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Polymeric Materials Enhanced with Antimicrobial Additives for Use in Dairy Milking Applications

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ABSTRACT

The transmission of mastitis infections within dairy herds, facilitated by biofilms on dairy liners, presents a significant challenge to animal health and dairy farm productivity. This study introduces novel engineered materials designed to reduce the prevalence of mastitis in dairy herds without escalating antibiotic drug usage. By integrating inherently antimicrobial materials into the polymer matrix of dairy liners, we aim to create a passive barrier against pathogenic organisms, thereby limiting disease transmission among animals. Specifically, the antimicrobial efficacy of Zinc Oxide (ZnO) embedded within a polymer matrix, akin to those utilized in commercial dairy liners, was assessed using two methods. The first method followed the non-GLP ASTM International Method E2180 to evaluate the antibacterial activity of treated articles against Staphylococcus aureus ATCC 6538. The results demonstrated a significant log reduction in bacterial count, achieving a 4.28 log reduction with a 2% (weight) ZnO incorporation and a 5.34 log reduction with 4% ZnO. The second method utilized was an augmentation of ASTM E2180-18 with the objective being to develop a pseudo-biofilm on the surface of the polymers by exposing them to raw dairy hospital parlor milk. Results show a decrease in microbiological growth with the addition of ZnO particles in the polymer matrix. This study underscores the potential of utilizing engineered materials with embedded antimicrobial agents to create surfaces that passively neutralize pathogenic organisms. By doing so, it is possible to inhibit disease transmission within dairy herds and decrease the dependency on antibiotics for treating communicable diseases in dairy cattle, thus contributing to sustainable dairy farming practices.

Keywords
Antimicrobial additives, Mastitis infections, Dairy farms.

Introduction

The persistent challenge of mastitis infections within dairy herds, exacerbated by biofilms on dairy liners, necessitates innovative solutions to safeguard animal health and enhance dairy farm productivity. This research examines the development of novel engineered materials, incorporating antimicrobial agents into the polymer matrix of dairy liners to establish a passive defense against pathogenic microbes, thus reducing the spread of mastitis without resorting to increased antibiotic interventions. Central to this investigation is the efficacy of Zinc Oxide (ZnO) as an antimicrobial agent when embedded within a polymer matrix resembling those used in commercial dairy liners. Employing the ASTM International Method E2180, this study assesses the antibacterial activity against *Staphylococcus aureus* ATCC 6538, demonstrating significant bacterial count reductions correlating with ZnO concentrations. Historically, the health-promoting properties of certain materials have been recognized long before the formal identification of microorganisms, yet the specific mechanisms by which these materials exhibit antimicrobial properties remain partly elusive [1]. Among the hypothesized mechanisms, the generation of hydroxyl radicals through the semiconductive properties of ZnO, direct cellular membrane interaction leading to cell death, and the cytotoxic effects of released zinc ions offer compelling explanations for ZnO's antimicrobial capacity [2-6].

While it's acknowledged that bacterial responses to antimicrobial surfaces vary, leading to different degrees of susceptibility and potential antimicrobial resistance (AMR), our focus on non-pharmacological approaches like ZnO embedment in dairy liners aims to mitigate such concerns by disrupting microbial cell functions without inducing widespread resistance. Notably, recent literature challenges prevailing notions about ZnO's primary antimicrobial mechanisms, suggesting a more intricate interplay of metabolic pathways in response to ZnO exposure [7].

This research represents a pivotal step towards redefining material science applications in agriculture by integrating passive microbial-resistant technologies into dairy operations. By examining the antimicrobial effectiveness of ZnO-embedded surfaces, this study provides valuable insights into strategies for reducing reliance on antibiotics in dairy herds, aligning with sustainable agricultural practices. Collaborating with industry stakeholders, this project endeavors to disseminate findings that can influence material innovations, marking a significant advancement in the quest for sustainable dairy farming solutions.

Experimental Methods and Materials

Materials

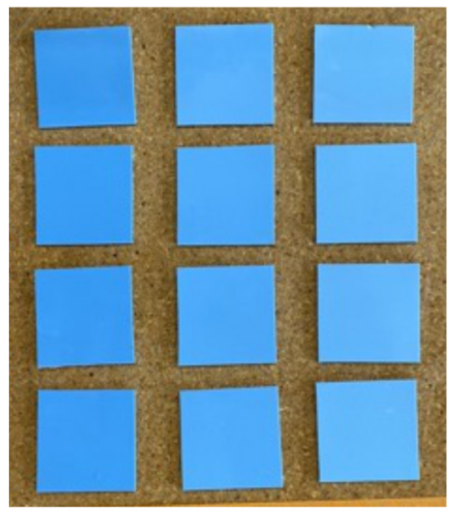
The critical role of milking liners as the sole point of direct contact between the milking machine and the cow emphasizes the importance of their production method for both mechanical and, in this case, antimicrobial properties. The interaction between the teat and the liner significantly influences the efficacy of the milking machine. Given the wide variety (hundreds) of commercial liners available globally and their complex chemical composition, which can include up to twenty different ingredients, maintaining high standards in dimensions and physical attributes across different batches is essential for optimal milking performance and reliability [8]. For this study, a platinum-cure silicone polymer (Smooth-Sil 950, Smooth-On, Inc., Macungie, PA, USA) was chosen as the test material due to its commercial availability, simple processing requirements, and similarity to the physical characteristics of proprietary polymers used in many common dairy liners. Zinc Oxide (ZnO 104, ZoChem, Bradbury, TO, CA), provided by Buffalo Technology Group, Ltd (Dimmitt, Texas, USA), served as the antimicrobial test agent.

Mold Design and Fabrication

A custom, 5-piece aluminum mold was designed using Fusion 360 (Autodesk, Inc, San Francisco, CA, USA) to cast two, 10 cm by 30 cm test sheets with a thickness of 3 cm, mimicking the wall thickness of critical features within the Pro-Square dairy liner (Pro-Square DPX CR, IBA Dairy Supplies, Sutton, MA, USA). The parts were machined from 6061-T6511 extruded aluminum (Online Metals, Seattle, WA, USA) using a Tormach 1100MX CNC Mill (Tormach, Inc, Madison, WI, USA). Special attention was given to the flatness of the interior faces and the thickness of the spacers, utilizing a fly cutter (Tormach TTS Super Fly Cutter) with a polished carbide insert for precision. Post-machining, the interior faces were polished to a semi-mirror finish and the mold was prepared with mold release (Ease Release 200, Mann Release Technologies, Macungie, PA, USA) before assembly.

Polymer Processing and Molding

A two-part silicone polymer was prepared at a 10:1 resin-to-hardener ratio, with ZnO added at 0, 2, and 4 % (by weight) concentrations to evaluate its antimicrobial efficacy. The mixture underwent a two-step blending process using a planetary mixer (ARE 310, Thinky USA, Laguna Hills, CA, USA) to minimize agglomeration. The mixed polymer was then injected into the mold, which was positioned at a 45-degree angle to facilitate air evacuation. The mold was subjected to a pressure chamber at ambient temperature under 50 psi for 24 hrs to reduce air pockets. Subsequently, the samples were released, washed, and cut into coupons for testing (Figure 1).



A B C

Figure 1: Polymeric Samples for Antimicrobial Testing including Control (A); 2% ZnO Additive (B); 4% ZnO Additive (C).

Trial 1: Antimicrobial Efficacy Testing – Standardized ASTM E2180-18

The antimicrobial activity of the samples was assessed following ASTM E2180-18, a standard test method for determining the activity of incorporated antimicrobial agents in polymeric or hydrophobic materials. This method evaluates the effectiveness of antimicrobials in materials by measuring the percent reduction in surviving populations of challenge bacterial cells at 24 hours compared to a non-treated control. The test involves maintaining an aqueous-based bacterial inoculum in close, uniform contact with the treated material in a “pseudo-biofilm” state. Calculations for average count of colonies, $\log_{10}$ reduction, and percent reduction were performed to analyze the data. The test microorganism(s) selected for this test was *Staphylococcus aureus* ATCC 6538. This bacterium is spherical-shaped, gram-positive, and facultative anaerobe. *Staphylococcus* species are known for their resistance to antibiotics like methicillin. *S. aureus* can cause various health conditions ranging from commensal skin colonization to severe diseases like pneumonia and toxic shock syndrome (TSS). It is frequently used as a model for gram-positive bacteria in various test methods. Although it is hard to disinfect, it does demonstrate susceptibility to low-level disinfectants. *S. aureus* is also a major cause of mastitis in dairy cattle. Mastitis is an inflammation of the udder tissue, which can be caused by various bacterial pathogens. *S. aureus* can cause chronic and subclinical infections in the udder, leading to reduced milk production and quality. The bacterium can also be transmitted to humans through the consumption of contaminated milk, causing a range of infections. Effective control measures, such as good milking hygiene and the use of antibiotics, can help prevent and treat mastitis caused by *S. aureus*.

Trial 2: Milk-Based Antimicrobial Efficacy Testing

To further relate the antimicrobial efficacy of the experimental polymers to the real-world application on farms, a second trial was designed using unpasteurized whole milk as the inoculum and nutrient broth. Raw milk was collected from a hospital milking parlor at a dairy in the Texas Panhandle. When cows in milk are diagnosed as being infected with mastitis, they are pulled from the primary milking parlor and instead milked at a secondary milking parlor often called the "hospital pen" or "hospital parlor" while under mastitis treatment protocols. The milk from these hospital parlors has an exceptionally high microbial load and is unfit for human consumption. Samples were taken from the bulk storage tank at the hospital parlor so that the bacterial colonies present in the milk were directly associated with known cases of mastitis in the Texas Panhandle. This methodology, while less documented than standardized cultures and inoculation protocols, allows the experimental polymers to be tested directly against the microorganism populations that are known to be associated with mastitis infection in the Texas Panhandle.

The protocol was developed as an augmentation of ASTM E2180-18 with the objective being to develop a pseudo-biofilm on the surface of the polymers by exposing them to a nutrient-rich media that includes microbial cells, biological and nonbiological matter – raw milk. Three samples were prepared by pouring 17.5 g of resin into an 88 mm diameter petri plate (VWR); samples included 2% (by weight) ZnO, 4% ZnO, and 0% ZnO (Figure 3). Samples were cured in a pressure chamber at 50 psi for 24 hours. Butterfield's Phosphate Buffered Dilution Water (BPBDW) was prepared by dissolving 34 g of KH_2PO_4 in 500 mL of steam-distilled water. The pH was adjusted to 7.2 with a 1 N NaOH solution. Following pH adjustment, the volume was brought to 1 L with distilled water. The solution was then sterilized by autoclaving at 121°C for 30 minutes. Each test plate was inoculated with 3 mL of unpasteurized raw milk from the hospital parlor of a Texas Panhandle dairy. A gentle swirling motion was used to fully wet the polymer surface of the sample with milk.

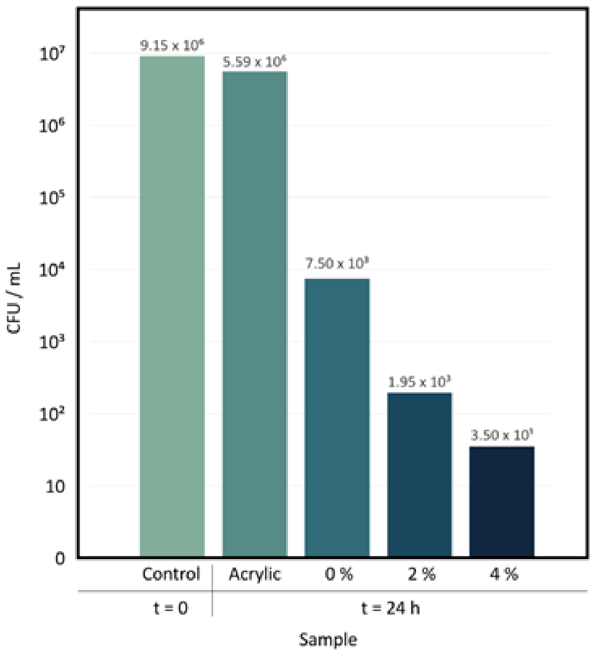
Samples were placed in a shallow pan with a Petri dish containing distilled water situated in the center. This assembly was loosely covered with aluminum foil and allowed to incubate at room temperature. Post-incubation, the plates were washed with 27 mL of BPBDW. A stainless-steel lab spatula was used to gently scrape the milk from the surface and sides of each plate. The effluent from each sample wash was collected in a 200 mL glass beaker. The beaker was then covered with wax film and swirled until all milk particles were dissolved. For each sample, serial dilutions were prepared by adding 9 mL of BPBDW into three Falcon tubes. One milliliter of the effluent, assumed to represent a 1:10 dilution of the initial inoculate concentration, was transferred into the first Falcon tube containing 9 mL of BPBDW. This mixture was shaken by hand for 25 strokes, each exceeding 1 inch in amplitude. Subsequently, 1 mL of this diluent was pipetted onto a Peel Plate (Charm Sciences Inc, Lawrence, MA, USA) to achieve a 1:100 dilution. Another 1 mL from this mixture was transferred to the next Falcon tube to prepare a 1:1000 dilution, which was also plated on a Peel Plate, and the process was repeated one more time to create a 1:10000 dilution. The inoculated Peel Plates were incubated at 32.5°C for 36 hours. The plates were then photographed (Figure 4) using a DFK33UX264 Camera (The Imaging Source, LLC, Charlotte, NC, USA) and images were analyzed using the Particle Analysis feature of ImageJ software (National Institutes of Health, USA) (Figure 5). This structured and detailed methodology provides clear and reproducible steps for the preparation, inoculation, and analysis of samples using BPBDW and serial dilution techniques for microbial analysis.

Results and Discussion

The incorporation of Zinc Oxide (ZnO) as an antimicrobial agent into the platinum-cure silicone polymer used for milking liners showed a significant reduction in bacterial count, indicating effective antimicrobial activity. The results, as detailed in Table 1 and illustrated in Figure 2, demonstrate that with an increase in the concentration of ZnO, there is a notable increase in the log reduction of bacterial count. Specifically, a 2% ZnO incorporation resulted in a 4.28 log reduction, while a 4% ZnO addition achieved a higher log reduction of 5.34. These results underscore the effectiveness of ZnO as an antimicrobial additive in the silicone

Table 1: *S. aureus* ATCC 6538 Percent Reduction and Log10 Reduction Compared to Control.

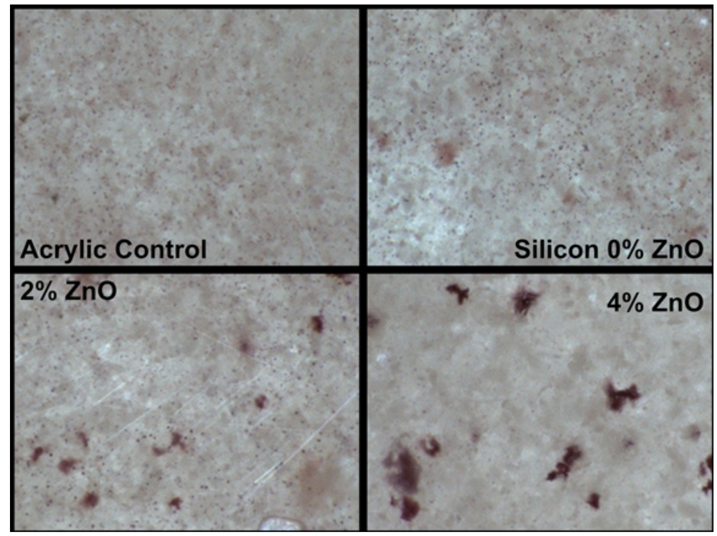
Contact Time	Test Article	Average CFU/mL	Percent Reduction of Microorganism Compared to Control	Log10 Reduction of Microorganism Compared to Control
Time Zero	Acrylic Control	9150000	N/A	N/A
24 Hours	Acrylic Control	5950000	N/A	
	0%	7500	0.998739496	2.9
	2%	195	0.999967227	4.48
	4%	35	0.999967227	5.23



polymer matrix used for creating dairy liners.

Figure 2: *S. aureus* ATCC 6538 Comparison of Average CFU/mL.

The milk testing results revealed a discernible threshold between 2% and 4% loading of zinc oxide (ZnO) after 24 hours of exposure, with an approximate rate of 2 square inches per mL milk (Figure 3). Images captured after 24 hours of exposure to the test materials and 36 hours of incubation provided visual evidence. Serial dilution by a factor of 10^4 was conducted on samples, followed by an assessment of colony number and average size. Notably, colonies exhibited an increase in size proportional to nutrient availability, as supported by previous studies [9]. A statistically significant difference in average colony diameter, corresponding to a greater number of colony-forming units (CFUs), was observed with increasing ZnO percentage. The initial milk inoculated was measured at approximately 2.81×10^8 CFU/ml. Subsequent exposure at room temperature for 24 hours indicated a reduction in CFUs, with the 4% ZnO sample showing a decrease to 1.76×10^7 CFU/ml, while the 2% ZnO sample had 1.37×10^8 CFU/ml. Despite a lower log reduction compared to water-based samples, noticeable efficacy was observed in the 4% ZnO samples, as



depicted in Figure 3.

Figure 4: Composite image showing comparative analysis of microbial colonies of sample effluent (1:10^4 dilution).

The experimental findings highlight the potential of ZnO as a powerful antimicrobial agent when incorporated into the material used for milking liners. The significant log reduction in bacterial count with ZnO incorporation not only indicates the effectiveness of ZnO in inhibiting bacterial growth but also suggests a dose-dependent relationship between ZnO concentration and antimicrobial activity. The observed 4.28 log reduction at 2% ZnO concentration and a further increase to 5.34 log reduction at 4% ZnO concentration support the hypothesis that higher concentrations of ZnO lead to greater antimicrobial efficacy. Moreover, the use of custom-designed mold and precision manufacturing techniques, as described in the Methods and Materials section, contributed to the reproducibility and reliability of the antimicrobial testing. The meticulous preparation and characterization of the polymer-ZnO composites underline the importance of controlled experimental

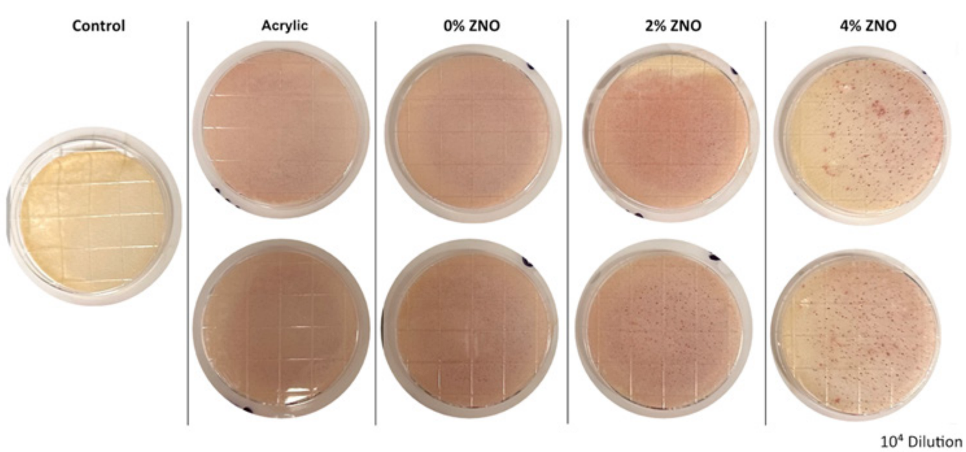


Figure 3: Comparative Analysis of Microbial Colonies of Sample Effluent (plated in duplicate).

conditions in evaluating the efficacy of antimicrobial additives.

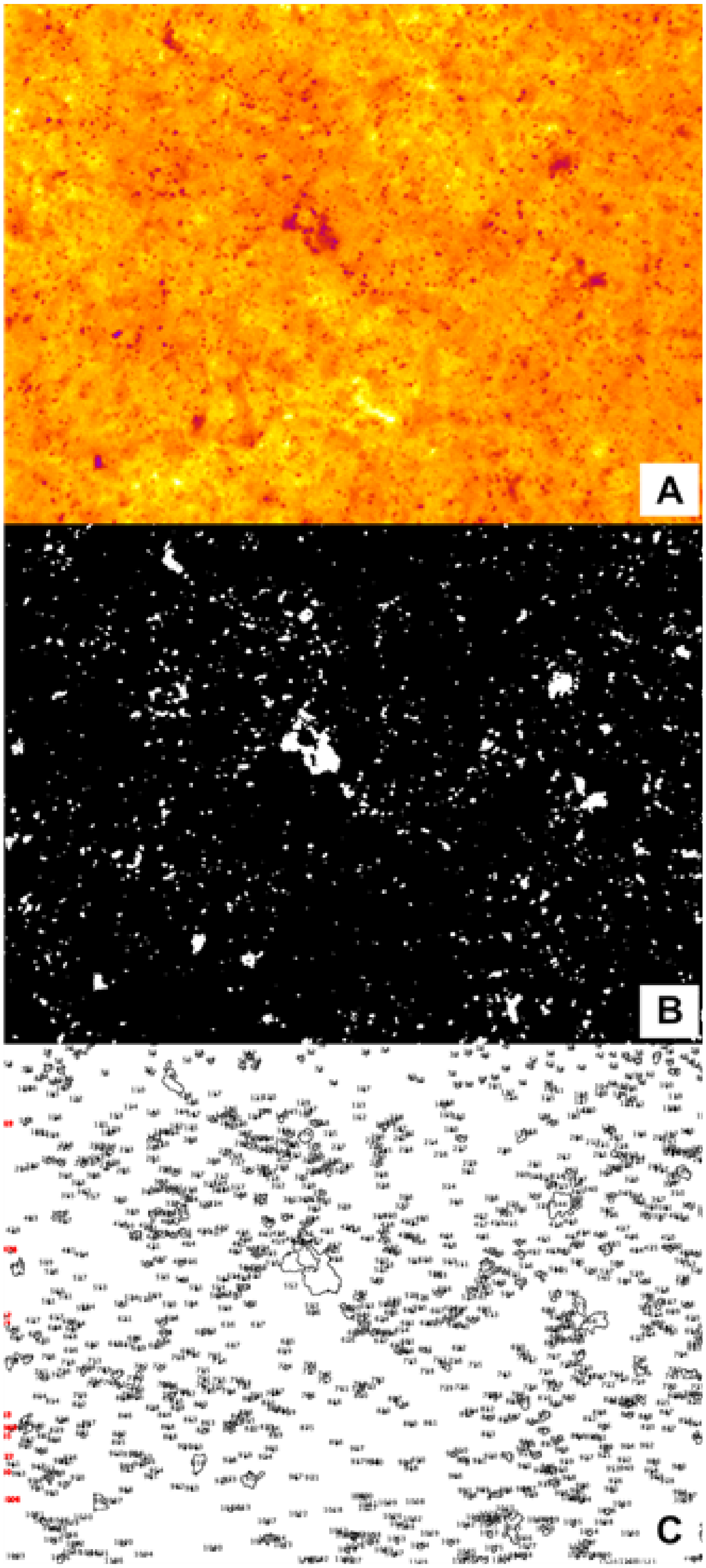


Figure 5: Analysis of Peel Plates using ImageJ Particle Analyzer, including heat mapping (A), threshold modification (B), and Particle Analysis (C).

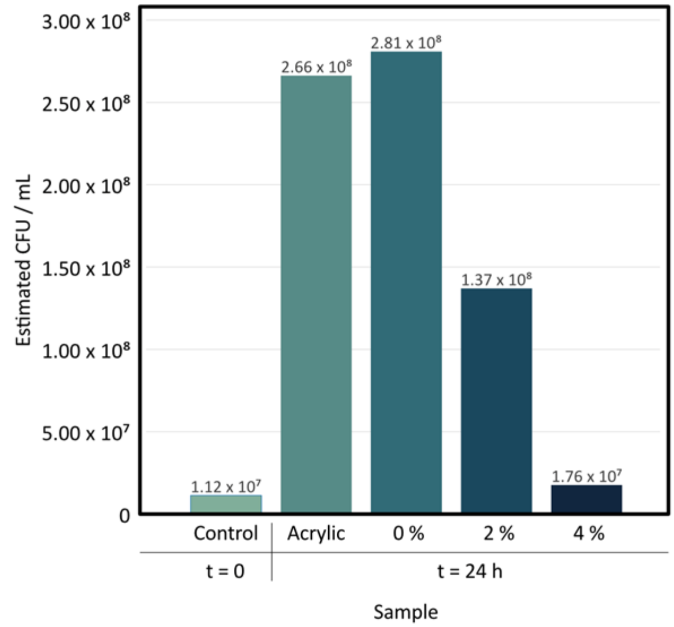


Figure 6: Raw Milk Overlay Comparison of Average CFU/mL.

Conclusions

The incorporation of ZnO into the platinum-cure silicone polymer used for milking liners presents a promising strategy for enhancing the antimicrobial properties of dairy equipment. This study contributes to the ongoing efforts to improve the safety and efficiency of dairy farming operations through innovative material solutions. Further investigations into the optimal concentrations of ZnO, its compatibility with different polymer matrices, and the economic viability of this approach will be crucial for its successful implementation in the dairy industry. These results are particularly relevant for the dairy industry, where the hygiene and safety of milking equipment are paramount. The interaction between the cow's teat and the liner is a critical point for potential microbial contamination, which can affect milk quality and animal health. By incorporating ZnO into the liners, there is a potential to significantly reduce the microbial load, thereby enhancing the overall hygiene of the milking process.

It is important to consider the implications of these findings on the design and manufacturing of dairy liners. The ability to incorporate antimicrobial agents directly into the liner material offers a proactive approach to hygiene, potentially reducing the reliance on post-milking disinfection procedures. However, further research is needed to assess the long-term stability of ZnO within the polymer matrix, its impact on the mechanical properties of the liners, and the safety of ZnO in contact with food products.

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