for bacterial growth and establishment. Thus, coagulase positive strains of *S. aureu*s are more virulent than those that are coagulase negative (Foster 1996). Coagulase negative *S. aureus* strains, however, are most commonly the culprits in prosthetic joint infections (Carek et al. 2001). Cassat et al. (2013) used a murine osteomyelitis model to investigate staphylococcal virulence factors and revealed a protease dependent *S. aureus* locus (Sae) that regulates factors contributing to intraosseous bacterial survival and pathologic bone remodeling. Of special interest is an enzyme secreted by nearly all strains of *S. aureus* called collagenase, which is responsible for degrading the peptide bonds in collagen (Dinges et al. 2000). McGregor et al. (1986) assayed organisms for protease activity and

found that *S. aureus* was capable of producing collagenase in aerobic conditions. Moreover, *S. aureus* secrete cysteine proteinases called staphopains (ScpA and SspB) that were found to degrade type-I collagen in a dose dependent manner (Ohbayashi et al. 2011).

Perhaps the most concerning characteristic of *Staphylococcus aureus* is its ability to develop antibiotic resistance. At one time the only antibiotic that effectively controlled all strains of *S. aureus* was vancomycin, however recently strains have been discovered to be less susceptible or resistant to this drug (Pechous et al. 2004). For instance, the minimum inhibitory concentration (MIC) of vancomycin to methicillin susceptible *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA) strains have been increasing (Gould et al. 2012). One reason for this antibiotic resistance is the formation of biofilms, a bacterial community embedded in a polysaccharide and techoic acid layer of glycocalyx that reduces the potential of antibiotic penetration (Brady et al. 2006; Rosa et al. 2015). As intuition suggests, the presence of MRSA in the blood is associated with a higher mortality rate than MSSA presence (Cosgrove et al. 2003). MRSA contributes to 40-60 percent of nosocomial (healthcare associated) infections and are now beginning to infect individuals in the community who do not have risk factors (Brady et al. 2006; Cassat et al. 2013). Together, these virulence factors produce an aggressive, opportunistic, pathogenic organism capable of inducing osteomyelitis.

Acute osteomyelitis is often treated with 4-6 weeks of pathogen specific antibiotics until the infection has arrested (Carek et al. 2001). Optimal route and duration of antibiotic therapy is undetermined and is taken on a case-by-case basis based on individual circumstances (Liu et al. 2011). If antibiotic therapy fails, or in cases of chronic osteomyelitis, debridement and ablation are often necessary (Carek et al. 2001; Brady et al. 2006). The goal of debridement is to remove dead bone and achieve re-vascularization so that recurring infection is avoided (Lew and Waldvogel 2004). In cases of extensive debridement, bone grafts, revascularization procedures, and muscle flaps may be used (Lew and Waldvogel 2004). Autografts that are taken from the patient's own body are typically thought of as the gold standard of bone grafts, however substitutes such as bone bank allografts from a donor are often necessary in certain patients with compromised bone or inadequate volume (Egol et al. 2015). Generally, bone grafting requires consideration of the healing ability of surrounding tissue and the ability of the graft to exhibit osteogenic, osteoinductive, and osteoconductive properties (Egol et al. 2015; Lichte et al. 2011). A 2-stage surgical approach is used in grafting infected extremities that includes inserting a polymethylmethacrylate cement and antibiotic combination in the bone as a spacer (phase 1) and then bone reconstruction (phase 2) (Egol et al. 2015; Lew and Waldvogel 2004).

Bone Biomechanics

Bone is a composite biological material that is comprised of an organic matrix of proteins (mainly type-I collagen), minerals (specifically a calcium phosphate crystal called hydroxyapetite), and water (Currey 2002). Macroscopically, bone material can form two different structures: cortical bone and cancellous (or trabecular/spongy) bone. Cortical bone and cancellous bone are made of the same bone materials, but there are key differences between the two. For example, the porosity of cortical bone can range from 5 to 30% while cancellous bone porosity is a much higher percentage (30 to 90%) due to wide spaces between trabeculae (Bonucci 2000). The diaphyses of long bones (e.g. femur and humerus) contain primarily thick cortical bone with little to no cancellous bone (Buckwalter et al. 1995).

Osteoblasts are the cells responsible for laying down the organic matrix, known as osteoid, in the process of bone formation, and then mineral is deposited in and around the collagenous matrix in the same orientation as the collagen fibrils (Currey 2002). Lamellar bone is arranged so that the mineralized osteoid material forms layered sheets (lamellae) around blood vessels. On the contrary, woven bone contains randomly oriented collagen fibrils and minerals because it is laid down very quickly compared to lamellar bone (Currey 2002). Fibrolamellar bone, which is found in large mammals (e.g. white-tailed deer), contains alternating layers of woven bone and lamellar bone (Currey 2002).

During day-to-day usage, most bones are repeatedly deforming due to physiological stresses. Biomechanical stress can be characterized as compressive, tensile, or shear and is defined as force per unit area (Turner and Burr 1993). Biomechanical

strain is expressed as a change length (deformation) relative to the original structural dimensions (Turner and Burr 1993). *In vivo* strain is a complex phenomenon involving compressive, tensile, and shear strain concurrently at different locations along the bone. As it pertains to biomechanics, a distinction is made between whole bone behavior and bone tissue as a material (Augat and Schorlemmer 2006) because material properties are intrinsic and independent of the bone's geometry. Cortical bone varies in measurements of stiffness depending on the direction of the stress due to bone's anisotropic character, that is, different mechanical properties of each orthotropic orientation (Cullinane and Einhorn 2002). In cortical bone, the stiffness of the bone is generally greater along the axis that is accustomed to experiencing load in physiological conditions (Cullinane and Einhorn 2002).

Rationale

To the best of my knowledge, there are no *in vitro* studies that specifically address the effects of bacterial infection on mechanical properties of cortical bone. Several researchers have developed experimental models for creating bone infection in rabbits (Del Pozo et al. 2009; Gaudin et al. 2010), rats (Rissing et al. 1985), dogs (Fitzgerald 1983), mice (Li et al. 2008), and sheep (Kaarsemaker et al. 1997), however these works focused on the pathogenesis and treatment of osteomyelitis *in vivo* and did not address the possible effect of osteomyelitis on bone quality. For example, El-Kamel and Baddour (2007) designed an *in vitro* experiment to test an implant delivery system's inhibition of MRSA in which they inoculated antibiotic containing implants in *S. aureus* broth for 4

days at 37°C and sampled colony counts to determine the bactericidal effect. They found that gatifloxacin (the antibiotic) releasing implants decreased the growth of MRSA compared to the control implants consistently over 4 days (El-Kamel and Baddour 2007). This is the extent to which *in vitro* experiments have been completed: testing antibiotics for treatment purposes. For this reason, it is of interest to address the effect that infection and treatment have on bone biomechanics.

Presently, according to the Food and Drug Administration's guidance for human cell, tissue, and cellular and tissue based product donors (HCT/Ps), eligible donors must not have had a diagnosis of sepsis "immediately preceding death," but there is no mention of previous osteomyelitis diagnosis as a donor restriction (USDA 2007). Furthermore, the American Association of Tissue Banks requires that bone-graft banks currently subject donors to an extensive physical and medical history review for risk of complications with transplantation before accepting a tissue, however the donor suitability screening only restricts tissue that has or is being treated for bacterial infection at time of examination (AATB 2016). More research available to elucidate effects of *S. aureus* infection on the biomechanical properties of bone may lead to tighter guidelines for screening in relation to osteomyelitis history.

As cortical bone mechanical properties depend on the quality and spatial arrangement of its collagen and mineral components (Augat and Schorlemmer 2006), my hypothesis is that *Staphylococcus aureus* infection of cortical bone cubes will result in decreased stiffness in all three orthogonal orientations. This experiment subjects bone to compressional stress to examine the linear relationship between stress and strain as a

measure of intrinsic bone stiffness, otherwise known as the modulus of elasticity, or Young's modulus (Cullinane and Einhorn 2002). Overall, 32 cubes were included in a group that was inoculated with *Staphylococcus aureus* (experimental group), and a 33 cubes were not inoculated, but immersed in sterile dH_2O in the same manner (control group). Each cube was tested in compression before and after processing to allow the sample to serve as its own control. The infected and uninfected groups' Young's moduli were ultimately compared to each other to determine significant differences.

Materials and Methods

Bone Sample

Four white tail deer humeri were donated from One Price Deer Processing Factory. The cause of death for all deer was not related to the musculoskeletal system and all humeri were intact with no sign of any pathology. Sex and age were unknown, but all bones had active growth plates, indicating these were juvenile individuals still in their growing phase. Sixty-five 2x2x2mm bone samples were cut using a low speed water-cooled diamond saw (TechCut 4 precision low speed saw, Allied Technologies). Thirty-two samples were cut from the cranial aspect and thirty-three from the caudal aspect of the bone. All cubes were cut parallel to the orthogonal axes of the bone (Fig. 1). Samples were stored frozen at -20°C in individual 1.5mL Eppendorf tubes containing a water soaked paper towel (Turner and Burr 1993). Samples were thawed in water at 4°C for 24 hours before testing.

Inoculum Preparation

The bacteria used in this study were *Staphylococcus aureus* (ATCC-12600) obtained from the American Type Culture Collection (Manassas, VA, USA). Upon arrival, the bacterial sample was immediately inoculated in nutrient broth (Difco™Nutrient Broth, BD) and incubated at 37°C for 48h. This initial inoculum was then stored as 1 mL stock cultures in 40% glycerol at -80°C. Storage of *S. aureus* in a concentration of 25-50% glycerol limits damage to the cells in the transition to -80°C which prevents the accumulation of mutations (Vitko and Richardson 2013). To prepare

for each experimental group's inoculation, 1 mL stock culture was added to nutrient broth and incubated at 37°C for 48h. 1 mL of this broth was then transferred to new sterile nutrient broth and incubated at 37 °C for 48h which was used as the test suspension for inoculating bone samples.
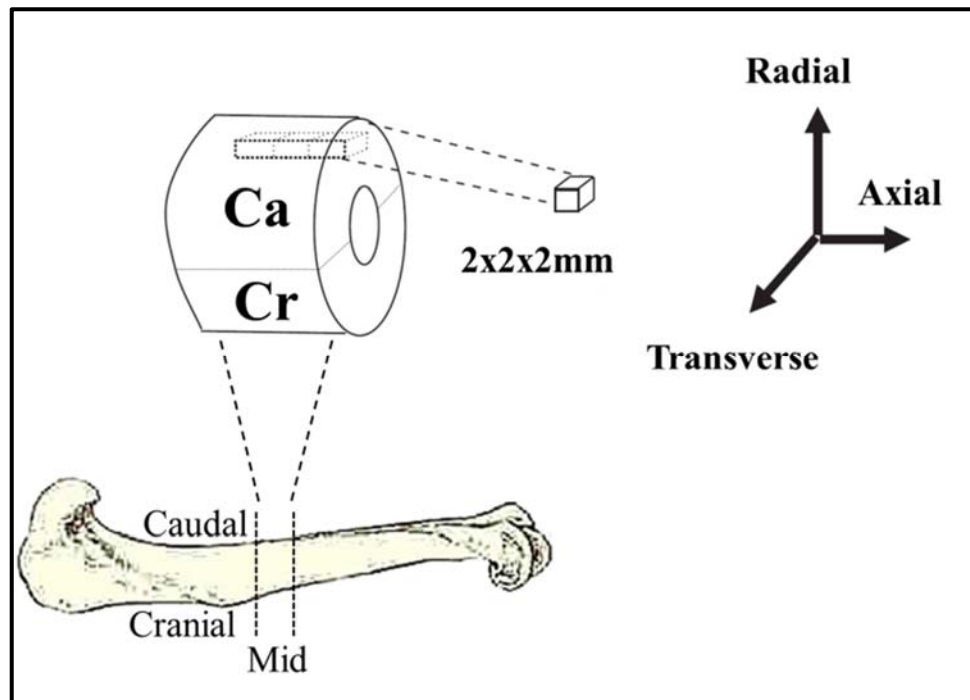
Figure 1: A schematic illustration of the deer humerus and the location from which the cubes were cut. The cubes (measured 2x2x2 mm) were prepared from the humerus mid-caudal and mid-cranial regions, parallel to the three primary axes of the bone (axial, radial, and transverse).

Experimental Timeline

Each experiment consisted of a group of 4-6 bone cubes and lasted 6 days from time of thawing to post compression testing. A recent study demonstrated that bone samples can be thawed and kept at 4°C for up to seven days with no significant difference in measurements of stiffness (Barrera et al. 2016). Figure 2 outlines the experimental timeline. Day 1 consisted of thawing the bone samples for 24 hours. During day 2,

Staphylococcus aureus was transferred to a new broth to grow for 48 hour. Concurrently, each bone cube was loaded in compression for the first time ("pre-test"). At the end of compression testing, each cube was set to sterilize in ethanol for 24 hours. The cubes were removed from ethanol and washed with sterile dH_2O several times on Day 3. On Day 4, the bone samples were rinsed once more with sterile dH_2O, surfaces swabbed and plated to verify sterility. All cubes from the group except one were inoculated with *S. aureus* for 48h at 37°C ("experimental group"). One cube was kept in sterile dH_2O and served as a control ("control group"). On day 6, all cubes were decontaminated with chlorhexidine gluconate and rinsed in dH_2O. One cube from the experimental group was crushed, made into a liquid solution with dH_2O, and plated on nutrient agar to test for bacterial growth. The surfaces of the other cubes were swabbed and plated on nutrient agar. Finally, all experimental bone samples and control samples were tested in compression. In order to keep the control and experimental groups at similar sizes, several cycles of the above protocol were run with just control cubes.
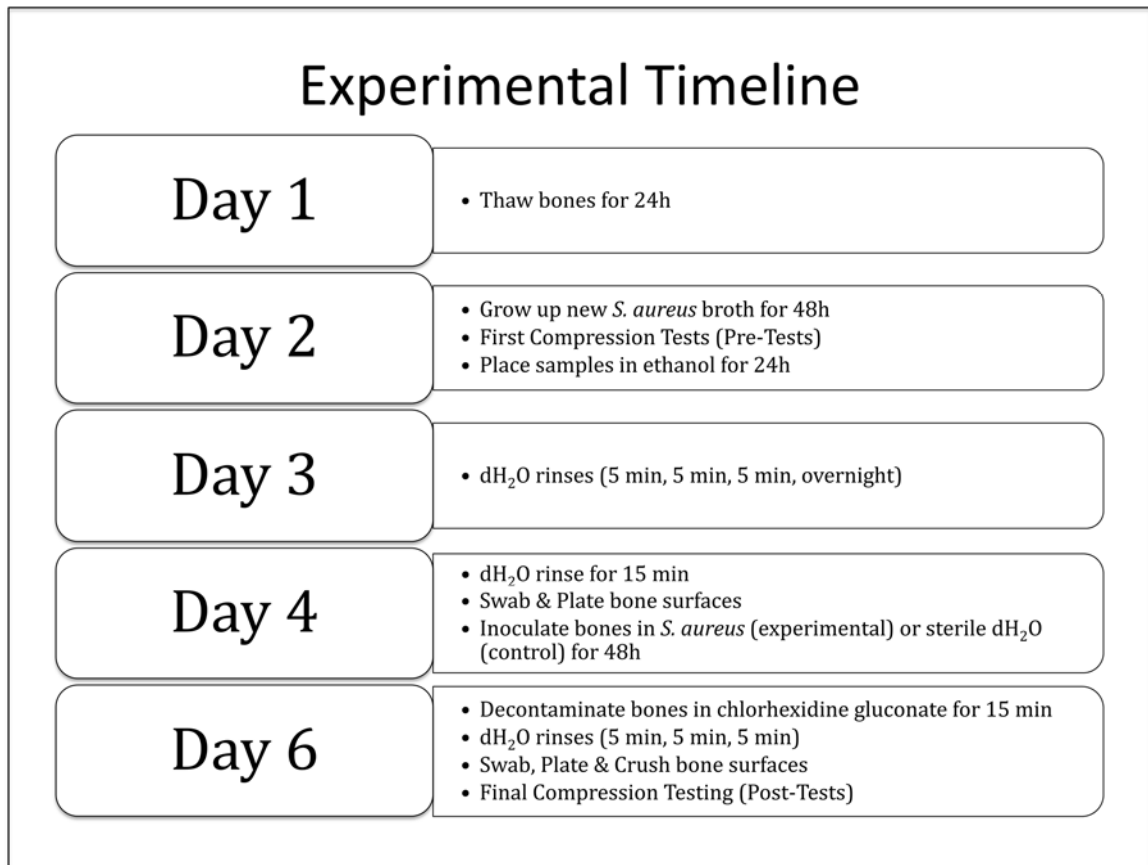
Figure 2: Overview of experimental timeline. Cubes were thawed for a total of 6 days throughout the experiment. Each experimental group consisted of 4-6 bone samples inoculated in *S. aureus* with one control sample inoculated in dH₂O per experimental group. Each control group consisted of 4-6 cubes inoculated in sterile dH₂O instead of *S. aureus*. This schedule was repeated until all 65 cubes were tested.

Sterilization

All bone cubes required sterilization before beginning the inoculation process. The most common methods of bone sterilization include a variety of chemical agents and irradiation (Haimi et al. 2008). Irradiation as a sterilization technique, however, has been shown to have a degrading effect on bone that significantly alters its biomechanical properties (Haimi et al. 2008). The ideal sterilization candidate for bone tissues undergoing mechanical testing is one that does not affect its mechanical properties.

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Ethanol is well known as a bactericidal, fungicidal, and virucidal agent (Rutala and Weber 2008). Evidence also suggests that bone preservation in ethanol contributes only a small change in mechanical properties if the bone is washed with sterilized water before the experiment (Turner and Burr 1993). Furthermore, a study by Linde and Sorenson (1993) determined no significant change in stiffness of trabecular bone after storage in 70% ethanol for 1, 10, and 100 days, supporting the use of ethanol as an alternative sterilizing agent.

A preliminary experiment was designed to examine the effect of ethanol on the stiffness of the samples. Four bone samples were measured in compression both before and after 24 hours in 91% ethanol and washing with sterile dH_2O. No significant difference in stiffness was found ($p \leq 0.05$). These results further confirmed the use of ethanol for sterilization in this study (see appendix A for details).

To ensure the bone cubes were void of any confounding microorganisms before treatment with the intended pathogen, all samples were individually placed in sterile 2 mL Eppendorf tubes containing 1 mL 91% ethanol under constant agitation (300 rpm) on a temperature controlled shaker (Eppendorf™ Thermomixer™ R) at 20°C for 24 h. To remove residual ethanol, samples were then washed four times (3 x 5 min; 1 x overnight 17.5h) in 1 mL sterile dH_2O under constant agitation (300 rpm) on the shaker at 20°C. Between each wash, samples were rinsed with 1mL sterile dH_2O and transferred aseptically via autoclaved forceps to new sterile Eppendorf tubes.

The next morning, samples were rinsed once more with 1mL sterile dH_2O, and washed (300 rpm) on the shaker at 20°C for 15 min. Each cube was then removed; all

surfaces were swabbed with a sterile cotton bud and plated on nutrient agar (Difco™Nutrient Agar, BD). The plates were incubated at 37°C for 48 h to test for sterility before inoculation.

Contamination

Bone samples were contaminated via submersion in 1 mL test suspension under constant agitation (300 rpm) on the shaker at 37°C for 48h. Each sample group that was tested was contaminated with broth that was grown in the same manner and for the same amount of time, so it was assumed that the same growth conditions would yield the same result for each testing batch. Previous relevant studies use contamination times of 10s - 45s in suspension (Hooe et al. 1996; Saegeman et al. 2009; Soyer et al. 2002). This short time period is thought to mimic surface contamination (Saegeman et al. 2009). Therefore, exposing cubes for 48 hours in *S. aureus* is sufficient to contaminate cubes in this study. One cube from each experimental batch (typically 4-6 cubes per batch) was not inoculated, but placed in sterile dH$_2$O, to serve as a biomechanical control.

Bacterial Penetration

In order to demonstrate that the bacteria were able to penetrate and contaminate all bone surfaces, two bone samples (from the same batch of white-tail deer humeri) were created by cutting a section of bone diaphysis in half along the sagittal plane so that each piece was semi-cylindrical (Fig. 3A). The sides of each bone piece were built up with dental acrylic (Jet™Denture Repair Acrylic, Lang Dental Manufacturing Co.) so that the

sample resembled a bowl-like structure. Samples were placed in dH_2O to verify that no leaks were present in the acrylic around the bone (i.e. no water entered the inside of the bowl). A small depression was machined in the inner center of the bone to remove the endosteum and to achieve a total cortical bone thickness of approximately 2 mm, mimicking bone sample thickness used in this study (Fig. 3A). The samples were placed on a holder inside a glass petri dish (Pyrex® 100 x 15mm). The holder was composed of two paperclips, bent so that two parallel beams were correctly positioned to cradle the bone sample. The holder was then adhered to the bottom of the petri dish with dental acrylic (Fig. 3B). The petri dish and holder were steam sterilized. The bone samples were sterilized by placement in sterile conical tubes containing 35 mL 91% ethanol under constant agitation (300 rpm) on a temperature controlled shaker (20°C) for 24 hours, mimicking the experimental conditions used in this study.

After 24 hours, the bone samples were removed from ethanol using sterile forceps and washed four times (3 x 5 min, 1 x overnight 15h) in 35 mL sterile dH_2O under constant agitation (300 rpm) at 20°C. The samples were rinsed with 35 mL sterile dH_2O between washes and transferred to new sterile conical tubes for each wash. After the overnight wash, samples were removed once more, rinsed with 35 mL sterile dH_2O, and washed (300 rpm) on the shaker at 20°C for 15 min. The surfaces of each sample were swabbed with a sterile cotton swab and plated on nutrient agar to confirm sterilization before beginning inoculation procedures.

Each sterile bone sample was set on its holder and each petri dish was filled with 85 mL sterile nutrient broth so that the bottom of the sample was in contact with the

broth. 10µL of a 48h *S. aureus* culture was dispensed in the depression of the inside of the experimental sample and 10µL of sterile nutrient broth was dispensed in the same manner to a negative control sample. The dishes were then incubated at 37°C. Every hour on the hour for five hours, an additional 10µL *S. aureus* or sterile broth was added to the experimental bone sample and negative control sample respectively. On the fifth hour, an additional 100µL was added to the inside of each sample. 200µL of broth underneath each bone was spread on nutrient agar after 24h, 36h, and 48h incubation to determine if any bacteria were present. The presence of bacteria in the broth underneath the experimental bone (but not the control bone) would indicate that *S. aureus* was successful in traveling through the porous structure of cortical bone (i.e. central canals, haversian canals, Volkmann's canals and canaliculi) to contaminate the broth that was initially sterile. A positive control was created by incubating 150µL of the experimental *S. aureus* culture in 85 mL sterile broth for 24h and then spreading 200µL of the infected broth on nutrient agar for comparison against the experimental and negative sample plates.
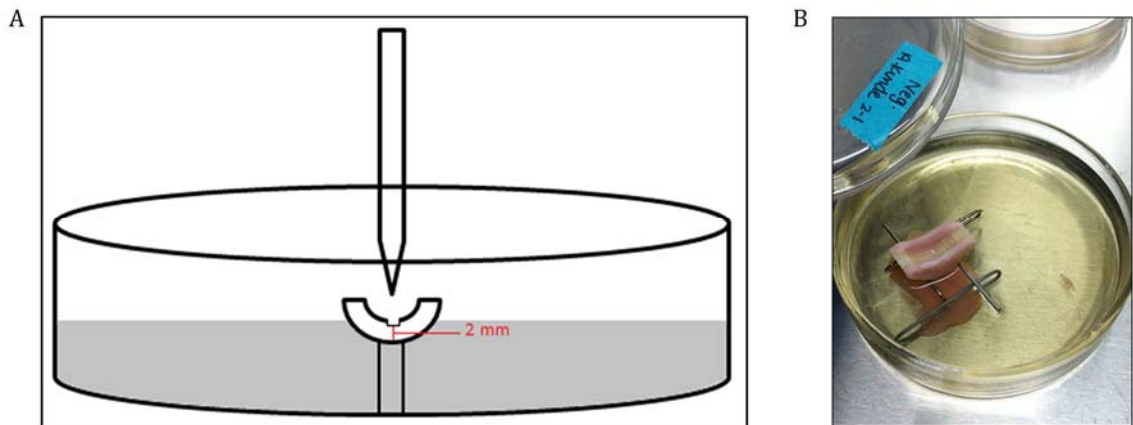
Figure 3: A) A schematic drawing representing the bacterial penetration set up. Note that the dental acrylic side walls are not present in the schematic drawing to reveal the depression. The bone sample was surrounded by sterile nutrient broth and contained a depression in the center allowing a 2mm thickness to which either sterile nutrient broth or *S. aureus* was added to the inside of the bone bowl. B) Experimental set up of schematic drawing shown in (A)

Decontamination

Antiseptic use was necessary to discontinue bone infection after 48 hours in order to prevent contamination of the equipment used for biomechanical testing. Two candidates were investigated for appropriateness based on previous research. Hooe et al. (1996) used 10% w/v aqueous povidone-iodine solution to disinfect bone samples contaminated with *Staphylococcus aureus* without changing their histological properties, and Soyer et al. (2002) confirmed this microbicidal effect with *Staphylococcus epidermidis*. Burd et al. (2000) successfully disinfected bone-tendon allografts contaminated with *Staphylococcus aureus* using 4% chlorhexidine gluconate. Preliminary tests were performed to verify the reported effects of these antiseptics. Compression testing was performed before and after bone cubes were soaked for 15 min in povidone-iodine and resulted in a significant decrease in stiffness, while cubes soaked for 15 min in

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4% w/v chlorhexidine gluconate did not exhibit this negative effect (see appendix B for details). In addition, five bone samples that were inoculated for 48h in *S. aureus* were successfully decontaminated after 15 min immersion in chlorhexidine (see appendix C for details). Thus, chlorhexidine gluconate was chosen as an appropriate antiseptic for this experiment.

Bone cubes were removed from *Staphylococcus aureus* test suspension aseptically via sterile forceps and placed directly in sterile 2 mL Eppendorf tubes containing 1 mL of 4% w/v chlorhexidine gluconate (Hibiclens™ Liquid Antiseptic Skin Cleanser, Fisher Scientific) under agitation (300 rpm) on the shaker at 20°C for 15 min. Each cube was then washed three times (3 x 5 min) in 1 mL sterile dH_2O under constant agitation (300 rpm) on the shaker at 20°C. Between each wash, samples were transferred aseptically via autoclaved forceps to new sterile Eppendorf tubes and rinsed with 1mL dH_2O.

After washing, the surfaces of each cube were swabbed, plated on nutrient agar, and incubated (37°C, 48h) to confirm the lysing of pathogenic bacteria. Soyer et al. (2002) previously used a grinding method to determine the decontamination efficiency of the chosen antiseptic. Hence, to verify that there were no residual bacteria inside the pores of the bone sample, one infected cube from each experimental group was crushed using sterile pliers, ground with a sterile pestle and mortar, mixed into solution with 1 mL sterile dH_2O, plated on nutrient agar, and incubated (37°C, 48h).

Compression occurs when two forces are applied to an object and directed toward each other acting upon the same line resulting in object deformation (Cullinane and Einhorn 2002). An Instron 5942 Single Column Table frame in compression mode was used as the universal testing machine in this study. Load and deformation data began collecting after a small preload of 5N was applied at the beginning of each cycle (measurements were taken every 0.1 s). The upper anvil of the machine loaded the bone sample against a lower stationary anvil at a rate of 50 μm sec^{-1} until a load of 140 N was measured (Fig. 4A, 4B). When the maximum load was reached, but still within the elastic region of the bone where no bone damage occurs, the upper anvil returned to its starting position before beginning another cycle.

In load control cycles where each cycle repeats the same magnitude and direction of load, the sample's deformation changes progressively with each load cycle (termed "creep") until the sample achieves a steady state (Olson et al. 2012). Thus, the sample was loaded 7 times to allow for this creep phenomenon, and the final 3 measurements were used to calculate the Young's modulus, or material stiffness value, for the sample in the loaded orientation. In each experimental group, the axial orientations of each cube were measured first, followed by the radial orientations of each cube, and finally the transverse orientations. Consequently, data was collected from three repeated measurements for each of the three orientations of every cube (Fig. 4C). Data for the axial orientation of one cube and transverse orientation of another cube in the

experimental group were lost due to computer error, so these orientations contain one less value than the radial orientation. All samples in each group were loaded at room temperature (18-22°C) and kept hydrated at 4°C between orientation measurements.

Each sample was mounted to the Instron machine using dental composite (Z250, 3M ESPE, St. Paul, MN, USA) to correct any minor incongruous cube surfaces that may have occurred during cutting. This allowed the sample to be loaded in pure compression and avoided any potential stress concentration and shear. The composite's stiffness value of 11 GPa is within the accepted range of cortical bone stiffness values, making it an acceptable method of adherence (Shahar et al. 2007; Barrera et al. 2016). First, a composite layer was adhered to the lower, stationary anvil of the Instron machine. The bone sample was then set on the composite such that the appropriate orthogonal orientation (axial, radial, or transverse) was correctly aligned with the anvils (Fig. 4D). Next, a composite layer was adhered to the upper, mobile anvil, and the anvil was slowly lowered, manually, until contact with the bone sample was made. Finally, the composite was polymerized for 60s with a hand-held LED curing light (GadgetWorkz Wood-pecker 5 W), and a small amount of water was added around the bone sample to simulate physiological conditions.
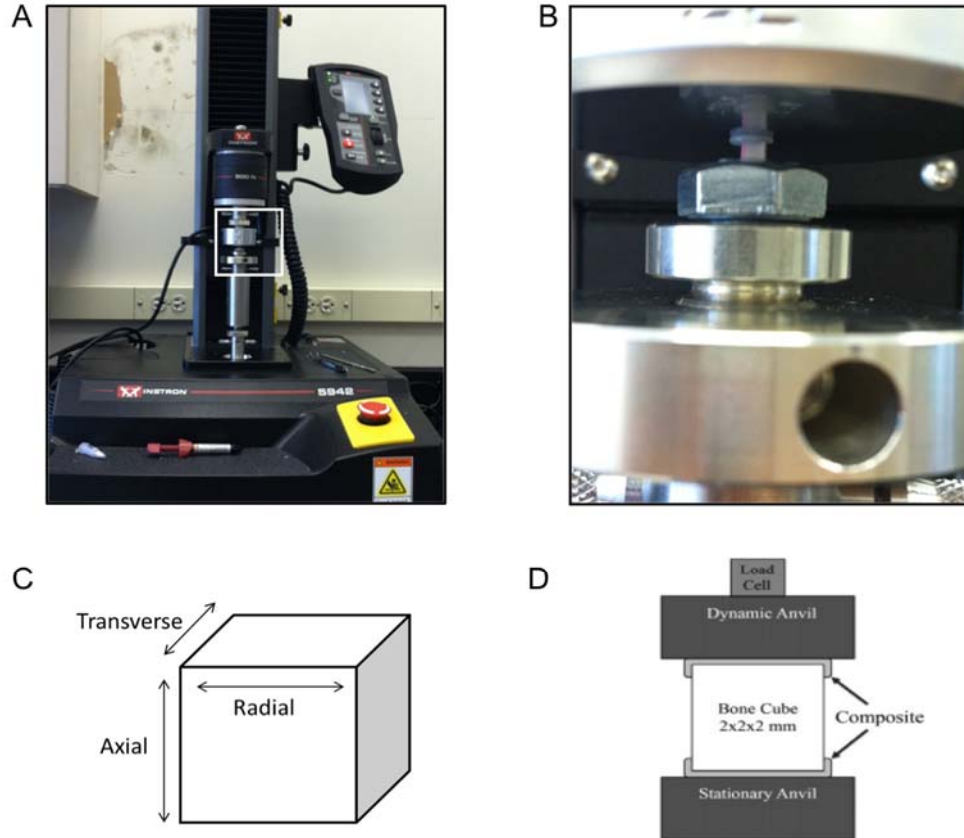
Figure 4: A) The universal testing machine (Instron 5942) in compression mode, used in this experiment. The white square frame marks the higher magnification inset shown in (B). B) A close-up showing a bone cube loaded between the upper and lower anvils. A composite layer can be seen between the upper and lower cube surfaces and its corresponding anvil. C) A schematic drawing of a bone cube with all three orthogonal orientations labeled. D) A schematic drawing of (B) showing a mounted cube between the lower stationary anvil and upper mobile anvil. A thin coat of composite was applied onto the cube's two loading surfaces to eliminate any possible stress concentration caused by small deviation from perfect parallel surfaces.

Data Analysis

The deformation of an object relative to its original length reflects the strain of the object while the force applied per area of that object is defined as stress (Olson et al. 2012). For each cycle, stress was calculated by determining the ratio between the force measured by the Instron machine and the area of the sample. Strain for each cycle was

calculated by determining the ratio between the displacement of the upper anvil and the height of the sample including composite thickness.

The linear, elastic relationship between stress and strain of an object gives a measure of the object's intrinsic stiffness, or Young's modulus (Turner and Burr 1993). For each experiment, the Young's modulus was obtained by calculating the slope in the elastic (40-100 N) region of the stress-strain curve after correcting for the area of the cube and addition of composite mounting. Data were analyzed by BlueHill 3 Software (Instron, USA). To verify the automated calculation by the software, we exported the raw data to excel files and re-calculated the values ourselves.

Statistical Analysis

Young's modulus data were found to be continuous, normally distributed, and display homogenous variation. Since each sample was tested twice (before and after treatment), the data from the experimental and control groups were analyzed using a one-tailed, paired *t*-test to determine whether there was a significant difference in cortical bone Young's modulus before and after treatment (*Staphylococcus aureus* inoculation or dH$_2$O) in each of the three orientations. Values smaller than 0.05 (p<0.05) indicate significant differences. Ultimately, the paired *t*-test results of each orientation were compared between experimental and control groups.

Results

Bacterial Penetration

Twelve hours after addition of *S. aureus*, the broth in the experimental petri dish showed signs of bacterial growth compared to the negative control evidenced by broth turbidity (Fig. 5A, 5B). The sample broth from the negative control that was spread on agar 24h after addition of sterile broth showed no bacterial growth after 48 hours of incubation at 37°C (Fig. 6A, 6B). The positive control sample revealed a bacterial lawn consistent with the description of *S. aureus* (Fig. 6C). In addition, the experimental sample broth that was spread on agar 24h after addition of *S. aureus* resulted in the presence of a bacterial lawn after 48 hours of incubation at 37°C and was confirmed to be *S. aureus* after gram staining and microscopic imaging (Fig. 6D).
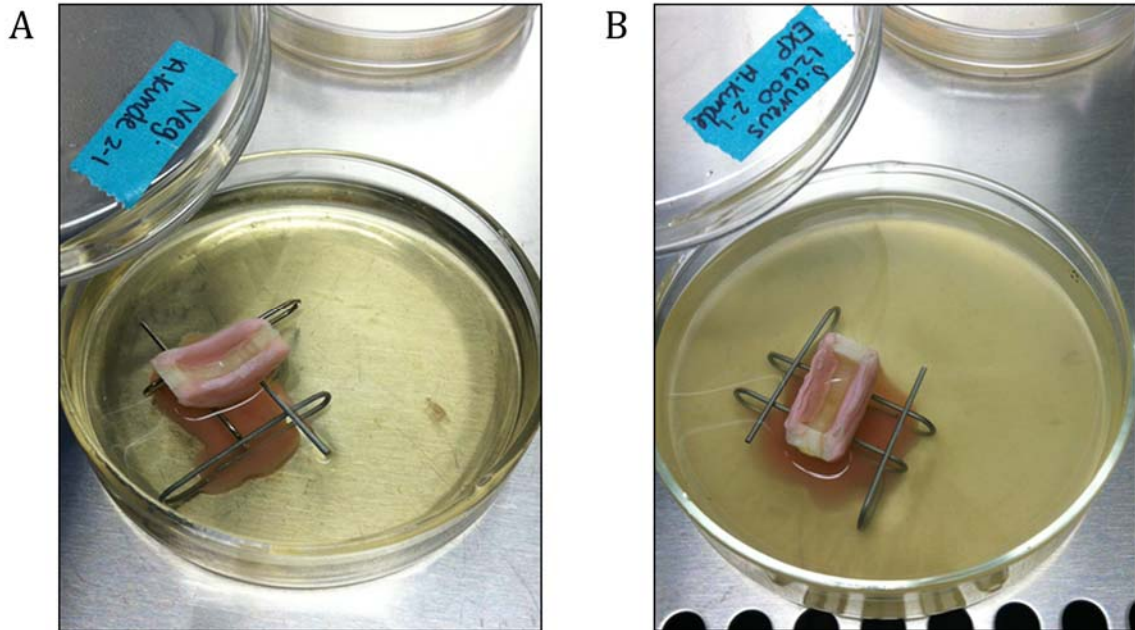
Figure 5: A) Negative control sample in the bacterial penetration experiment to which 150 µL sterile nutrient broth was added to the inside of the bone and incubated at 37°C. B) Experimental sample in the bacterial penetration experiment to which 150 µL *S. aureus* was added to the inside of the bone and incubated at 37°C. Photos were taken after 24 hours of incubation and show difference in broth turbidity indicating the presence of bacteria in the experimental sample.
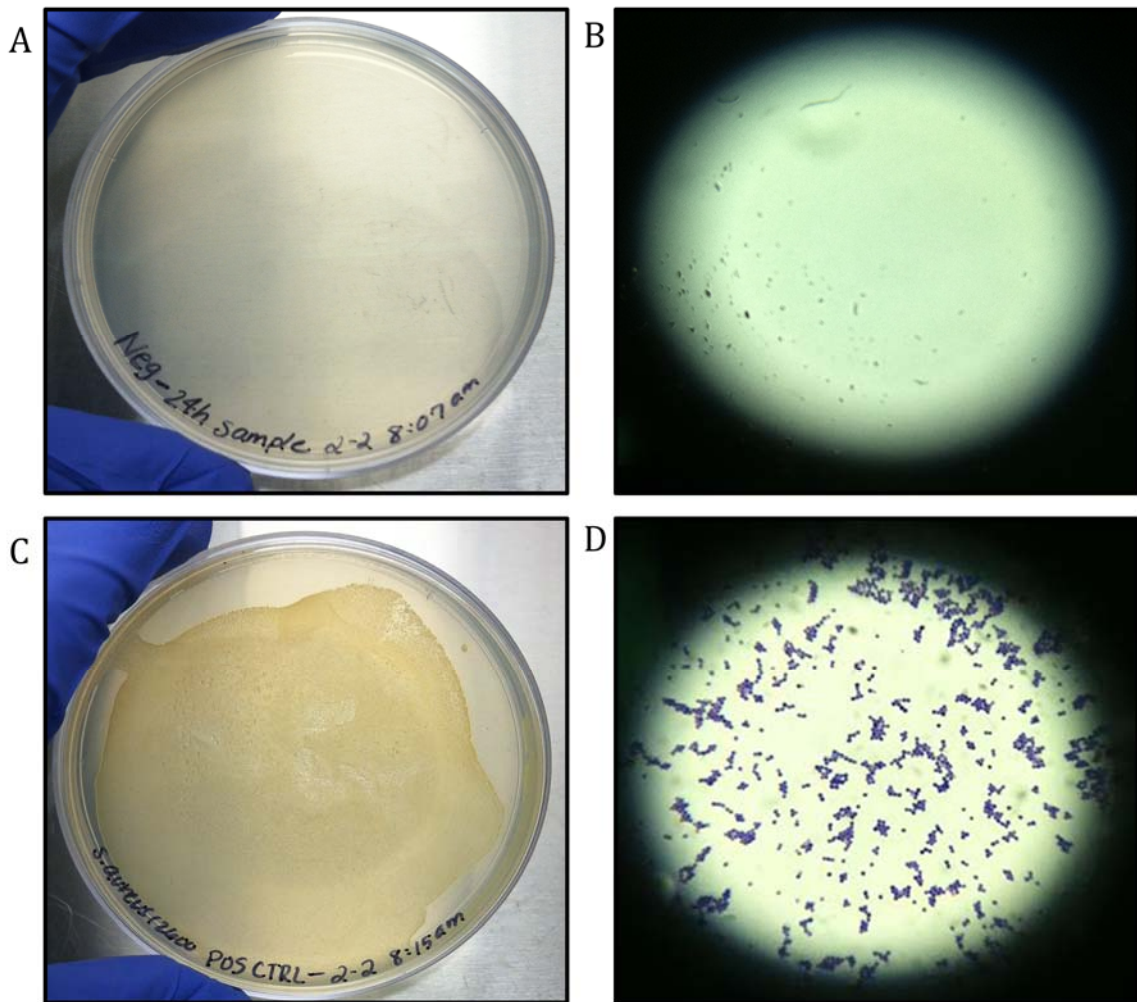
Figure 6: A) 200 μL broth from the negative control petri dish was spread on a sterile agar plate 24h after addition of broth. No growth was seen after 48h incubation at 37°C. B) Sterile nutrient broth showing no bacterial growth at 1000X magnification C) 200 μL broth from positive control sample was spread on sterile agar plate. Bacteria were present after 48h incubation at 37°C. D) 200 μL broth from the experimental petri dish was spread on a sterile agar plate 24h after addition of *S. aureus*. Gram stain performed after 48h incubation at 37°C revealed presence of *S. aureus* at 1000X magnification.

Young's Moduli Before Treatment

Cortical bone stiffness was highest in the axial orientation for both the control and experimental groups (24.4 ± 4.4 and 27.1 ± 5.1 GPa, respectively) (Fig. 7A and 7B). The transverse orientation of both control and experimental groups followed in stiffness values (16.8 ± 3.3 and 18.8 ±3.8 GPa, respectively) (Fig. 8A and 8B). The radial orientation displayed the lowest stiffness values for both control and experimental groups (14.6 ± 2.8 and 16.2 ± 3.6 GPa, respectively) (Fig. 9A, 9B and Table 1).

Young's Moduli After Treatment

After inoculating the experimental bone cubes in *S. aureus* at 37°C for 48h, the axial orientation showed no significant change in stiffness (27.1 ± 5.1 and 26.9 ± 4.8 GPa before and after treatment respectively, p>0.05). Similarly, the control axial orientation did not exhibit any significant change in stiffness after 48h immersion in sterile dH_2O at 37°C (24.4 ± 4.4 and 23.2 ± 3.7 GPa before and after treatment respectively, p>0.05) (Fig. 7A, 7B and Table 1).

After inoculating the experimental bone cubes in *S. aureus* at 37°C for 48h, the transverse orientation showed a significant decrease in stiffness (18.8 ± 3.8 and 17.6 ± 4.6 GPa before and after treatment respectively, p<0.05). In contrast, the control transverse orientation did not exhibit a significant change in stiffness after 48h immersion in sterile dH_2O at 37°C (16.8 ± 3.3 and 16.3 ± 3.9 GPa before and after treatment respectively, p >0.05) (Fig. 8A, 8B and Table 1).

After inoculating the experimental bone cubes in *S. aureus* at 37°C for 48h, the radial orientation showed a significant decrease in stiffness (16.2 ± 3.6 and 14.6 ± 3.2 GPa before and after treatment respectively, p<0.01). Similarly, the control radial orientation exhibited a significant change in stiffness after 48h immersion in sterile dH$_2$O at 37°C (14.6 ± 2.8 and 13.0 ± 2.1 GPa before and after respectively, p<0.01) (Fig 9A, 9B and Table 1).

	Axial			
	Control Group		Experimental Group	
	n	Mean (SD)	n	Mean (SD)
Pre-Test	32	24.4 (4.4)	32	27.1 (5.1)
Post-Test	32	23.2 (3.7)	32	26.9 (4.8)
	Transverse			
	Control Group		Experimental Group	
	n	Mean (SD)	n	Mean (SD)
Pre-Test	32	16.8 (3.3)	32	18.8 (3.8)
Post-Test	32	**16.3 (3.9)**	32	**17.6 (4.6)**
	Radial			
	Control Group		Experimental Group	
	n	Mean (SD)	n	Mean (SD)
Pre-Test	32	**14.6 (2.8)**	33	**16.2 (3.6)**
Post-Test	32	**13.0 (2.1)**	33	**14.6 (3.2)**

Table 1: Average Young's moduli (GPa) in the axial, transverse, and radial orientations for cubes tested before and after inoculation in sterile dH$_2$O for 48h at 37°C (control group, left) and cubes tested before and after inoculation in *S. aureus* for 48h at 37°C (experimental group, right). Values that are significantly different before and after treatment are bolded.

Figure 6: Box plot diagram showing the distribution of the A) control group's Young moduli (GPa) in the axial orientation before (pre-test) and after (post-test) 48h inoculation in sterile dH$_2$O at 37°C and the B) experimental group's Young moduli (GPa) in the axial orientation before (pre-test) and after (post-test) 48h inoculation in S. aureus at 37°C. The horizontal line inside the boxes is the median. Box hinges represent 25th and 75th percentiles. Whiskers represent minimum and maximum measured values. The results revealed no significant difference in both control and experimental groups before and after treatment (p >0.05).

Transverse Orientation

Figure 7: Box plot diagram showing the distribution of the A) control group's Young moduli (GPa) in the transverse orientation before (pre-test) and after (post-test) 48h inoculation in sterile dH₂O at 37°C and the B) experimental group's Young moduli (GPa) in the transverse orientation before (pre-test) and after (post-test) 48h inoculation in *S. aureus* at 37°C. The horizontal line inside the boxes is the median. Box hinges represent 25th and 75th percentiles. Whiskers represent minimum and maximum measured values not including outliers. An outlier is defined as a data point that is located 1.5 times IQR above the upper quartile and below the lower quartile. Outliers are represented by a circle sign. An extreme value is defined as a data point that is located 3 times IQR above the upper quartile and below the lower quartile. Extreme values are represented by a star. (These outliers appear as such only in the figure, no values were excluded from statistical calculations). The results revealed no significant difference before and after treatment in the control group (p >0.05). In contrast, a significant difference was found before and after treatment in the experimental group (p<0.05). * indicates significance (p<0.05).
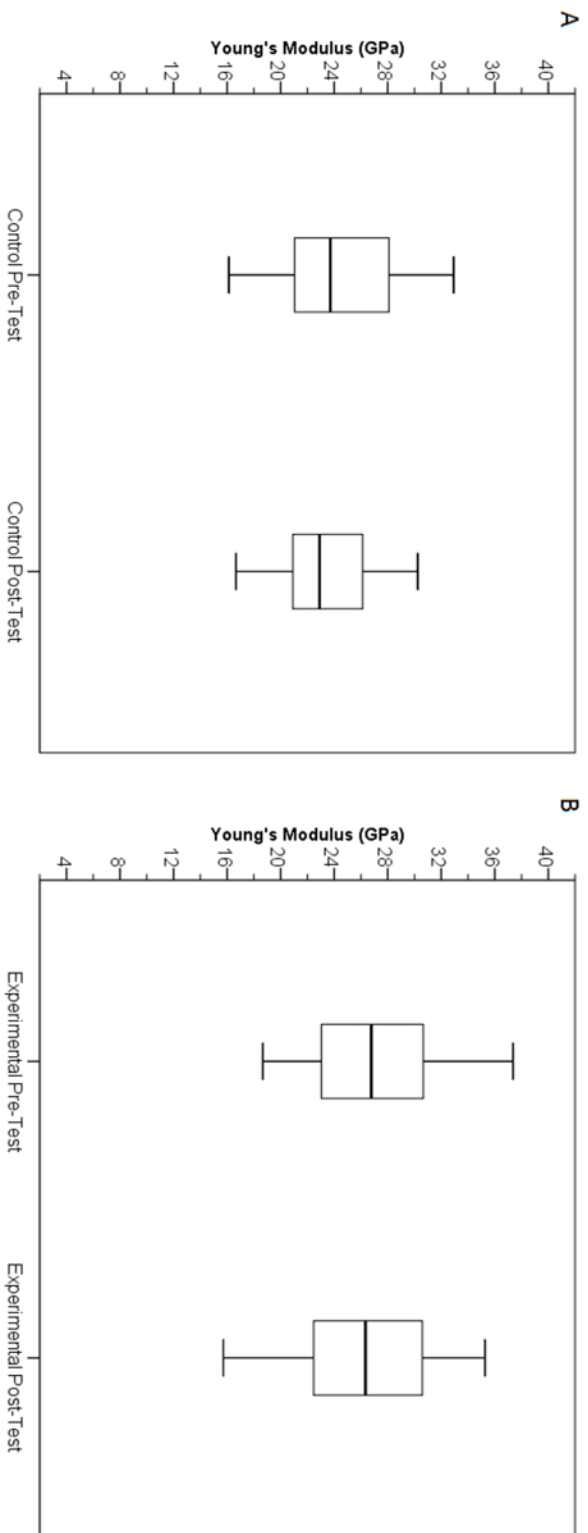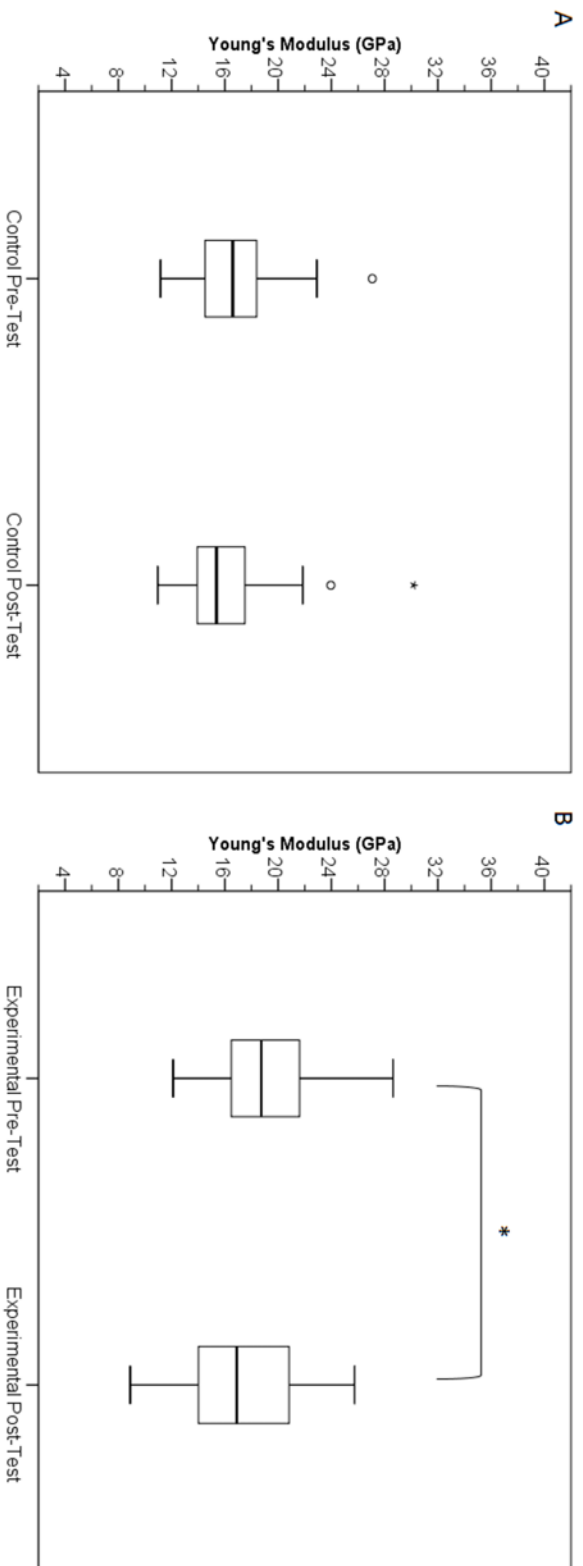
33

Radial Orientation
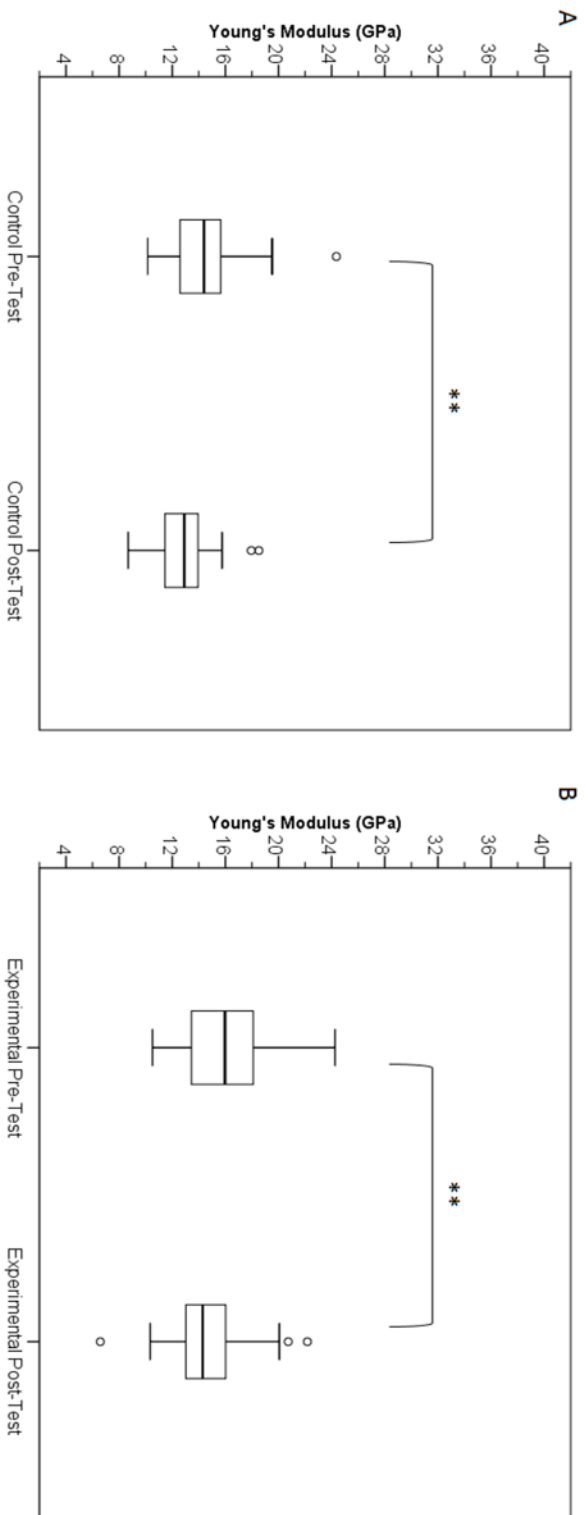
Figure 8: Box plot diagram showing the distribution of the A) control group's Young moduli (GPa) in the radial orientation before (pre-test) and after (post-test) 48h inoculation in sterile dH_2O at 37°C and the B) experimental group's Young moduli (GPa) in the radial orientation before (pre-test) and after (post-test) 48h inoculation in *S. aureus* at 37°C. The horizontal line inside the boxes is the median. Box hinges represent 25th and 75th percentiles. Whiskers represent minimum and maximum measured values not including outliers. An outlier is defined as a data point that is located 1.5 times IQR above the upper quartile and below the lower quartile; outliers are represented by a circle sign (These outliers appear as such only in the figure, no values were excluded from statistical calculations). The results revealed a significant difference in both control and experimental groups before and after treatment (p< 0.01). ** indicates significance (p<0.01).

Discussion

The purpose of this study was to determine if any stiffness changes occur in cortical bone after bacterial inoculation with *Staphylococcus aureus*. I hypothesized that a significant stiffness decrease would occur in the axial, radial, and transverse orientations of the bone after exposure to bacteria. For the most part, the results refute my hypothesis and show no significant difference in Young's modulus between pre-test and post-test values for either the experimental (infected) or control (uninfected) groups. No significant difference was found between the pre-test and post-test values for the infected and control cubes in the axial direction. The axial direction corresponds to the physiological direction of loading. Therefore, these results are a strong indicator that an acute infection with *S. aureus* does not significantly decrease bone stiffness and does not negatively affect bone material structure. A significant Young's modulus decrease was found between pre-test and post-test values for the infected (experimental) and control cubes in the radial orientation. That this significant difference was detected in both the control and experimental groups implies that the difference in stiffness is not due to *S. aureus* infection. A possible reason for the small (but statistically significant) decrease in stiffness is the natural deterioration of bone structure when it is left for a prolonged time at or above room temperature (five days in this study). A previous study (Barrera et al. 2016) also showed a decrease of up to 5% in bone stiffness after cortical bone cubes were kept for 7 days at 4°C, yet this difference was found to be non-significant. This study reports an average decrease of 10% in the radial direction for both the control and experimental groups. Seeing that the cortical bone cubes in this study were kept at much

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higher temperatures than in the previous study (20-37°C vs. 4°C), it is possible that this led to faster decomposition and significant stiffness differences in the radial orientation. Finally, a significant Young's modulus decrease was also found between pre-test and post-test values in the transverse orientation, yet this significant difference was exclusive to the infected (experimental group). That only the transverse orientation demonstrates a reduction in stiffness after *S. aureus* was introduced implies that other factors may be at play here. One possible explanation is again that our cortical bone cubes were left at room temperature and 37°C for prolonged periods during the five days between mechanical testing (pre and post-treatment). Nevertheless, the stiffness for the infected cubes decreased by only 6.4% after treatment. While this value was found to be statistically significant, it is questionable if it is biologically significant, especially considering the findings of Barrera et al. (2016) that 5% decrease in stiffness is not statistically significant and of this study that the axial and radial orientations did not exhibit decreased stiffness due to *S. aureus* infection.

Bone is a composite hierarchical material in which collagen fibers and hydroxyapatite crystals are interconnected by way of dilational bands to form tough mineralized collagen fibrils. In the next level of organization, mineralized collagen fibrils are arranged into fibril arrays where adjacent arrays are interlocked through sacrificial bonds. Consequently, any damage to the bone material structure (e.g. collagen-mineral interphase or bonds between adjacent arrays) would affect its mechanical behavior as a whole and we would expect to find a concurrent decrease in stiffness in all three orthogonal orientations (axial, radial, and transverse). Furthermore, since long bones are

loaded mostly along their long axis, the decrease in bone mechanical properties (such as stiffness) is expected to affect the axial orientation predominately. The phenomenon of decreased material properties in all orthogonal orientations is supported by Barrera et al. (2016). While they did not find any significant decrease in stiffness after cortical bone cubes were maintained for seven days at 4C, all three orthogonal orientations did show decreased trend in stiffness, indicating that any small damage that may have occurred to the bone material during these seven days affected all three orientations simultaneously. Likewise, Russell et al. (2012) reported a general decrease in cortical bone stiffness of all three orthogonal orientations after exposure to gamma irradiation. Indeed, in agreement with Barrera et al. (2016) and Russell et al. (2012), we found a small decrease (5-10%) in all three orientations for both the infected and control groups. In some cases this decrease was not significant (e.g. control and experimental groups of the axial orientation and control group of the transverse orientation), and in other cases it was (e.g. control and experimental groups of the radial orientation and experimental group of the transverse orientation). If a biologically significant decrease in stiffness due to *S. aureus* infection would have occurred, we would expect to see it in all three orthogonal orientations, especially in the axial orientation.

Another finding of this study was that *S. aureus* are capable of traveling through the microscopic pores in cortical bone structure (approximately 50µm for osteon central canals) even within the short timeframe exposure of our experiment (48h). As part of this study, I performed a bacterial penetration preliminary experiment that was instrumental in providing a basis for the subsequent study. I demonstrated that in a 48-hour period of

inoculation, the bacteria were not only surrounding the surface of the bone, but able to travel through the small pores of the cortical bone samples. Since the broth in the experimental sample showed bacterial growth, I was confident that the bones were sufficiently contaminated during the allotted inoculation time. These results were essential in ruling out the possibility that the bones were only contaminated on their surfaces. A recent study by de Mesy Bentley et al. (2017) showed that *S. aureus* entered the canaliculi of the bone and migrate toward lacunae *in vivo* using immunoelectron microscopy and BrdU labeling. Interestingly, de Mesy Bently et al. (2017) discovered a motility technique employed by *S. aureus* that involves their deformation from cocci into submicron rod-shaped bacteria that are 20% of their original diameter. These bacteria migrated *in vivo* using haptotaxis, durotaxis, and asymmetric binary fission (de Mesy Bently et al. 2017). Moreover, they were able to create an *in vitro* model showing bacterial migration through a 0.5 μm porous membrane in a 6.5 hour time period that further validated the ability of bacterial to move through cortical bone. These results support my findings that *S. aureus* are able to fully integrate into cortical bone.

To the best of my knowledge, there are no previous studies that discuss the biomechanical properties of cortical bone after bacterial infection. Many studies examine osteomyelitis secondary to fracture, but work describing fracture risk after recent bacterial infection is limited. A study by Rittmann and Perren (1974) questioned the change in bone biomechanics as a result of local infection. This particular study applied an internal fixation technique to fractured tibia or femur shafts of mountain sheep and measured interfragmentary compression of the plate under infection using strain gauge

elements during fracture healing. *Staphylococcus aureus* isolated from known human cases were injected locally into osteomy sites created in sheep set with different fixation techniques. Strain gauges measuring compression data across osteomy sites revealed decreased readings at the end of the healing process. These decreased results however cannot be contributed to bacterial infection alone as an internal fixation device was used for healing which could have acted as a confounding variable. On the contrary, Wang et al. (2001) measured Young's modulus in human femur beams after heating at specific temperatures between 37°C and 200°C to denature collagen. Three-point bending tests revealed less than 10% variation in Young's modulus in the axial orientation that was found to be insignificant across all levels of collagen denaturation. The results of Wang et al. (2001) are particularly related to this study as my hypothesis was, in part, based on *S. aureus'* ability to degrade collagen cross linkage and cause decreased stiffness. The findings of Wang et al. (2001) support my conclusion that *S. aureus* does not contribute to decreased bone stiffness.

Studies do, however, report low success rates in eradication and increased need for subsequent surgery in patients with periprosthetic infection due to MRSA. In patients suffering from periprosthetic joint infection due to MRSA from 2002 to 2007, a low eradication rate of 37% was reported after treatment with debridement and retention of prosthesis (Parvizi et al. 2009). Similarly, in a retrospective review of patients with acute periprosthetic joint infections due to MRSA treated with open irrigation and debridement with component rentention (ODCR) from 1990-2007, subsequent infection related surgery was necessary in 84% of patients (Bradbury et al. 2009). Without presence of

infection, Platzer et al. (2010) reported successful fracture healing of 87% in patients treated with periprosthetic fractures from 1992 to 2008. Similarly, Holder et al. (2014) reported an 82% fracture union rate in patients treated with periprosthetic femoral fractures from 2004 to 2009.

While I am unaware of studies that have tested the mechanical properties of cortical bone subjected to acute osteomyelitis, several studies have looked at the mechanical properties of fibrolamellar bone. Barrera et al. (2016) provide a summary of previous studies testing stiffness of bovine femora and tibiae fibrolamellar bone. In the axial orientation, our pre-test Young's modulus values (24.4 and 27.1 GPa for the control and experimental groups respectively) fall within the range of previously reported axial stiffness values of 21.0 GPa (Martin & Boardman 1993) to 30.3 GPa (Reilly et al. 1974). In the radial orientation, our pre-test Young's modulus values (14.6 and 16.2 GPa for the control and experimental groups respectively) are also within previously reported radial stiffness range of 11.6 GPa (Van Buskirk & Ashman 1981) to 17.3 GPa (Lipson & Katz 1984). Likewise, in the transverse orientation, our Young's modulus pre-test values (16.8 and 18.8 GPa for the control and experimental groups respectively) are within the range of previously reported transverse stiffness values of 14.6 GPa (Van Buskirk & Ashman 1981) to 21.4 GPa (Lipson & Katz 1984).

Barrera et al. (2016) is the only other study to test cortical bone cubes from white-tailed deer in compression, albeit they used cortical bone from the femoral proximal diaphysis while I have used cortical bone cubes from the humerus mid-diaphysis. The Young's modulus value of 21.6 GPa reported by Barrera et al. (2016) in the axial

orientation was slightly lower than my pre-test Young's modulus values in the axial orientation (24.4 and 27.1 GPa for the control and experimental groups respectively). The radial Young's modulus value of 14.9 GPa reported by Barrera et al. (2016) falls in between my pre-test Young's modulus values in the radial orientation (14.6 and 16.2 GPa for the control and experimental groups respectively). Similarly, the transverse Young's modulus value of 17.6 GPa reported by Barrera et al. (2016) also falls in between my pre-test Young's modulus values in the transverse orientation (16.8 and 18.8 GPa for the control and experimental groups respectively). The higher stiffness values in this study compared to Barrera et al. (2016) are likely due to the differences in stresses experienced by femur and humeri. The forelimbs in quadrupedal mammals support 60% of their body weight (Kilbourne and Hoffman 2013), so humeri (tested in this study) are subjected to higher stresses and can generally be expected to exhibit higher stiffness values compared to femora (tested by Barrera et al. 2016). These varying stress profiles lead to mineralization and remodeling differences that could also contribute to the different stiffness values. Taken together, my results, in agreement with all previous studies, describe fibrolamellar bone's orthotropic behavior with the axial orientation exhibiting the greatest stiffness. As majority of loads are experienced in the axial orientation of the humerus mid-diaphysis, the axial data is perhaps the most revealing in terms of biological significance.

A limitation of this study remains that many of *S. aureus* virulence factors that contribute to osteomyelitis such as leukocidins and coagulase act by interfering with host immune response, which was not reproduced here. *In vivo,* the material properties of

bone may be affected by leukocidins and coagulase as they promote biofilm growth and do not allow nutrients to the access the bone resulting in damage. Also, the Sae locus that is responsible for regulating virulence factors that are cytotoxic to osteoblasts most likely have an effect on bone material properties *in vivo*. These mechanisms were not a part of this study, but factors that still may be at play during *in vitro* inoculation include adhesins and collagenase. Looking forward, it would be of interest to examine bone material properties with all factors at play in an *in vivo* murine model like that of de Mesy Bentley et al. 2017 to mimic direct contamination with *S. aureus*. In addition, the bacterial strain used for this study (ATCC 12600) was chosen because it has been proven to be easily eradicated in laboratory settings. It may be possible that use of a more virulent strain of *S. aureus* could have more detrimental effects on bone structure. For instance, it is possible that a more virulent strain may adhere tighter to bone material and secrete degradative enzymes to a greater extent than seen in this study. Another limitation of this study is that our cubes were in thawed conditions one day more than recommended by the American Association of Tissue Banking that requires osteoarticular tissue processing be completed within 5 days of recovery (AATB 2016). Furthermore, the bone samples in our study were exposed to *S. aureus* for 48 hours because of the thawing time constraint. In experimental animal models of osteomyelitis, previous studies have infected bones for up to 7 days to mimic acute osteomyelitis (Patel et al. 2009).

In summary, I measured stiffness in all three orthogonal orientations of humeri cortical bone and confirmed that fibrolamellar bone exhibits orthotropic behavior. All values recorded were in accordance with those previously reported. The results show no

significant decrease between pre and post-test stiffness values for the axial and transverse orientation of the uninfected group. This is in line with the Barrera et al. (2016) that showed no significant decrease in bone stiffness after exposure to thawed conditions for 7 days. Though there was a significant difference between pre and post-test stiffness values for the radial orientation in the control group, this difference was small (about 10%). In fact, for the experimental group the decrease in stiffness values for all three orientations were also less than 10% even when they were statistically significant, so statistical significance may not indicate biological significance in this case; especially when the most important orientation (axial) was found statistically insignificant. Seeing that (1) the axial orientation showed no significant difference between experimental pre and post-test stiffness, (2) the radial orientation showed a significant difference in both control and experimental pre and post-test stiffness indicating that the bacteria is not responsible for this difference, and (3) the results only indicate a statistically significant decrease in the transverse orientation due to bacterial infection, the logical conclusion is that *in vitro* infection with *S. aureus* does not contribute to stiffness change in cortical bone. It seems likely that any decrease in stiffness of the cubes in this study was caused by subjection to temperatures as high as 37°C during the thawing period. Overall, the null hypothesis was supported, and there is no clear evidence of change in material properties after 48h inoculation with *S. aureus.* In light of these findings, current tissue collection screening methods employed by bone-graft banks seem to be adequate.

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Ethanol Testing

Four bone cubes (2x2x2 mm) cut from the distal femoral diaphysis of white tailed deer were loaded in compression using an Instron 5942 machine. Young's modulus values were recorded for each cube in the axial, radial, and transverse orientations. Immediately following biomechanical testing, each cube was placed in an Eppendorf tube containing 1 mL 91% ethanol for 24 hours under constant agitation (300 RPM) on a temperature-controlled shaker at 20°C. To remove residual ethanol, samples were then washed four times (3 x 5 min; 1 x overnight 16h) in 1 mL sterile dH_2O under constant agitation (300 rpm) on the shaker at 20°C. Between each wash, samples were rinsed with 1mL sterile dH_2O and transferred aseptically via autoclaved forceps to new sterile Eppendorf tubes. The next morning, samples were rinsed once more with 1mL sterile dH_2O, and washed (300 rpm) on the shaker at 20°C for 15 min. All bone surfaces were then swabbed with a sterile cotton tip and plated on nutrient agar at 37°C for 48h to confirm sterility. The samples were then loaded once more in compression to obtain post-test Young's modulus values. The purpose of this preliminary test was to examine the effect of bone's intended exposure to alcohol on stiffness of cortical bone. The results were analyzed with a non-parametric Wilcoxon signed-rank test to determine differences between pre and post-test stiffness in all three orientations. No significant difference was observed in the axial, radial or transverse orientations ($p>0.05$) (Table 2), thus the use of 91% ethanol for sterilization in this experiment is supported.

	Ethanol					
	Axial		**Radial**		**Transverse**	
Cube	*pre-test*	*post-test*	*pre-test*	*post-test*	*pre-test*	*post-test*
1	18.6	22.6	13.6	13.7	16.6	14.8
2	18.6	21.2	14.1	12.5	10.1	12.0
3	15.9	14.4	14.1	12.3	9.0	10.1
4	17.7	16.5	12.1	11.4	9.9	9.3

Table 2: Average Young's moduli (GPa) in the axial, radial, and transverse orientations for cubes tested before (pre-test) and after (post-test) 24h exposure to 91% ethanol at 20°C and 300 RPM. Wilcoxon signed-rank test analysis revealed no significant difference in any orientation between pre-test and post-test groups ($p > 0.05$).

Appendix B

Povidone Iodine and Chlorhexidine Gluconate Testing

Five bone cubes (2x2x2 mm) cut from the mid femoral diaphysis of white tailed were loaded in compression using an Instron 5942 machine. Young's modulus values were recorded for each cube in the axial, radial, and transverse orientations. Immediately following biomechanical testing, four cubes were each placed in an Eppendorf tube containing 1 mL 4% w/v chlorhexidine gluconate (Hibiclens™ Liquid Antiseptic Skin Cleanser, Fisher Scientific) for 15 min under constant agitation (300 RPM) on a temperature-controlled shaker (20°C). The remaining two cubes were each placed in an Eppendorf tube containing 1 mL povidone iodine (Povidone-Iodine Solution USP, 10% w/v, Ricca Chemical) for 15 min under constant agitation (300 RPM) on a temperature-controlled shaker (20°C). To remove any residual solution from the samples, each cube was washed three times (3 x 5 min) in 1 mL sterile dH_2O under constant agitation (300 rpm) on the shaker at 20°C. Between each wash, samples were transferred aseptically via autoclaved forceps to new sterile Eppendorf tubes and rinsed with 1mL dH_2O. The cubes were loaded once more in compression after exposure to either solution. The purpose of this preliminary test was to determine which of the two agents would be suitable for this project. The results were analyzed with a non-parametric Wilcoxon signed-rank test to determine differences between pre and post-test stiffness in all three orientations. No significant difference was found between pre and post-test stiffness of the cubes that were exposed to povidone-iodine ($p > 0.05$) (Table 3). Likewise, no significant difference was observed in the axial, radial, and transverse orientations of cubes exposed to

chlorhexidine gluconate (p>0.05) (Table 4). Though no statistical difference was found

for either of these treatments, the arithmetic difference in stiffness was greater for

povidone iodine than chlorhexidine (up to 56% difference with iodine). These results

suggest that chlorhexidine gluconate is an acceptable disinfectant as it did not damage the

material properties of the samples.

| | Povidone Iodine | | | | | |
| | Axial | | Radial | | Transverse | |
Cube	*pre-test*	*post-test*	*pre-test*	*post-test*	*pre-test*	*post-test*
1	25.5	11.1	13.8	12.7	12.9	10.8
2	25.3	14.5	15.5	11.3	11.0	9.2

Table 3: Average Young's moduli (GPa) in the axial, radial, and transverse orientations
for cubes tested before (pre-test) and after (post-test) 15 min. exposure to 10% aqueous
povidone iodine solution at 20°C and 300 RPM. Wilcoxon signed rank test analysis
revealed no significant difference in any orientation between pre and post-test groups
(p>0.05).

| | Chlorhexidine Gluconate | | | | | |
| | Axial | | Radial | | Transverse | |
Cube	*pre-test*	*post-test*	*pre-test*	*post-test*	*pre-test*	*post-test*
1	24.5	25.2	18.0	16.3	26.5	28.9
2	24.8	26.4	17.6	15.5	18.4	17.0
3	26.5	28.9	18.8	24.3	24.2	25.5

Table 4: Average Young's moduli (GPa) in the axial, radial, and transverse orientations
for cubestested before (pre-test) and after (post-test) 15 min. exposure to 10% aqueous
povidone iodine solution at 20°C and 300 RPM. Wilcoxon signed rank test analysis
revealed no significant difference in any orientation between pre and post-test groups
(p>0.05).

Appendix C

Chlorhexidine Gluconate- Antimicrobial Activity Test

Five bone cubes (2x2x2 mm) were each inoculated in an Eppendorf tube with 0.5mL of nutrient broth containing *S. aureus* (ATCC 12600) and placed on a shaker at 37°C. In a BSL-2 hood, bone samples were removed from the broth after 48 hours and the surfaces were swabbed with a cotton tip to be plated on agar (Fig. 10A). This served as a control – showing bacterial growth on untreated bone. Immediately after swabbing, each bone sample was set in a microtube containing 0.5 mL chlorhexidine for 15 min under constant agitation (300 RPM) on a temperature-controlled shaker (20°C). Each sample was then removed from chlorhexidine, and surfaces were swabbed to be plated on nutrient agar for 48h at 37°C (Fig. 10B). This step verified all bacteria on bone surfaces were killed. To remove any residual amount of chlorhexidine from the samples, each cube was washed three times (3 x 5 min) in 1 mL sterile dH_2O under constant agitation (300 rpm) on the shaker at 20°C. Between each wash, samples were transferred aseptically via autoclaved forceps to new sterile Eppendorf tubes and rinsed with 1mL dH_2O. Each cube was then crushed with sterile pliers and ground with a sterile mortar & pestle (separate one for each cube). 1 mL of sterile dH20 was pumped into each mortar to create a solution that was plated on agar with an inoculating loop (Fig. 10C). This step verified all bacteria on the bone's internal surfaces (e.g. central canals and canaliculi) were killed. The chlorhexidine solution and the dH2O after all 3 rinses for each bone sample were also plated. This served to verify that no bacteria survived (but were washed off the bone). The results of this experiment were successful in showing the presence of

S. aureus before treatment and its elimination after 15 min of chlorhexidine immersion at 20°C. This demonstrates the use of Chlorhexidine as an antimicrobial agent for this project.
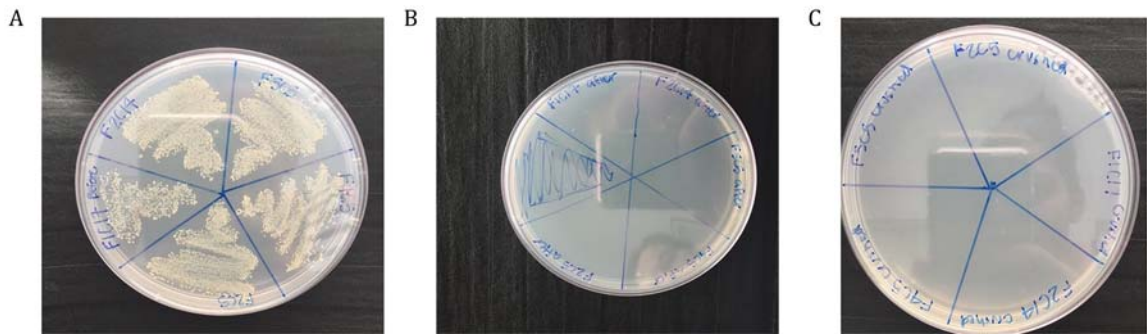

Figure 10: A) *S. aureus* 12600 from the surface of each bone sample before being introduced to chlorhexidine solution. B) Surface of each bone sample swabbed on nutrient agar after 15 minutes immersed in chlorhexidine at 20°C. C) Crushed bone samples spread on nutrient agar after inoculation, immersion in chlorhexidine, and rinsing with dH₂O. All photos show plates after 48 hours of incubation at 37°C.