INVESTIGATION ON THE ANTIBACTERIAL EFFICACY OF TITANIUM ALLOY COATING COMPARED TO THE BULK TITANIUM ALLOY AND STEEL

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Investigation on the Antibacterial Efficacy of Titanium Alloy Coating Compared to the Bulk Titanium Alloy and Steel

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

The advancements in medical operations and sciences have improved patient lives, but device-related infections and bacterial contamination remain significant concerns. Escherichia coli is a prominent bacterium causing various infections. Commonly used antibacterial materials in the health industry for surgical operations include silver, copper, zinc, titanium, and steel exhibit antibacterial properties due to their durability, corrosion resistance, and diverse applications. This study aimed to compare the antibacterial properties of titanium (Ti64), steel, and titanium coating on aluminum to inhibit the growth of Escherichia coli. The experiment utilized serial dilution and colony counting techniques to assess bacterial growth on the materials. Results showed that titanium has better antibacterial properties, with Ti64 coating on aluminum also displaying effectiveness but to a less extent. In contrast, steel was the least effective. The study highlights the need for further research to understand the underlying mechanisms of antibacterial behavior in these materials and their long-term efficacy in surgical operations, ultimately contributing to improved infection control in medical settings.

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1. INTRODUCTION

The advancements in medical operations and sciences have brought with them challenges and hurdles over the years, which have impacted human lives. With the great improvements that medical devices bring to the quality of patient lives, device related infection and inflammation is still a main complaint despite strict operating procedures [1]. Bacterial contamination and subsequent biofilm formation on various surfaces also have significant implications in healthcare, food processing, and environmental hygiene [1]. One noteworthy application involves the utilization of biomaterials in the field of medical and healthcare. For instance, biomaterials play a pivotal role in crafting medical devices like joint replacements, dental implants, heart valves, prosthetic limbs, tissue engineering constructs, and drug delivery systems [2]. These biomaterials encompass a variety of materials, including metals, polymers, biological substances such as collagen and hyaluronic acid, ceramics, and even composites [2]. Each surgical procedure on the human body demands a specific type of biomaterial to ensure optimal functionality and safety. For example, orthopedic implants commonly employ metals like titanium, stainless steel, and cobalt-chromium alloys due to their exceptional durability. In contrast, soft tissue implants such as heart valves frequently employ polymers like polyethylene, polyurethane, and silicone [2].

Upon initial introduction into the host body, implanted biomaterials may become coated with various substances such as serum proteins, aqueous humor, mucosal secretions, microbiota, and extracellular fluids, depending on their location within the body [3]. This renders the surface of the implanted biomaterial highly susceptible to microbial and bacterial attachment. Postoperative infections have been attributed to microbiota from the host body, the operating

room environment, and the implant surface itself. Bacterial attachment to the implant surface is facilitated by host proteins that coat the implant [3].

In cases where antibacterial coatings or surface modifications are absent, specific bacteria species can release signaling molecules to communicate and alter their metabolic states, forming a biofilm. This biofilm consists of proteins, DNA, and membrane structures that shield the bacteria, rendering them resistant to drugs [3]. To mitigate infections and reduce bacterial attachment to implanted surfaces, surface modifications can be implemented to confer antibacterial and antimicrobial properties. This, in turn, can reduce the need for re-hospitalizations, additional interventions, and post-implantation antibacterial treatments [3]. Figure 1 provides a visual representation of how bacteria can adhere to a surface and the protective benefits of surface modifications. According to *Wang et al.*, the ideal antibacterial surface is required to achieve the following functions: (1) prevent initial adhesion of bacteria, (2) kill all bacteria that have managed to surmount the anti adhesion measure used, and (3) thirdly, to clear dead bacteria *[4]*.

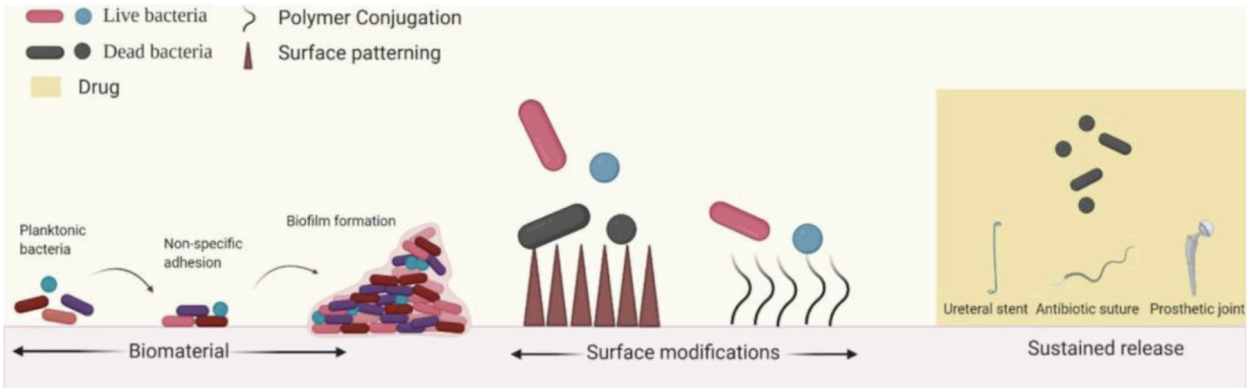

Figure 1. Biofilm Formation and Surface Modification: A diagram that displays the stages of biofilm formation and strategies to prevent infection. Modifying surface properties of the biomaterial such as biocidal patterning and polymer conjugation can prevent biomaterial associated infections in humans [3].

One prominent bacterial species of concern is Escherichia coli (E.coli), which is known to cause gastrointestinal infections, urinary tract infection, neonatal meningitis, and foodborne illnesses [5].In recent years, the exploration of advanced materials with antimicrobial properties has gained significant attention [2]. Titanium alloys, such as Ti-6Al-4V (Ti64), have been widely used in various applications due to their excellent biocompatibility, mechanical properties, and corrosion resistance [6]. Furthermore, surface modifications, such as the application of Ti64 coatings, have been developed to enhance the performance and antimicrobial properties of materials [6]. The most commonly used methods for applying Ti64 coatings include plasma immersion ion implantation, chemical and physical vapor deposition, sol-gel, plasma spraying, plasma electrolytic oxidation, powder metallurgy coating, and anodization [7].

Ti-based alloys are favored for biomedical applications due to the formation of a protective titanium oxide film, which prevents corrosion and enhances biocompatibility [6]. However, the oxide film formation that makes Ti-based implants biocompatible also makes it susceptible to bacterial infections [8]. The formation of a thin titanium oxide layer under physiological conditions provides a substrate for protein and cell adhesion, leading to bacterial colonization and biofilm formation [8]. Therefore, surface modifications or coatings are necessary to meet clinical requirements in minimization contamination, particularly in preventing infections, as titanium and its alloys alone are not that effective [8]. According to Wu *et al.*, the micro superhydrophobic nanostructure of titanium alloys enhances its antibacterial property, which further reduces the contact area for bacterial adhesion [9]. In addition, surface modifications with titanium lowers the surface energy which helps to improve the self-cleaning effect and facilitates the detachment and removal of bacteria cells [9].

Steel (alloy of iron and carbon) possesses biocompatibility and mechanical properties that make it very versatile in medical application. Figure 2 below, displays the unique properties of steel with different applications and how it possesses the desired characteristics for each different operation. Steel can be used for a wide range of applications including orthopedic implants, bone fixation, artificial heart valves, and much more [10]. Although steel possesses good durability, non-toxicity, and cost-effectiveness, bacteria can still survive and form biofilm on the surface of steel [11]. Therefore, to reinforce and strengthen the properties of steel, it is typically accompanied with another material on its surface like copper, zinc, or silver. Adding copper, zinc, silver, or titanium to steel can lead to an effective antibacterial material to be used in medical environments. Due to steel's already existing biocompatibility and mechanical properties, when it is combined with a coating that expresses strong antibacterial properties its properties become enhanced [11]. A study conducted by Pang *et al.* showed that the surface topography, chemical composition, and energy were crucial to the antibacterial ability of steel [12]. The results of Pang's experiment showed that the smoother the surface of steel is, the higher the antibacterial rate was, which is attributed to smoother surfaces making it more difficult for bacteria to adhere onto and grow [12].

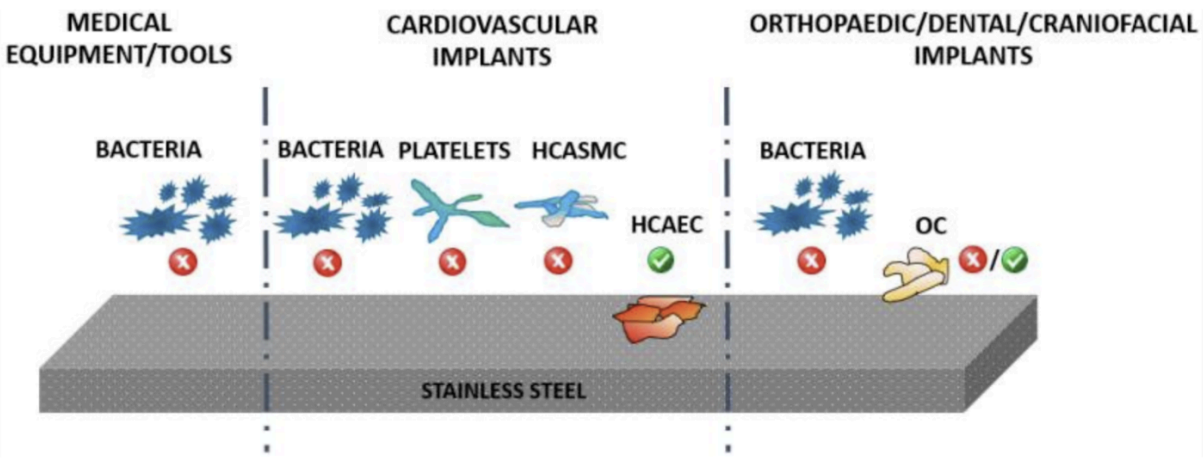

Figure 2. Applications of Steel in Medicine: A schematic of the applications of steel in medicine and the desired response of bacteria and human cells (platelets, HCAEC–human coronary artery endothelial cells, HCASMC–human coronary artery smooth muscle cells, OC–osteoblast cells) [10].

Both steel and titanium possess antibacterial properties, as do some other metals such as copper, silver, gold, and zinc. However, there are some metals such as aluminum, that do not have noteworthy antibacterial properties [13]. Since aluminum metal itself lacks inherent antibacterial properties, its oxides and certain aluminum-based compounds can exhibit antibacterial effects when engineered or treated accordingly [14]. In the food and health industry, aluminum can be utilized to inhibit bacterial growth when it is used as a subcomponent to a primary antibacterial material [14]. The cost of molded titanium and titanium composites is expensive compared to the cost of steel, aluminum, and other metals. Therefore, applying titanium coating on aluminum is much more accessible and affordable than titanium metal, and it enhances the aluminum properties [15]. Arthroplasty, craniofacial, maxillofacial dental inserts, careful instruments, medical services items, and external/internal prosthesis are some examples of metal titanium coating applications [15]. As a result, with its wide applications in the healthcare field, assessing its effectiveness in comparison to full titanium metal will further shed light on the antibacterial properties of titanium metal and coating with aluminum.

The primary objective of this experiment is to conduct a comparative analysis of the antibacterial properties exhibited by three distinct test materials: a Ti64 metal plate, Ti64 coating on aluminum, and a steel metal plate. The aim is to determine which material demonstrates the least growth of E. coli bacteria. There are a multitude of techniques used to measure bacterial growth that include direct microscopic count, serial dilution and plate count, dry weight measurement, and biomass measurement. Bacterial growth on antibacterial materials is also sometimes measured using optical density (OD). Optical density involves monitoring the growth of bacteria by measuring turbidity of a liquid culture using a spectrophotometer [16]. As the bacterial population increases, the culture becomes cloudier, which results in increasing optical density readings that are then correlated with cell density using a calibration curve [16]. Another notable method for calculating bacterial growth is metabolic activity measurement. This method is used by assessing the reduction of a colorimetric substrate or the production of gasses can be used to estimate bacterial growth [17].

Bacterial growth measurements will be obtained utilizing serial dilution calculations, which will provide a range of bacterial concentrations to allow for a clear understanding of which material is more potent at high/low bacterial concentrations. Subsequently, the diluted samples will be spread onto bacterial culture plates for incubation and subsequent colony counting. The number of colony forming units (CFU) per OD per mL will be estimated by multiplying the colony count by the dilution factor.

2. MATERIALS AND METHODS

For this study, three distinct materials were employed to assess their antibacterial properties: steel (S), Ti64 components (T1), and Ti64 coatings on aluminum (C). Each sample was two 1 cm X 1 cm square (Figure 3).

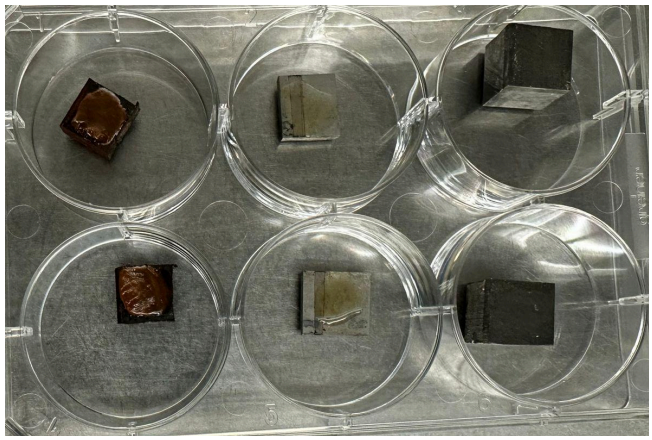

Figure 3. Sample Materials: Sample materials used for experimentation (S1, S2, T1, T2, C1, C2).

To cultivate the bacterial culture, a standardized suspension of Escherichia coli (E. coli) with a concentration of approximately 1 mg/µl was utilized. A total of 48 agar petri dishes were used as containers for the materials during the experiment, as depicted in Figure 4. Eight agar plates were allocated for each of the six materials. The inoculation of the bacterial culture onto the material surfaces was accomplished using sterile loops constructed from sterile metal wire. These loops facilitated the streaking of the bacterial suspension. It is worth noting that the selection and preparation of these materials were carefully undertaken to decrease the sources of error across all samples that can be caused by contamination, different sample sizes, or even a difference in bacterial concentrations. Furthermore, two samples of each material were prepared.

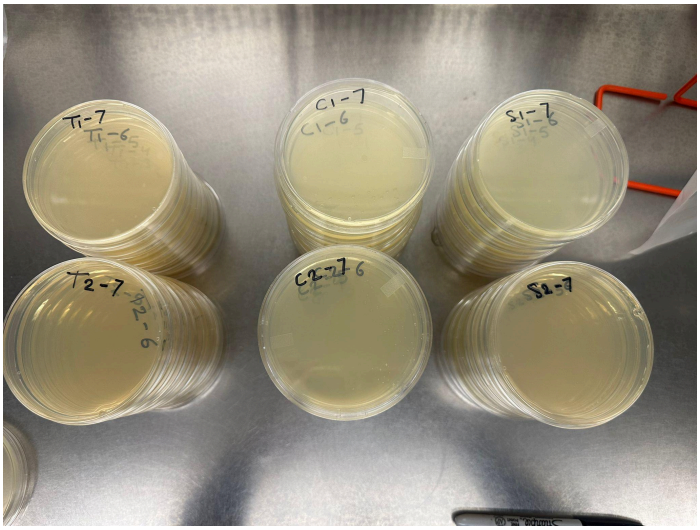

Figure 4. Agar Plate Preparation: Agar plates organized and labeled T1, T2, S1, S2, C1, C2.

2.1. Preparation and Serial Dilution

To initiate the experiment, eight tubes were designated and filled with phosphate-buffered saline (PBS), with each tube receiving 900 microliters following aseptic techniques. These tubes were sequentially labeled from #1 to #8, with the first tube representing a 1-10 dilution, and subsequent tubes representing a logarithmic series dilution (1-100, 1-1000, and so forth). The PBS was transferred into the tubes using a 1ml pipette pen with two stops, ensuring no contact with the inside of the cap. E. coli cultures were then aspirated into one of the PBS-filled tubes using a p200 pipette, followed by three pipetting cycles and mixing via a vortex genie 2.

Serial dilutions were performed by transferring 100 µl of the mixture from one tube to the next, ensuring thorough mixing before each transfer. Additionally, six sterilized metal samples (two each of steel, titanium, and titanium coating on aluminum) were prepared and placed in a tray, with corresponding metals paired together. These metals were labeled accordingly, designating one pair as controls (without E. coli) and the other pair with overnight E. coli culture (100 microliters). Each agar plate was divided into eight sections using a sharpie marker, starting from the most diluted to the least diluted, to visualize bacterial growth based on varying

concentrations. Subsequently, a p200 pipette was used to spot 3 drops (10 microliters each) of the PBS-E. coli mixture onto each section of the agar plate, allowing the spots to dry before proceeding. The bacterial growth and plate division are depicted in Figure 5.

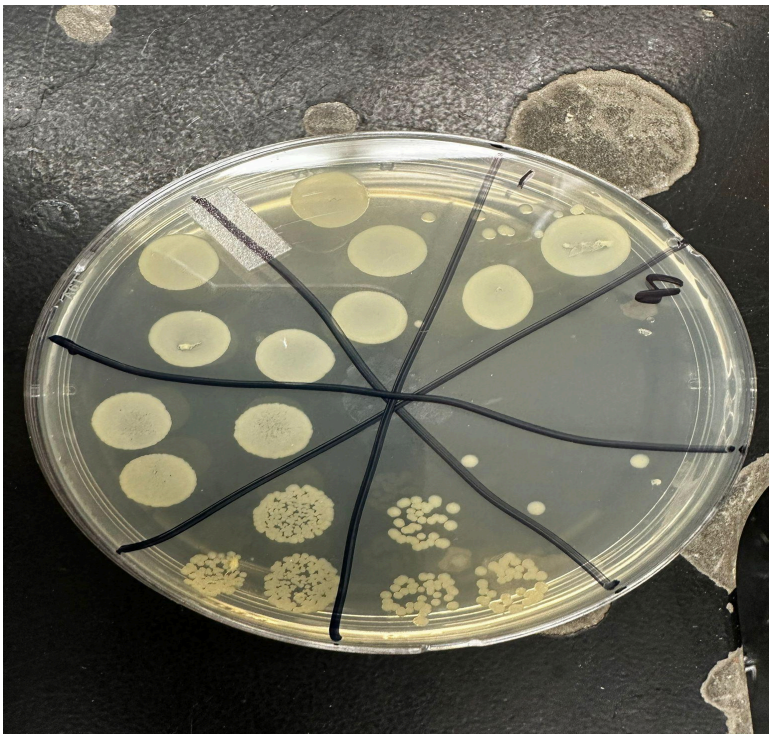

Figure 5. Bacterial Growth Test: Bacterial growth based on different concentrations (1 is the most concentrated and 8 is the least). Section 8 in the image displays the smallest bacterial growth, while section 1 has the largest colonies.

2.2. Inoculation and Agar Plate Setup

To continue the experiment, the process was repeated, filling 48 tubes with 900 microliters of the PBS-E. coli mixture. Cotton swabs were employed to collect samples from the PBS mixture, followed by serial dilutions for all eight tubes for the first steel sample (S1) and subsequently for the second steel sample (S2). This procedure was replicated for the Ti64 coating on aluminum (C1, C2) and titanium Ti64 (T1, T2) samples. After completing the dilution process for all 48 tubes, 48 agar plates were prepared and labeled according to the serial dilution of the samples. A turntable was utilized to arrange the agar plates, and they were labeled accordingly

for steel (S1-0, S1-1, S1-2, S1-3, S1-4, S1-5, S1-6, S1-7) and repeated for the second steel sample (S2).

The same procedure was replicated for the Ti64 coating on aluminum (C1-0, C1-1, C1-2, C1-3, C1-4, C1-5, C1-6, C1-7) and titanium Ti64 (T1-0, T1-1, T1-2, T1-3, T1-4, T1-5, T1-6, T1-7) samples, as well as for all C2 and T2 samples. Subsequently, all 48 agar plates with inoculums were incubated for 24 hours at room temperature.

2.3. Colony Counting and Data Collection

Following the incubation period, the inoculum and all 48 agar plates were removed from the incubator, and bacterial colony growth was assessed, as demonstrated in Figure 6 below. Colonies were marked as dots on the plates, and counts were made, including those that were either too numerous to count (TNTC) or too few to count (TFTC). The colony counts are presented in Table 1.

	Dilution							
Sample	**10^{0}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**
S1	TNTC	TNTC	384	158	19	3	2	TFTC
S2	TNTC	354	193	31	TFTC	4	TFTC	TFTC
T1 - 2	TFTC	TFTC	TFTC	TFTC	TFTC	TFTC	TFTC	TFTC
C1	67	6	3	TFTC	TFTC	TFTC	1	TFTC
C2	1	1	TFTC	TFTC	TFTC	TFTC	TFTC	TFTC

Table 1. Experiment Colony Count at Different Concentrations: TFTC—Too few to count and TNTC—Too numerous to count.

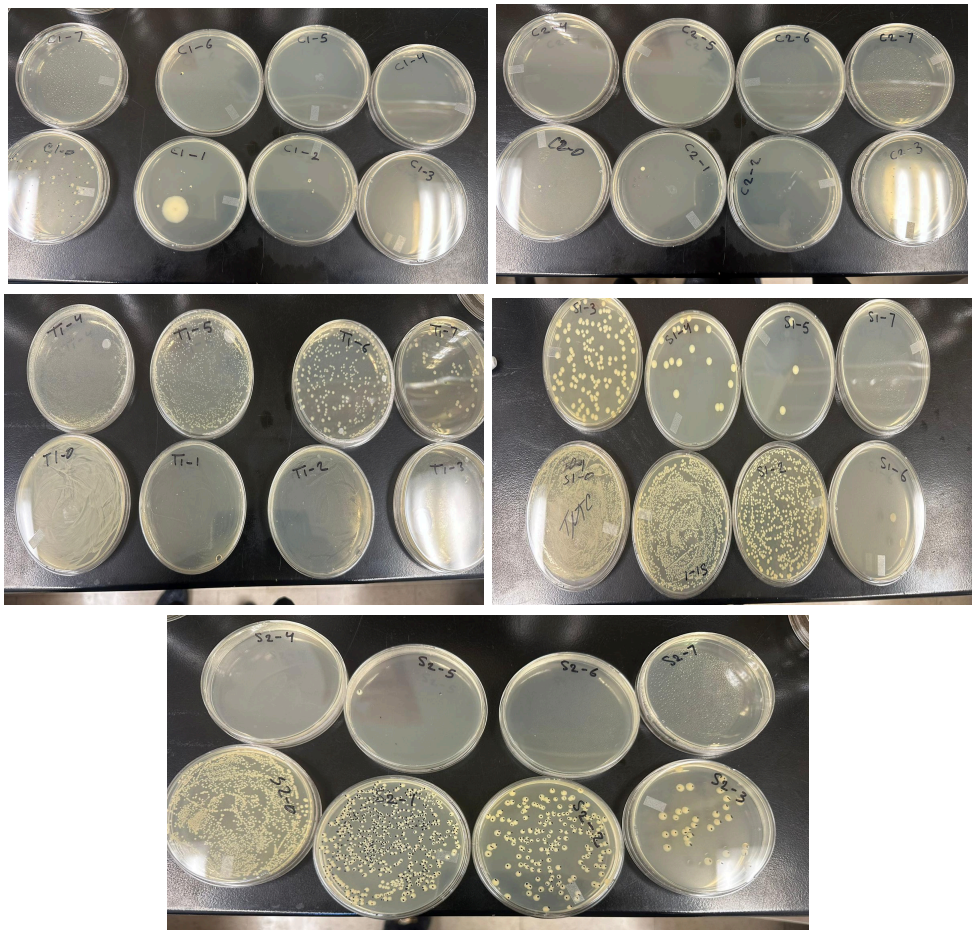

Figure 6. Experiment Colony Counting: Bacterial growth can be observed in the experimental agar plates (top left to right: C1, C2, T1, T2, S1 S2).

Calculations for the serial dilutions for each sample were performed (see Appendix A). Observations pertaining to the materials and their respective bacterial growth were also recorded including the bacterial growth trends, colony sizes, and if there was any contamination. It was ensured that the serial dilution technique yielded results with bacterial counts lower than the initial inoculum. These procedures and subsequent analyses aimed to assess the effectiveness of different materials in inhibiting bacterial growth, providing valuable insights for potential applications in healthcare settings.

3. RESULTS

The antibacterial potency of the materials can be observed through the absence or slow growth of E. coli colonies surrounding the experimental samples [18]. Additionally, the relative effectiveness of the antibacterial coatings can be ascertained by comparing the sizes of bacterial colonies or zones of inhibition across different materials [19].

The average diameter of bacterial colonies or zones of inhibition for each experimental material was calculated as a quantitative measure of bacterial growth inhibition for each material. By comparing the average diameters of bacterial colonies or zones of inhibition of these materials, their antibacterial effectiveness can be identified. The material that demonstrates substantial inhibition of E. coli growth or exhibits the smallest bacterial colony diameter is considered the optimal antibacterial material for healthcare facility applications.

Table 1 presents the findings from the analysis of bacterial growth on the various materials used at different dilution levels. The table reveals the quantitative assessment of bacterial colonies for each material at distinct dilution factors. Steel 1 demonstrated bacterial counts that are too numerous to count (TNTC) at the most concentrated (undiluted) level (10^0) and the first dilution (10^{-1}). At 10^{-2} dilution, there are 384 bacterial colonies counted, and the count decreases progressively with higher dilutions. Similarly, Steel 2 displayed a similar trend to Steel 1, the bacterial counts are TNTC at the most concentrated level (10^0) and decrease with dilution. At 10^{-1} dilution, 354 bacterial colonies are counted, and the count further decreases with higher dilutions.

Ti64 (Sample 2) consistently exhibited low bacterial loads, with all bacterial counts for this sample being too few to count (TFTC) at all dilution levels tested. On the other hand both the aluminum samples with titanium coatings demonstrated effective antibacterial properties but

not to the extent of the full titanium metal sample. Ti64 Coating 1 at the most concentrated level (10^0) displayed 67 bacterial colonies. The count decreases with dilution, and at 10^{-6} dilution, there is only 1 bacterial colony detected. Similarly, Ti64 Coating 2 at the most concentrated level (10^0), there is only 1 bacterial colony. The count decreases with dilution, and no colonies are detected at 10^{-3} or higher dilutions.

Overall, the table shows the effect of dilution on the bacterial counts for each material. Some materials show high bacterial counts at lower dilutions, indicating high bacterial load, while others show lower or negligible counts, indicating lower bacterial load. The material with the lowest bacterial counts across most dilution levels is likely to be the most antibacterial. In this case, T2 (Ti64 Sample 2) stands out as the most antibacterial material since its bacterial counts are too few to count (TFTC) at all dilution levels. The bacterial counts across dilutions indicates that T2 has the least bacterial colonization and exhibits strong antibacterial properties. The material with the highest bacterial counts, especially at lower dilution levels, is likely to be the least antibacterial. In Table 1, S1 (Steel Sample 1) shows relatively high bacterial counts, including 384 colonies at the 10^{-2} dilution level. The bacterial counts across dilutions suggest that S1 is the least antibacterial among the tested materials.

4. DISCUSSION

The results obtained from this study showed that when tested under the highest dilutions of 10^0 and 10^{-1}, the titanium metal sample outperforms the antibacterial effectivity of steel and Ti64 coating on aluminum metal. The presence of Ti64 coating on aluminum also showed more effectiveness in antibacterial properties than steel metal. From in vitro antibacterial experimentation conducted by Bolzoni *et al.*, they showed that titanium alloys can display up to 98.2% antibacterial rate against E.coli [20]. Given the experiment's limited size, Ti64 alloy proved to be a more effective antibacterial material compared to both steel and Ti64 coating on aluminum, aligning with findings from the existing literature reviews. Once Ti64 is added as a coating to aluminum it has a remarkable effect on its mechanical behavior and physical properties, and the combination of the two materials leads to the formation of new microstructural features [20]. The microstructural features that emerge include intermetallic compounds that contain both Ti and aluminum, influence structure of both materials, and lead to alloying effects. Alloying effects pertain to the modifications in material properties that arise when two or more elements or metals are blended to produce an alloy, thereby impacting the physical, mechanical, and chemical attributes of the material [20].

Steel was the least antibacterial out of all the materials used in this experiment, even though it is deemed to be greatly antibacterial and is used in a wide range of applications in the industry. The highest counts of E.coli bacterial colonies were consistently obtained in this experiment in the steel samples.

The potential variables that may influence the results encompass factors such as porosity, surface charge, topography, and the presence of coatings. While the current study and existing literature provide insight into the steel, aluminum (titanium coating), and Ti64 antibacterial

properties, further research is necessary to understand the underlying mechanisms of antibacterial behavior in these materials. Different testing conditions, such as varying temperature, pH or pressure, as well as different surface modifications can be factors to study for their effects on the material's antibacterial behavior.

5. CONCLUSION

Device-related infections remain significant concerns in the healthcare industry, due to many antibiotic-resistant bacterial strains. E. coli is a prominent bacterium causing various infections. Titanium alloys are widely used in medical applications due to their biocompatibility. Similarly, steel possesses biocompatibility and mechanical properties, making it versatile in medical applications. The study findings underscore the pivotal role of material selection in mitigating bacterial growth, especially in surgical operations and healthcare settings. Among the tested materials, Ti64 (T2) emerged as the most antibacterial, consistently demonstrating bacterial counts too few to count (TFTC) across all dilution levels. In contrast, Steel (S1) exhibited relatively high bacterial counts, notably at and before the 10^{-3} dilution level. Furthermore, the introduction of a titanium alloy coating on aluminum demonstrated antibacterial properties, making it a promising candidate for applications where bacterial contamination needs to be minimized.

While titanium alloy, Ti64 , showcases superior antibacterial properties compared to steel, it's worth noting that steel offers its own set of advantages, including cost-effectiveness, ease of fabrication, and the potential for surface modifications. These qualities make steel a viable option in healthcare where antibacterial properties may be complemented by other factors. The choice of material is critical when designing medical devices and surfaces to combat bacterial colonization. This study provides valuable insights into the antibacterial properties of steel, Ti64, and Ti64 coating on aluminum, offering guidance for selecting materials that enhance patient safety and contribute to infection control efforts in healthcare environments. Future research should investigate the influence of variations in both surface roughness and temperature on the antibacterial properties of steel, Ti64, and titanium-coated aluminum.

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APPENDIX. SERIAL DILUTION CALCULATIONS

S1 (First Steel Sample):

- Initial concentrations: 384×10^{-2}, 15.8×10^{-2}, 0.19×10^{-2}

- Simplified to the same power: 384×10^{-2}, 15.8×10^{-2}, 0.19×10^{-2}

- Sum of the concentrations: $(384 + 15.8 + 0.19) \times 10^{-2} = 399.99 \times 10^{-2}$

- Converted to scientific notation: $399.99 \times 10^{-2} = 133.33 \times 10^{-2}$

- Average of the three numbers: 133.33×10^{-2}

- 10 microliters used from the E. coli mixture

- Final concentration: $133.33 \times 10^{-2} \times 10^{8} = 133.33 \times 10^{6}$, which is $< 3.7 \times 10^{9}$

- The average of the three numbers was compared with the E. coli solution to verify the correctness of the serial dilution.

S2 (Second Steel Sample):

- Initial concentrations: 25.4×10^{-2}, 1.93×10^{-2}, 0.031×10^{-2}

- Sum of the concentrations: $(25.4 + 1.93 + 0.031) \times 10^{-2} = 27.361 \times 10^{8}$

- 10 microliters used from the E. coli mixture

- Final concentration: 27.361×10^{8}

- Since two identical samples were used, the average is calculated: $(0.13 + 2.73) \times 10^{9} / 2 = 1.43 \times 10^{9}$, which is also $< 3.7 \times 10^{9}$

C1 (First Titanium Coating on Aluminum Sample):

- Initial concentrations: 67×100, 0.1×10^{-1}, 0.03×10^{-2}

- Sum of the concentrations: $(67 + 0.1 + 0.03) \times 10^{3} = 2.237 \times 10^{9}$

- Since 10 microliters were used from the E. coli mixture, the final concentration is 2.23×10^{9}, which is less than 3.7×10^{9}.

C2 (Second Titanium Coating on Aluminum Sample):

- Initial concentrations: 1×100, 0.1×10^{10}

- Sum of the concentrations: $(1 + 0.1) \times 10^2 = 0.55 \times 10^8$

- Since 10 microliters were used from the E. coli mixture, the final concentration is 0.55×10^8, which is less than 3.7×10^9.

In this experiment, serial dilution was performed for each sample to determine the concentration of E. coli solution, and the results were compared to a threshold concentration of 3.7×10^9 to assess the correctness of the dilution.