

Simulation studies to profile development and control of mixed-culture static and dynamic biofilms during extended raw beef processing operations using combinations of nanobubble water and peracetic acid

by

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Abstract

Biofilms are the dominant mode of bacterial growth in nature, known for their exceptional resilience and ability to withstand common cleaning and sanitizing procedures, thereby posing significant challenges for the food industry in maintaining safety and quality standards. Multiple studies have investigated technologies to eradicate and control biofilm development on hard surfaces, however, few have taken into consideration the effect of organic residue accumulation and natural microflora presence in simulated beef processing conditions—even though mixed-species biofilms remain the most common form encountered in real-world settings. This research evaluates the application of nanobubble water (NW)—recognized for its potential antimicrobial properties—in combination with peroxyacetic acid (PAA)—a strong oxidizer commonly used in meat processing—to control mixed-species biofilm development on stainless steel (S304) and rubber (Buna-N) surfaces over extended processing times. Experiments were conducted under simulated beef processing conditions at 4°C, using either tap water (TW) or NW combined with 0, 200 or 400 ppm of PAA. Solutions contained organic matter added in the form of beef purge and were replenished daily with 20% (v/v) new solution and purge. Two important meat industry scenarios were simulated: static biofilm development (Chapter 3) and dynamic biofilm development using the Centers for Disease and Control (CDC) biofilm reactor (Chapter 4). Both planktonic (free in solution) and attached bacterial cells (biofilm on coupons) were assessed for Aerobic Plate Count (APC) and Enterobacteriaceae (EB) populations. Treatment solutions were inoculated with a cocktail of five USDA-approved non-pathogenic *Escherichia coli* strains (surrogates for pathogenic Shiga toxin-producing *E. coli*), while control samples remained non-inoculated. The static study tested the efficacy of antimicrobial solutions with 5% or 10% (v/v) beef purge over an extended period of 10 days. Results showed that

organic matter content significantly affected biofilm development in 200 ppm PAA solutions ($P < 0.0001$), reaching 4.5 log APC/coupon at 10% purge compared to 2.0 logs at 5% purge. At 400 ppm PAA, APC biofilm development was higher ($P = 0.043$) in TW solutions, but similar ($P = 0.16$) in NW solutions between organic levels. There was no difference ($P > 0.05$) between using TW or NW in the treatment solutions against planktonic cells or biofilm control in the static setting. The dynamic study tested treatment solutions at the 10% beef purge level over a 5-day period. Results showed that 200 ppm PAA, regardless of water type, inhibited EB biofilm formation but allowed approximately 2.5 log CFU/coupon of native mixed-culture biofilm (represented by APC Petrifilm counts) to develop on both coupon materials. 400 ppm PAA solutions completely inhibited both APC and EB biofilm growth on coupons. The use of NW alone (no PAA added) resulted in a 0.7 log reduction of APC biofilm after 5 days compared to TW alone, demonstrating its slight effect against biofilm development ($P = 0.0269$). In general, PAA at ≥ 200 ppm inhibited biofilm development by native meat microflora on S and R surfaces under both static and dynamic, extended duration, refrigerated conditions encountered in meat processing, with a strong influence by the level of organic loading present. This finding serves to support the continued use of PAA which is commonly applied in beef manufacturing operations but suggests that additional biofilm control can be realized if the PAA is applied in NW versus typical municipal TW.

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Chapter 1 - Introduction

Americans relish ground beef and utilize it in a wide array of food products, ultimately consuming nearly 27 pounds per capita annually (Shahbandeh, 2024). Ground beef and hamburger are among the most popular foods in the United States, with 40 to 45 percent of beef consumed as ground beef (Cooperative Extension Service at University of Arkansas System, 2025). In beef processing operations, chilled carcasses are fabricated into primal and subprimal units destined for wholesale and retail marketing. During fabrication, primals and subprimals undergo trimming steps, thus, generating large volumes of raw beef manufacturing trimmings which ultimately are utilized in comminuted/non-intact meat products such as ground beef and sausages. In 2022, a total of 8.4 billion pounds of beef trimmings, including both domestic and imported, were used in ground beef manufacturing in the U.S. (Gerdt, 2023). These trimmings are available in lean-to-fat ratios of 90/10 to 50/50, or even XF (extra-fat), with supply material pricing higher as lean composition increases. Trimmings of different fat content are blended and ground to produce consumer ground beef and hamburger products of specified lean content, from 70 to 90 percent (Gerdt, 2023).

Commercially manufactured ground beef and hamburger frequently includes Lean Finely Textured Beef (LFTB) in the formulation. Here, the lower value high fat boneless beef trimmings, having 50% or more fat content, undergo a specific lean recovery process involving grinding, heating, centrifuging, exposing to food-grade ammonia gas (anhydrous ammonia) and quick freezing to obtain the LFTB which has a lean percentage between 94 and 97% (Greene, 2012; Moon et al., 2016). LFTB is frequently added to commercial ground beef formulations at a rate of 10-20 percent by weight to adjust to desired target lean composition (Greene, 2012). The LFTB added to ground beef increases the overall carcass yield by producing approximately 45 kg of lean

meat that would otherwise be wasted or undervalued, thereby significantly improving the economics of ground beef manufacturing. To enhance the microbiological safety of the resulting LFTB, ammonia gas is applied to the meat, which contains a naturally high water content (Unruh et al., 2016). This produces ammonium hydroxide, an approved and commonly used process aid in several food products (Greene, 2012), which causes an elevated meat pH that helps to control pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. (Moon et al., 2016).

The use of LFTB faced widespread public backlash after a 2012 ABC World News Report on the quality of this ground beef additive, particularly after former USDA scientist, Jim Zirnstein, announced that this “pink slime” was used in dog food and cooking oil and was a “cheaper filler” (Runge et al., 2018). Due to the negative and sensational information projected by the media on LFTB, huge economic damages affected ground beef manufacturers as sales decreased, while the primary LFTB producer (utilizing a proprietary manufacturing and equipment process), Beef Products Inc. (BPI, Dakota Dunes, SD), shut down three of their four LFTB production plants (Yadavalli & Jones, 2014). Over a two-month period, strong media influence played a huge role in the demise of LFTB use in ground beef products, including ground beef destined for the national school lunch program and several large fast-food chains, even though the USDA maintained that the technology was safe and wholesome (Greene, 2012). BPI settled a defamation lawsuit with ABC News out of court in 2017, receiving a \$1.9 billion damage payout (Drovers, 2020). More recently, BPI changed its name to “empirical foods” and diversified its product line and pursuit of technology innovations (Feedstuffs, 2019). The company gained USDA approval in 2018 to label and market their recovered lean beef as “ground beef” directly, as opposed to a previous designation as a “qualified component of ground beef”, meaning that the lean meat can be further

processed and used and labeled as just ground beef based upon its composition and nutritive make-up (Shaffer, 2018); thus, broadening the product's potential food applications and reducing consumer misperceptions.

Meat companies seek to satisfy customers and consumers and strive to provide them with products that satisfy their needs. In some cases, ground beef that is LFTB-free is specified. Therefore, the need for new approaches to produce affordable, high quality lean beef emerges. A start-up commercial beef manufacturing company (American Beef Processors of Oregon; ABPO) has developed and patented a novel ground beef production process to produce high-lean ground beef from high-fat commercial beef trimmings (Garwood, J., 2021; Garwood, J., 2022). The proprietary manufacturing process involves the use of nanobubble water (NW) to enhance the physical separation of lean from fat tissues. Nanobubbles, being extremely fine gas bubbles with diameters below 500 nm, exhibit unique characteristics, including high stability, high zeta potential, and high gas dissolution rate (Phan et al., 2020). Scientists discovered that these characteristics allow nanobubbles to be effective in flotation processes where separation is based on density of the components (Fan et al., 2010; Li & Zhang, 2022). The patented ABPO production system (Figure 1.1) involves dicing beef trimmings, rapid freezing of the cubes, “milling” of the frozen cubes to shatter the tissue components and circulating NW through a closed stainless steel conduit system, allowing the lean portions to separate from the fat based upon differences in the density and buoyancy of these components. Lean meat is recovered and de-watered using decanting centrifuges and used in ground beef production, potentially offering a higher quality alternative to traditional LFTB.

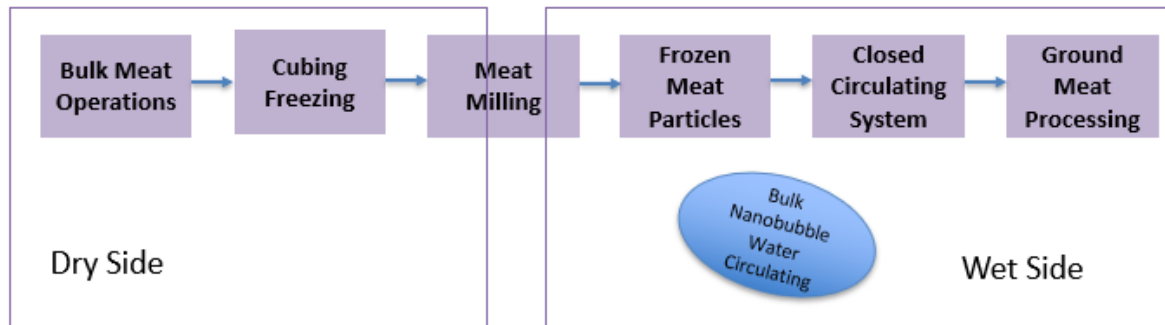


Figure 1.1 Proprietary meat processing flow diagram

While this innovative process brings excitement to the ground beef manufacturing business in that it maintains the beef trimmings (ultimately cubed, frozen and shattered) at a low temperature with no application of ammonium hydroxide, microbiological considerations must be proactively addressed. The microbiological status of the overall proprietary manufacturing process, and ultimately the finished ground beef produced, depends upon the quality of incoming boneless beef trim, along with the sanitation and hygiene of the processing environment from start to finish. Biofilm development on food contact equipment in this closed, continuously circulating system presents a challenge for effective safety and sanitary control. Bacteria can thrive in communities that attach to surfaces while producing a protective polymetric matrix that provides higher resilience to the embedded microorganisms against external stress, which makes biofilms harder to eradicate (Olanbiwoninu & Popoola, 2023). Biofilms are being identified as a more difficult challenge and a bigger concern to the food industry than in the past (even though they have received a great deal of attention for decades); actually, several foodborne outbreaks have been traced back to problems related to biofilm formation on equipment and surfaces in food industries (Srey et al., 2013). The microbiological shelf life of final ground beef can also be negatively impacted if biofilms are allowed to persist on equipment. Indeed, both spoilage and pathogenic microorganisms have the ability to produce biofilms and persist as sessile bacteria, entrapped in

the protective matrix, causing numerous safety and quality problems. Shiga toxin-producing *Escherichia coli* (STEC) serogroups are important human pathogens associated with gastroenteritis and kidney failure and can be strong biofilm formers (Vila et al., 2016; Zhou et al., 2022). Being a ubiquitous microorganism, various types of *E. coli* can be found in a variety of food products but is especially associated with meat products like ground beef, with approximately 25% of its meat-associated strains being biofilm formers (Barilli et al., 2020). Unfortunately, several foodborne outbreaks and product recalls continue to be attributed to STEC, especially when biofilm formation is involved in food industries, increasing the risk of food contamination. Recently, the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) announced on November 20, 2024 that Wolverine Packing Company (Detroit, MI) was involved in an *E. coli* O157:H7 recall of 167,000 pounds of fresh ground beef with an associated food-borne illness outbreak; seven total STEC-related beef recalls occurred in 2023-2024 (U.S. Department of Agriculture Food Safety and Inspection Service, 2024). Growing in concern from a public health and regulatory standpoint are *Salmonella* contamination risks in ground meat and poultry products (Food Safety Magazine, 2025). Such incidents can lead to huge financial losses to the companies and illustrate the seriousness of enteric pathogen contamination in fresh ground beef manufacturing.

Food processing plants struggle to attain food safety assurance even with the implementation of good cleaning and sanitation programs and HACCP systems, as well as end-product testing compliance checks. Traditional ground beef generation from boneless beef trimmings does not typically include a validated antimicrobial step whereby all meat components are exposed to an effective antimicrobial treatment. LFTB as a specific component is ammoniated, however, LFTB only accounts for a small percentage of the overall ground beef formulation.

Therefore, the addition of an antimicrobial intervention step in ground beef processing that impacts all incoming trim component streams would, conceptually, aid in the reduction of this risk.

In ABPO's novel commercial beef manufacturing process, the trim treatment process which uses recirculating NW fluid is designed to operate over multiple days and therefore requires that the system includes inherent disinfection capabilities to prevent microbial accumulation during the extended processing period. To mitigate the risk of biofilm formation and ensure safe production, food-grade peracetic acid (PAA) is added to the circulating NW in the proprietary beef manufacturing process (Garwood, J., 2021). Fresh antimicrobial solution (NW plus PAA) is continuously added to replenish a portion of the circulating fluid throughout the processing day. PAA is an approved food-grade antimicrobial that is widely utilized in meat and poultry processing, with demonstrated microbial control features particularly in beef and pork carcass and sub-primal washing operations (Pozuelo et al., 2021). It is also widely utilized in the poultry processing industry for carcass washing and chilling. This antimicrobial method not only could potentially mitigate the risk of biofilm formation, but could also contribute to a cost-efficient process compared to traditional antimicrobial practices characterized by extended cleaning with interruptions of the production process, a shortened life span of production equipment and a high cost of wastewater treatment (Flemming, 2011).

This thesis reports findings on the effectiveness of the novel proprietary manufacturing system utilizing air-based NW infused with differing concentrations of PAA to control static and dynamic biofilm development on key materials used in the system design (i.e., stainless steel and rubber). The experimental protocol was designed to mimic actual ground beef processing at the manufacturing site by 1) adding organic material to the circulating fluid, 2) maintaining refrigerated solution temperatures, 3) allowing mixed culture biofilm development originating

from the natural beef (purge) native microbial populations, 4) replenishing NW+PAA solutions daily at a 20 percent (v/v) rate, and 5) allowing biofilm development over an extended processing period with no sanitation shutdown. USDA-approved non-pathogenic *E. coli* surrogate cultures (5 strains) were inoculated into each treatment fluid system to simulate and quantify enteric pathogen presence and potential incorporation into the native mixed-culture biofilms on stainless steel (S) and rubber (R) surfaces. Two separate studies were conducted focusing on control of biofilm on S and R surfaces comparing different treatments of NW and regular tap water (TW) containing different concentrations of PAA.

The first study focused on statically developed biofilms grown over a 10-day period at 4°C, while the second study used a Centers for Disease Control and Prevention (CDC) biofilm reactor to simulate a dynamic growth environment, incorporating a shear force factor that more closely resembled real-world conditions inside the circulation conduit, over a 5-day period at 4°C. The main research questions addressed in both studies include the following:

1. Is there a definable water type (NW or TW) with a specific PAA concentration that seems to limit biofilm development during extended chilled manufacturing time in the presence of 5% (low) and 10% (heavy) purge build-up?
 - a. From a process sanitation and food safety standpoint, is it practical to attempt continuous processing of beef materials for extended periods, such as 5 or 10 days, considering the potential for biofilm accumulation?
2. Is there a significant difference between the use of NW or TW in combination with PAA at all concentrations?
3. Is there a notable difference in biofilm development levels on S and on R materials in different solutions?

- a. Under these extended processing conditions, should sanitation procedures focus more heavily on equipment surfaces which would mostly be stainless steel, or should conveyor belting and/or gaskets be the primary target for enhanced cleaning and sanitation efforts on a daily basis?
4. Does the level of organic matter in the system significantly influence biofilm accumulation in the system?
 - a. Should the level of organic matter be considered a critical process control point with a set limit not to be exceeded during extended processing run times?
 - b. Should the PAA concentration be adjusted according to the organic matter level in the system?
5. Is there a significant difference between statically generated biofilms and dynamic biofilms in terms of the overall biofilm accumulation on materials throughout the 5-day period and the effectiveness of PAA solutions on controlling biofilm formation?
6. Can pH of the circulating solution containing PAA be used as a real-time process monitoring tool to predict organic level accumulation in the system and thus safe operation levels during processing?

The current research discussed herein focuses on microbial control in the organically loaded circulation fluid used to separate lean and fat beef components, and on the food-contact material surfaces encountered within the circulation conduit. As mentioned earlier in this section, control of microbial pathogens on the meat particle surfaces themselves is also a key element in the overall design of this novel system. Other research conducted in the K-State Food Safety and Defense Laboratory quantifies the effectiveness of these treatment solutions for eliminating *E. coli*

surrogates, STEC and *Salmonella* attached to beef particles entering the recirculating portion of the overall ground beef manufacturing system and will be published separately.

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Chapter 2 - Literature Review

2.1. Biofilms

Bacteria, whether pathogenic or spoilage, have the ability to form communities that attach to surfaces and produce a protective polymetric matrix. This exceptional mode of growth and survival is known as the ability to form a biofilm and is, in fact, the “dominant mode of bacterial growth in nature” (Rumbaugh & Sauer, 2020, p.571). In fact, biofilms are the oldest form of life with fossils dating back to 3.5 billion years ago, and their existence nowadays proves that they are also considered the most successful form of life on Earth (Flemming, 2011). Antonie van Leeuwenhoek, the father of microbiology, first observed biofilms in the 17th century on dental plaque (Donlan, 2002). This discovery was transformative for the field of microbiology, as it led scientists to realize that the sessile form of bacteria demonstrates increased resilience, growth and activity compared to their planktonic form (Donlan, 2002). Furthermore, it prompted scientists to develop advancements in microscopy (electron microscopy) as well as in genetic analysis to better characterize a biofilm structure and the genes behind its formation and attachment (Donlan, 2002).

Biofilm Structure and Characteristics

The formation of a mature biofilm involves a strictly controlled, sequential process that begins with the initial contact of individual planktonic bacterial cells with an available surface. The process continues only if the attachment is capable of maintaining stability; once sustained, it becomes irreversible. Planktonic bacterial cells then aggregate and form the structure of the mature biofilm (Rumbaugh & Sauer, 2020). As the bacterial cells grow in multilayered cellular aggregates, they secrete an extracellular material, known as the extracellular polymeric matrix, that acts as both a protective layer for the embedded sessile bacteria and a resilient structure,

enhancing nutrient recruitment and surface adhesion (Alvarez-Ordóñez et al., 2019; Lopez et al., 2010).

The extracellular polymeric matrix (EPM), also referred to as the extracellular polymeric substance (EPS), is usually composed of polysaccharides, proteins, extracellular DNA (eDNA) and lipids (Bremer et al., 2015). The composition and structure of the EPS depend on the different bacterial species in the biofilm, the available substrates in the environment, as well as the external growth conditions (Bremer et al., 2015; Lopez et al., 2010). The advanced properties that a bacterium gains in a biofilm compared to its planktonic, free-living form are attributed to this protective matrix which serves as the essence for biofilm maturation and rigidity (Flemming et al., 2016). In fact, Zhang et al. (2014) tested the role of the EPS in mature biofilm formation by studying microbial cells from suspended sludge and biofilm. They discovered that the EPS changes the surface characteristics of the microbial cells by increasing the hydrophilicity and decreasing the zeta potential, thus promoting cell aggregation (Zhang et al., 2014). Several other experiments first observed reduced structural complexity and increased susceptibility to antimicrobial agents with the disturbance or agitation of the EPS (Stoodley et al., 2002). Since the EPS structure is the key player in revealing biofilm properties, scientists have studied its morphology, and the genes involved in its creation. For example, the presence of special proteins known as curli fibers and the accumulation of exopolysaccharide cellulose are both associated with EPS morphology in *Escherichia coli* and *Salmonella* biofilms (Serra et al., 2013). Serra et al. (2013) were pioneers in showing the spatial organization of the different components that contribute to biofilm formation by using scanning electron microscopy (SEM) to observe *E. coli* macro-colony formation. The *E. coli* biofilm shows distinct layers. In the top layer, curli fibers are accumulated around the tightly packed cells with a detected, prominent RpoS-dependent gene expression, including the gene

CsgD. On the other hand, the bottom layer predominantly consists of rod-shaped bacterial cells surrounded by flagella (Serra et al., 2013). This observation helps in characterizing biofilm structures and in better understanding their formation as well as the functions of their different components.

As the attached cells multiply and grow into microcolonies, the EPS structure enclosing the bacterial cells also increases and creates a dense community of cells. This specific structure plays an important role in advancing cell-to-cell communication. The cluster of bacterial cells within a biofilm, viewed as a closed and tightly packed system, is a unique structure that creates a denser concentration of signaling molecules that can more easily reach bacterial cells within the community, thus facilitating communication. On the contrary, signaling molecules are less concentrated in solutions where planktonic, free-floating cells are available in an open, less dense environment, thus showing reduced signal reachability (Flemming et al., 2016). At the level of biofilm formation, quorum sensing—a bacterial communication mechanism—becomes critical for biofilm regulation including complete maturation and persistence (Bremer et al., 2015). Quorum sensing is a chemical communication involving signaling molecules produced by bacteria called autoinducers that control gene expression, favoring genes involved in EPS formation, nutrient retention, antibiotic resistance, and other genes coding for biofilm specific phenotypes (Mukherjee & Bassler, 2019). Even though quorum sensing is viewed as an essential form of communication to attract cells into a biofilm contributing to biofilm maturity and expression of specific genes, sessile cells in a biofilm also use quorum sensing mechanisms to release themselves back to their planktonic form through dispersion—the last stage in a biofilm life cycle (Bremer et al., 2015; Rumbaugh & Sauer, 2020). After the biofilm reaches maturity, several factors may affect its

stability and persistence. These factors, such as environmental changes, might compromise the biofilm's rigidity, triggering the detachment of bacterial cells from the matrix (i.e., dispersion).

Factors Affecting Biofilms

The initial contact of bacterial cells with a specific surface is the first step which will depict the mode of growth of the bacteria in a specific environment. This event is uncertain and can be reversible depending on several factors that affect the initiation of the biofilm structure as well as its subsequent growth and maturity (Bremer et al., 2015). pH is one key factor that can affect cell adhesion in biofilm formation—the process of cells transitioning from planktonic to sessile form. These effects can vary among different bacterial species. Some bacterial species grow planktonically at a different optimal pH level than in their biofilm mode of growth, such as *Salmonellae*, while other bacteria are not affected by pH, such as *Pseudomonas fluorescens* (Speranza et al., 2011). Ariaifar et al. (2016) studied *Salmonella* Virchow biofilm formation at different pH and temperature levels and concluded that both an elevated pH level and an increased temperature led to a higher biofilm formation rate and an increased overall biofilm production. Borges et al. (2018) also confirmed that the highest percentage of the *Salmonella* strains they studied grew best at 37°C compared to lower temperatures.

Different strains within a bacterial species can react differently to pH, temperature, and other factors, influencing bacterial growth and maturity. This highlights the importance of considering several bacterial strains and incorporating mixed-strain cultures while researching biofilm formation within a specific bacterial species (Speranza et al., 2011). Under normal growing conditions, some bacterial strains are characterized with low-biofilm formation, while others are high-biofilm formers and tend to transition more often to sessile cells when compared to other strains (Borges et al., 2018; Çam & Bicek, 2023). In fact, the sessile bacterial mode of life is often

described as a survival mode for bacteria when the conditions are not optimal for their planktonic mode of life. For example, since the EPS can enhance nutrient acquisition, bacterial cells tend to transition to sessile mode whenever nutrient depletion increases in their environment (Speranza et al., 2011). Additionally, the nature of the surface plays a role in the sustained attachment of planktonic bacterial cells. Understanding the surface properties and their effect on bacterial attachment allows a better approach to biofilm control, since attachment is the first step of biofilm formation (Song et al., 2015). Surface charge, surface energy, roughness, and topography, as well as stiffness are all surface material properties that should be considered when evaluating biofilm formation on hard surfaces. As surface roughness increases, bacterial cells have greater contact with the surface, thus enhancing the likelihood of cell attachment and consequently favoring biofilm formation (Song et al., 2015; Teughels et al., 2006). Borges et al. (2018) and Agarwal et al. (2011) noted that the bacterial intrinsic properties, such as the ability to produce flagella, fimbriae, membrane proteins and other attachment aids, are far more important than the extrinsic factors related to the environment in which the bacteria are attaching.

Indeed, in a study performed to assess the initial attachment of eight different *Escherichia coli* O157:H7 strains on both stainless steel and high-density polyethylene surfaces (HDPE) at different temperatures (15°C and 4°C), while mimicking the ground beef processing environment, the results showed no significance difference in the attachment of bacterial cells related to the type of surface material or the different temperatures (Dourou et al., 2011). Therefore, some bacterial species tend to naturally form biofilms whenever minimal conditions are present for the cells to attach, secrete EPS and create the biofilm, regardless of the environmental conditions or surface properties. However, the presence of natural microflora as well as organic material certainly has shown effects on biofilm formation by several pathogenic bacteria. For example, *E. coli* O157:H7

biofilm formation was higher on stainless steel and HDPE coupons in the presence of ground beef, which represented the natural organic and inorganic substrates found in beef processing facilities, compared to growth in the presence of sterilized tryptic soy broth (Dourou et al., 2011). The attachment of cells was enhanced due to the presence of food residues, enhancing the capability of cells to stick to surfaces with the build-up of organic layers. In a similar study, food residues, particularly meat exudate at 35°C, significantly enhanced the formation and microbial composition of *Escherichia coli* and *Staphylococcus aureus* biofilms on stainless steel surfaces, demonstrating the critical influence of substrate and temperature under simulated industrial conditions (Dutra et al., 2018).

The presence of organic residue can attract the growth of naturally present microorganisms associated with specific types of food. When studying biofilm formation, incorporating mixed bacterial species, including both pathogenic and spoilage strains, provides a more accurate representation of real industrial environments. The attachment of a certain bacterial species can be influenced by the presence of other microorganisms in the surrounding environment or on the potential surface of attachment, as well as by environmental conditions such as agitation or flow, which can introduce competition between bacterial strains (Srey et al., 2013). Synergistic interactions between bacterial species can occur when dealing with mixed-culture biofilms. The presence of *Acinetobacter calcoaceticus* significantly enhances the survival and persistence of *E. coli* O157:H7 while forming biofilms, where the former provides increased protection to the latter from disinfectants that would have otherwise been lethal to a single-culture of *E. coli* O157:H7 biofilm (Habimana et al., 2010). The dynamic surroundings in which biofilms form also affect the overall biofilm generation in terms of quantity and quality of biofilms as well as bacterial species dominance in multi-species biofilms. Habimana et al. (2010) investigated the influence of

multi-species biofilm on *E. coli* growth in a dynamic environment and discovered that shear force posed an obstacle on *E. coli* O157:H7 biofilm growth, whereas the presence of *A. calcoaceticus* cells created a protective embedment for *E. coli* O157:H7 against shear force. Multiple studies have demonstrated that the presence of other microorganisms can be supportive for enhanced biofilm growth in dynamic environments where shear force can be an obstacle for bacterial attachment.

Biofilms in Food Processing Facilities

Biofilms in food processing environments, including dairy, meat, poultry, fish, produce and ready-to-eat (RTE) industries, present significant challenges by persisting in these settings and contributing to multiple outbreaks and operational issues (Srey et al., 2013). Factors such as extended production cycles, high-volume production, vulnerable hard surfaces, plant complexity, inadequate cleaning protocols, and ineffective sanitizers or antimicrobials contribute to creating an ideal environment for biofilm formation on food processing lines (Carrascosa et al., 2021). Some of the most common bacterial species found to persist in beef-producing facilities include lactic acid bacteria such as *Carnobacterium* sp. and other spoilage bacteria such as *Raoultella* sp., while some species of *Pseudomonas*, *Commamonas*, and *Lactobacillus* are among the most associated with beef products (Ercolini et al., 2006; Nan et al., 2024). In fact, Wagner et al. (2021) analyzed food-contact and non-food contact surfaces in a meat processing facility and identified 29 different bacterial genera across the phyla *Proteobacteria*, *Acinetobacteria*, *Firmicutes*, and *Bacteroidetes* in ten biofilm-positive samples. They observed that *Brochothrix* (80% prevalence), *Pseudomonas*, and *Psychrobacter* (each at 70% prevalence) were genera most often encountered. Additionally, several foodborne pathogens are strong biofilm formers in the meat industry. *Salmonella* sp., *Listeria monocytogenes*, Shiga-toxin producing *E. coli* (STEC), *Campylobacter*

jejuni, and *Staphylococcus aureus* are among the most detrimental pathogens that can grow in biofilms in meat processing industries, leading to challenging eradication attempts, increased risks of product recalls, and thus significant economic losses (Giaouris & Simoes, 2018).

As bacteria favor surfaces with debris and moisture to attach, several surfaces, materials, and equipment can act as harboring niches for biofilm formation in a commercial food processing system. Biofilms most frequently accumulate in areas that are more difficult to clean and sanitize or that usually do not receive a sufficient amount of disinfectant and/or scrubbing during routine cleaning, such as drains, pipelines, gaskets, valves, conveyor belts, and damaged or old materials (Giaouris et al., 2014; Giaouris & Simoes, 2018; Wang et al., 2018). Stainless steel type 304 is the most commonly used hard surface material in most food processing industries; however, several studies confirmed that it provides a suitable surface for bacterial attachment and biofilm growth (Carrascosa et al., 2021; Dutra et al., 2018; E. Giaouris et al., 2005; Oliveira et al., 2010; H. Wang et al., 2013). Rubber is another common surface material used in food processing equipment and production environments. It is frequently used for equipment seals and gaskets, with Buna-N being one of the most common types (Ronner & Wong, 1993). Ronner & Wong (1993) found that biofilms of *L. monocytogenes* and *S. typhimurium* formed on Buna-N rubber surfaces were more resistant to sanitizers compared to those on stainless steel surfaces. This raises concerns for the food processing facilities to emphasize critical attention when addressing biofilm formation particularly on rubber surfaces.

Challenges of Biofilm Eradication

Food processors should use a proactive approach to prevent biofilms from initially forming on surfaces; however, processors find it difficult to completely eliminate biofilm risks in their facilities. As several factors might have an influence on the ability of microorganisms to form

biofilms on surfaces, different factors also can affect the process of biofilm removal from these surfaces. The food industry faces several challenges while establishing cleaning protocols to ensure an effective cleaning and sanitation system that will address all the necessary areas in a food processing facility including biofilm eradication. Notably, mechanical energy during cleaning (e.g., scrubbing, use of ultrasonic treatments, cold plasma technology) increases the efficiency of biofilm detachment and removal from surfaces (Oliulla et al., 2024). The use of disinfectants such as quaternary ammonium compounds (QAC), chlorine, peroxides (peracetic acid and hydrogen peroxide), ozone, iodine, aldehydes (formaldehyde, glutaraldehyde, paraformaldehyde), or phenolics is fundamental in a food company's sanitation protocol to provide an antimicrobial effect against microorganisms (Carrascosa et al., 2021; Srey et al., 2013). Bacteria living in a biofilm community generally are more likely to survive stressful conditions, such as nutrient depletion, dehydration, pH changes, or the presence of other inhibitors such as antimicrobials (Rumbaugh & Sauer, 2020). Due to the complexity of the biofilm structure, embedded sessile bacteria become more resistant to those antimicrobials and thus harder to eradicate compared to planktonic bacterial cells (Alvarez-Ordóñez et al., 2019). Current research speculates that several mechanisms together make sessile bacteria in biofilm communities more resistant to antimicrobial treatments. Starvation-induced slow growth, the presence of dormant and persister cells, and the natural biofilm matrix's ability to sequester and restrict the penetration of antimicrobial agents contribute to this strong resistance (Rumbaugh & Sauer, 2020). Indeed, biofilms possess an intrinsic antimicrobial resistance due to the nature of the EPS as well as the presence of the nutrient-depleted persister cells that naturally have a defense mechanism against antibiotics (Barman et al., 2024; Wang et al., 2018). Moreover, a biofilm's physiochemical properties, morphology, maturity, and the components of its EPS can influence its resistance to disinfectants. When trying to inactivate

viable bacterial cells of *Pseudomonas fluorescens* (a common spoilage microorganism in meat and poultry) enclosed in biofilm, Wang et al. (2018) discovered that mature biofilms showed higher resistance to all of the applied chlorine-based disinfectants due to the thicker EPS that formed with time. Acknowledging that biofilm eradication is a serious problem, several food industries aim to address this issue with the purpose of decreasing the risk of undesirable outcomes in their facilities. The presence of untreated biofilms in food processing facilities can lead to decreased microbial shelf-life and elevated risks of microbial persistence and harmful biological hazards in the final products, as well as increased end-product recalls and possible foodborne outbreaks (Alvarez-Ordóñez et al., 2019).

2.2. Laboratory Biofilm Development: Static & Dynamic Biofilms

Biofilm research has expanded tremendously over the past two decades, becoming a major focus for scientific advancements. In fact, while biofilm research was basically non-existent between 1976 and 1980 with zero publications, the number of published biofilm studies rose from 3 in 1981 to 170 in 1996 and continued to significantly increase reaching 3251 publications in 2013 (Høiby, 2014). Since then, we observed a surge in the number of publications from 5,683 in 2015 to an impressive 10,772 in 2024 (9 years, Web of Science, search word: biofilm, 21 January 2025). Laboratory biofilm studies are based on the development of biofilms on target surface materials by providing the necessary growth parameters (nutrients, time, temperature) to target microorganisms, in addition to choosing one of the two main conditions: static development or dynamic development.

Static biofilm development refers to the growth of biofilm where low or no shear forces are introduced in the environment, allowing planktonic cells to attach to surface materials of interest and form aggregates naturally in undisturbed conditions. Some common methods used for

static biofilm growth include multi-well plates, petri dishes, agar plates, and surface material coupons, which are incubated at specific temperatures with the provision of specific nutrients in a fixed immobile environment (Borges et al., 2018; Çam & Bicek, 2023; Oja et al., 2014; Pant et al., 2025). On the other hand, dynamic biofilm growth involves some level of agitation in the environment in the form of shear force with a constant flow rate, allowing turbulence to impact biofilm attachment and growth over time. The most widely adopted technique to grow biofilms in a dynamic environment is using a rotary biofilm reactor, such as the CDC Biofilm Reactor (Azeredo et al., 2017). The CDC bioreactor is used in official ASTM standard methods such as E2562-22 which is related to the quantification of *Pseudomonas aeruginosa* biofilm (ASTM, 2022). The CDC bioreactor (Figure 2.1) consists of a 1-L glass vessel with an effluent spout, designed to hold approximately 400 mL of liquid with a baffled magnetic stir bar placed at its bottom center to provide continuous shear, as well as a polyethylene lid holding eight independent rods. Each rod accommodates three removable 1/2-inch (12.7 mm) diameter coupons (BioSurface Technologies Corporation, 2022). This bioreactor can operate in two modes: a batch phase, where a single batch of media and inoculum is added through the inoculum port, or a continuous flow phase, where nutrient tubing lines connect to a carboy filled with the nutrient broth, allowing consistent pumping to replenish the bioreactor with fresh growth medium at a defined rate. The CDC bioreactor allows the testing of robust biofilm attachment on multiple types of coupon surfaces at once and is a reusable, autoclavable system (BioSurface Technologies Corporation, 2022).

The development of biofilms in a static environment is beneficial when addressing the first stages of biofilm attachment to a specific surface material and identifying the early attachment period of different bacterial species; e.g. a 2-hour attachment phase was observed for four bacterial

species (*S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*) grown under static conditions (Oja et al., 2014). Similarly, Dourou et al. (2011) grew biofilms on stainless steel and high-density polyethylene tubes under static conditions in order to assess the effect of substrate presence and difference in temperature ranges on *E. coli* O157:H7 initial cell attachment (Dourou et al., 2011). However, when comparing biofilms formed in a static setting (48-well plate) versus a dynamic continuous setting (CDC bioreactor), Pant et al. (2025) detected a significantly higher cell concentration and consequently a higher tolerance to the sanitizer sodium hypochlorite in biofilms grown in a continuous dynamic system (Pant et al., 2025). The stronger tolerance to the sanitizer was associated with higher exopolysaccharide production by dynamic biofilms which means that more EPS was formed with the presence of a flow rate, facilitating nutrient access and strengthening attached bacterial cells (Fang et al., 2017; Pant et al., 2025). Since biofilms acquire a stronger and more rigid structure that is not easily detached from surfaces when grown in a dynamic environment, researchers grow biofilms in dynamic environments when focusing their studies on evaluating the efficacy of a certain antimicrobial or sanitizer. In fact, Ivers et al. (2024) used the CDC bioreactor to grow mature multi-strain *Salmonella* biofilms before treating them with multiple sanitizers to assess their efficacy, while taking into account additional conditions (application time and surface type), explaining that biofilm formation is a dynamic process in nature (Ivers et al., 2024). In reality, biofilms can form under both static and dynamic food processing scenarios. For example, a food processing belt continuously receiving an antimicrobial spray application or an enclosed fluid conduit in a dairy processing plant would support dynamically produced biofilms. Meat processing red water pooling in a corner of a cutting table during extended production or water collecting in a condensation pan in a RTE processing facility would represent statically produced biofilms.

The quantification of cells in a mature biofilm is achieved after effective viable cell detachment followed by enumeration methods. However, since biofilms form rigid structures and embed bacterial cells under a thick EPS structure, specific methods for cell detachment must be applied to obtain a final accurate cell count. Several studies have relied on washing the biofilm-containing surface with saline solution multiple times (preferably three times) in order to detach loosely-attached cells and then using Crystal Violet stain to measure the optical density which correlates to the biofilm's bacterial cell concentration or density (Ariafar et al., 2016; Borges et al., 2018; Çam & Bicek, 2023). Other methods that researchers have relied on to detach biofilms from surface coupons include the immersion of coupons in solutions of phosphate buffered saline (PBS) and relying on either vortexing for a specific duration to dislodge the cells (Abdallah et al., 2015; Rosado De Castro et al., 2017; Wagner et al., 2021), or placing the samples in an ultrasonic bath (Dutra et al., 2018; Todorčić et al., 2023) and then performing serial dilutions for enumeration methods. In addition to that, an ASTM method E2871-19, also known as the Single Tube Method, is a standard laboratory method used for efficacy testing of antimicrobials against biofilms (ASTM, 2019; Goeres et al., 2019). This method involves a series of vortexing and sonication, each performed for thirty seconds and repeated three times, to detach and analyze bacterial cells after treatment with a sanitizer. This method is commonly used in several research studies (Goeres et al., 2019; Ivers et al., 2024).



Figure 2.1 Standard CDC Biofilm Reactor®. Source: BioSurface Technologies Corp.

2.3. Nanobubble Technology: Mechanisms and Applications

Nanotechnology, an innovative transformative field, provides promising advancements in multiple diverse industries by offering nanoscale solutions to improve efficiency, precision and sustainability in applications. Among these innovations, nanobubbles, also referred to as ultrafine bubbles, have demonstrated promising potential in science and technology in fields such as water treatment, agriculture, healthcare, and food processing. Some of the earliest research to understand the impact of bubble size on their stability in solutions began in 1950, with a rich timeline to the present of advancements in understanding, developing and applying nanobubble technology (Alheshibri, et. al., 2016). Attard and his colleagues first proposed nanobubbles in 1994 (Ball, 2012) based on findings from research to understand the attraction forces between two hydrophobic surfaces in water (Parker et. al., 1994). Numerous applications of these tiny gas-filled bubbles, along with different methods and equipment for generating them, have since been developed and patented. For example, Jeanologia patented an “e-flow” device to transmit chemicals using minimal water quantity in manufacturing of “stressed” denim jeans. The demand

for nanobubble use across several fields has risen by 5% globally (Chaurasia, 2023). This technology's market share has been growing since 2016 at a fast rate, with a projected €145 million share in the EU by the year 2030 (Foudas et al., 2023). Nowadays, several companies from all over the world are actively involved in the generation of nanobubbles, such as Moleaer Inc. (Hawthorne, CA) which aims to “empower industries worldwide to use water more efficiently and effectively” (Moleaer, n.d.). Nanobubbles are considered as a potentially powerful tool for modern technological advancements in food safety and food quality due to their versatile and unique properties.

Properties and Unique Characteristics

Nanobubbles are tiny gas-filled molecules showing several remarkable characteristics in aqueous solutions. Stable bulk nanobubbles can be generated by different methods such as cavitation, electrolysis, nanopore membranes, and compression of macro-bubbles, which all yield the same result (Foudas et al., 2023). However, cavitation is the method most commonly used for industrial purposes, since it is considered more efficient and can supply higher concentrations of nanobubbles at a higher needed rate (Foudas et al., 2023; Javed et al., 2023). The two most popular types of cavitation are acoustic and hydrodynamic, which are both based on the principle of pressure fluctuations to produce bubbles. Acoustic cavitation happens when ultrasonic waves lower vapor pressure, while hydrodynamic cavitation occurs when decreased fluid flow pressure induces vaporization, forming bubbles that subsequently collapse due to the pressure increase (Fan et al., 2010; Javed et al., 2023). Typically, the type of gas used for nanobubble formation, such as O₂, O₃, CO₂, N₂, or air, can show an impact on their formation, stability or other general properties. In fact, the uniqueness of each gas's performance is related to its ability to produce OH⁻ ions at the water/gas interface giving the bubble a unique negative surface charge that correlates with an

important characteristic of nanobubbles known as zeta potential (Foudas et al., 2023; Wang & Wang, 2023). The zeta potential is an important indicator of the stability of nanobubbles in solution, where a higher absolute value of zeta potential indicates a higher stability and a lower chance of nanobubble collapse in solution (Wang & Wang, 2023). The uniqueness of nanobubbles resides in their extremely small size that allows them to differ from micro and macro bubbles in various aspects. Larger bubbles, such as micro and macro bubbles, usually float on the surface of a solution and eventually collapse at a quick rate compared to nanobubbles. The phenomenon that helps nanobubbles remain dispersed in solution and prevents them from rising to the surface is termed Brownian motion (Foudas et al., 2023). According to ISO 20480-1:2017 which covers fine bubble technology, nanobubbles have a size ranging from 1 μm to 100 nm or less and are classified as stable for weeks and months in this specific size range (Foudas et al., 2023; Kyzas & Mitropoulos, 2021). With respect to detection and sizing methods, in general, using laser diffraction-based methods is preferred. Currently, the most frequently used and preferred research techniques include laser particle size analyzer, nanoparticle tracking analyzer, and dynamic light scattering (Montazeri et al., 2023).

The stability of nanobubbles can be influenced by several factors, including pH, temperature, type of gas used, and the presence of surfactants. Ke et al. (2019) researched the effect of pH and salt on bulk nanobubbles in solutions and determined that the concentration of nanobubbles is the highest in alkaline solutions (pH= 9.5) followed by acidic water solution (pH = 4.5), and are least stable in salt solutions (Ke et al., 2019). Similarly, air-based nanobubbles produced by hydrodynamic cavitation showed an increase in concentration and a decrease in size when the pH increased from 4 to 6.7, indicating a higher stability in more alkaline solutions which was explained as being due to better stabilization of the electric double layer by OH^- ions

(Montazeri et al., 2023). Moreover, several studies suggested that an increase in temperature favors a higher concentration of nanobubbles in solution as well as a decreased bubble size, which indicates a better stability (Li et al., 2021; Montazeri et al., 2023). The longevity and stability of nanobubbles in solution are remarkable characteristics, as they are considered stable even with temperature fluctuations. Montazeri et al. (2023) explained that the effect of temperature on nanobubbles is determined by two mechanisms related to ion formation in solutions: the self-ionization of water and the ion mobility mechanism, both of which lead to the formation of OH^- ions, increasing the absolute value of the zeta potential and resulting in a higher concentration of stable nanobubbles in solution. Indeed, the unique characteristic of nanobubbles which allows them to produce highly reactive oxygen radicals (such as hydroxyl ions) that are also known as reactive oxygen species (ROS) makes nanobubbles effective in applications requiring advanced oxidation processes, such as water treatment and biofouling control (Phan et al., 2020).

Nanobubble Application in Non-food Industries

The use of nanobubbles began with the purpose of improving water quality, while contributing to more efficient and sustainable practices. The unique characteristics of nanobubbles allow them to change the properties of water and to create better sustainable uses of those solutions. In fact, nanobubble usage in water treatment has shown promising results in decreasing the use of chemicals and improving the overall water quality by offering a more efficient water purification technique (Atkinson et al., 2019; Singh et al., 2024). Due to the generation of ROS, nanobubble water is able to oxidize pollutants, impurities and even pathogens in water without the addition of harmful chemicals such as chlorine (Atkinson et al., 2019). Sumikura et al. (2007) found that using ozone-based nanobubbles reduces more than 70% of the ozone dose needed to inactivate 2 logs of coliform bacteria in wastewater. Nanobubbles show improved environmental sustainability in

various other processes such as cleaning, surface coating, water reservoir aeration, carbon footprint reduction, as well as in biofouling and flotation processes (Chaurasia, 2023; Singh et al., 2024; Wu et al., 2008; Xiao et al., 2020; Zhu et al., 2016). Nanobubbles can create turbulence in liquid solutions leading to strong agitation coupled with the release of hydroxyl radicals when the bubbles collapse contributing to an efficient removal of suspended particles as a proper membrane defouling technique (Foudas et al., 2023). Recently, the carbon-neutral approach in water treatment has gained a lot of attention for the purpose of tackling environmental pollution. Nanobubble technology promotes a carbon-neutral solution in gas flotation techniques utilizing microbubbles and nanobubbles through achieving high separation efficiency, limited sludge production and reduced energy consumption; indeed, using nanobubbles offers potential for minimal chemical use and more efficient processes (Singh et al., 2024). Another successful environmental application of nanobubbles is the decontamination of sediments coming from lake and river pollution where oxygen delivery is achieved through oxygen and ozone nanobubbles, providing a better alternative to conventional oxygenation methods (Foudas et al., 2023).

Nanobubble Application in the Food Industry

Seeing the potential that nanobubble technology holds in the advancement of several fields, the food industry's interests have led to a growing body of research related to the application of nanobubbles to improve food safety and food quality, as well as optimizing food processes. For instance, incorporating nanobubbles in the production of liquid foods such as milk has shown better performance in membrane filtration processes due to the viscosity reducing effect of nanobubbles, facilitating the reduction of membrane fouling (Babu & Amamcharla, 2023). Nanobubbles were shown to enhance the process of spray drying in production of milk powders through creating highly porous water-communicating channels (Babu & Amamcharla, 2022).

Multiple other food quality advancements have been related to the incorporation of nanobubbles to enhance diverse approaches such as freezing and crystallization of foods, emulsions and foams, as well as general sensory attributes (Phan et al., 2020).

However, our primary focus in the following studies concentrates on examining the impact of nanobubble application for the aim of advancing food safety. As they have proven effective in treating and purifying wastewater, as well as in preventing biofouling by the inhibition of protein adsorption onto surfaces, nanobubbles are able to address the critical challenge of biofilm formation on surfaces in food industries (Agarwal et al., 2011; Xiao et al., 2020). Shiroodi et al. (2021) demonstrated that nanobubble water (produced using O₂ gas) was an effective treatment for microbial biofilms as well as an effective inhibitor of biofilm formation on hard surfaces of stainless steel and plastic by preventing attachment of cells. In fact, nanobubble solution alone (no added antimicrobial) reduced *Vibrio parahaemolyticus* adhered to stainless steel and plastic surfaces (1.4 and 2.5 log CFU/cm², respectively, within 2 minutes of exposure), with complete elimination on these surfaces (>7 log cycle reductions) after 5 minutes of exposure. These researchers found *E. coli* biofilm reductions from 2.5 to 4.6 log CFU/cm² depending on the surface type and increasing exposure times up to 10 minutes (Shiroodi et al., 2021). For *Listeria innocua* biofilms, more resistance was observed to nanobubbles applied alone, with a range of reductions from 1.3 to 3.8 log cycles recorded. In Shiroodi et al.'s study, biofilms were generated on plastic and stainless steel coupons submerged in a nutrient broth culture medium.

Moreover, nanobubbles have been found to enhance the efficacy of antimicrobials that are commonly used alone to reduce microbial loads or tackle biofilm formation (Burfoot et al., 2017; Shiroodi et al., 2021; Ushida et al., 2017). Nanobubbles produced with air, CO₂ and N₂ can produce hydroxyl radicals that react with the organic and inorganic components of the biofilm's EPS,

enhancing the antimicrobial and antibiofilm properties of peracetic acid, resulting in greater log reductions in both fresh and aged *L. monocytogenes* biofilms on polypropylene and stainless steel surfaces compared with antimicrobial solutions that did not contain nanobubbles (Unger et al., 2023). Similarly, in a study performed to determine whether the incorporation of nanobubbles amplifies the effectiveness of different antimicrobial solutions (peracetic acid, chlorine, citric acid and lactic acid), Singh et al. (2021) revealed a significant positive impact of nanobubbles on both chlorine and peracetic acid when used against *E. coli* O157:H7 and *L. monocytogenes*. Unger et al. (2024) attributed the improved antimicrobial potency of both peracetic acid and chlorine in combination with nanobubble water against fresh and aged *E. coli* biofilms on polypropylene, silicone, and stainless steel surfaces to be related to the advanced physical characteristics of nanobubbles (increased stability, negative zeta potential, large specific surface area, high gas dissolution rate, and generation of free radicals).

Undoubtedly, pathogen control is one of the most interesting targets of nanobubble research especially with the critical need for reducing the quantity of general biocides and antimicrobials used due to the rising challenge of antimicrobial resistance among foodborne pathogens (Donaghy et al., 2019). Therefore, some studies have already begun to demonstrate the effectiveness of combining nanobubble technology with existing strategies and technologies. A study conducted by Rafeeq & Ovissipour (2021) obtained promising results regarding a strong synergistic relationship between nanobubbles and ultrasound for reducing pathogens (*E. coli* O157:H7 and *L. innocua*) in cell suspensions and on spinach leaves, where a greater log reduction was obtained when both technologies were combined. Another study focusing on the incorporation of complementary technologies to maximize the inactivation efficiency of plasma-activated water and ultraviolet photolysis against *E. coli* cells in chicken meat revealed that treating with

micro/nanobubbles in combination with the two technologies produced an optimal inactivation (Moonsub et al., 2024). Additionally, studies on shelf-life extension and preservation effect of nanobubbles in the seafood industry have proven this emerging technology as a promising one. The ability of nanobubbles to maintain safe microbial levels and reduce oxidation without affecting sensory attributes of fresh foods makes nanobubble application an attractive alternative to conventional preservation techniques. For instance, a study on the preservation of fresh yellowfin tuna (*Thunnus albacares*) evaluated the impact of nitrogen nanobubbles compared to the conventional preservation method (using ice) and revealed that treatment with nanobubbles resulted in low dissolved oxygen levels, reduced histamine content (an indicator for decline of fish quality), as well as a safe mercury level (a dangerous heavy metal), all while having no effect of organoleptic characteristics (Saputra et al., 2020). Saptura et al. (2020) further confirmed that nitrogen nanobubbles enhanced the preservation of fresh tuna by achieving low total microbial and *E. coli* counts within required safe limits, as well as absence of *Salmonella* sp. Similarly, the application of CO₂ micro-nanobubbles in the washing process of tilapia fillets demonstrated a significant reduction of aerobic bacteria with the addition of NaOCl and NaCl at a contact time of 32 minutes, revealing that this combination can extend storage time of those filets by more than 7 days at 4 ± 2 °C (Naewkanya & Petiraksakul, 2024).

Minimal studies have been conducted on the use of gas nanobubbles to prevent or reduce the formation of biofilms to improve the quality and safety of raw meat products. Moreover, some of the observed limitations in the currently available studies include the lack of incorporating mixed-species biofilms as well as accounting for the effect of the organic build-up from the meat residue in meat processing facilities that can both have significant impacts on the treatment and control of biofilms.

2.4. Peracetic Acid as an Antimicrobial

Peracetic acid, also known as peroxyacetic acid (PAA), is a type of chemical disinfectant listed by the Centers for Disease Control and Prevention (CDC) under its infection control protocol and is an approved sanitizer and disinfectant by the United States Food and Drug Administration (FDA) (21 CFR 178.1005-1010). Initially, PAA was patented in 1950 for usage in treating vegetables and fruits against spoilage microorganisms and was then officially used in disinfecting wash water in the fresh produce industry (Organic Materials Review Institute, 2000). Nowadays, PAA is widely used in food industries as a disinfectant in wash and rinse water for raw and processed fruits and vegetables, meat and eggs as well as a sanitizer on food contact surfaces with specific limitations set by the FDA (Organic Materials Review Institute, 2000). PAA is mostly used in combination (blended commercial products) with other compounds such as hydrogen peroxide and acetic acid in the meat industry for spraying, washing, or dipping red meat and poultry carcasses, parts, trims and organs as well as in chiller water and brines; it is allowed at different limits depending on the intended use, with a typical maximum concentration of 2000 ppm (U.S. Department of Agriculture Food Safety and Inspection Service, 2024a). Practically, the most common methods of application of PAA on meat and poultry carcasses include spraying, immersion, rinsing, and surface washing which can be done in an automated or manual manner (Rossi et al., 2023).

PAA ($C_2H_4O_3$) is produced from the acid-catalyzed reaction between acetic acid and hydrogen peroxide, which is considered a very strong oxidizing agent (Pechacek et al., 2015). With a higher oxidation potential than chlorine sanitizers but less than ozone, PAA can inactivate vegetative bacterial cells, endospores, yeast, and mold spores with its primary mode of action relying on the oxidation of the cell's outer membrane (Organic Materials Review Institute, 2000).

This potent biocide follows the typical mode of action of several oxidative biocides aiming at shattering the chemiosmotic and transport functions of the cell membrane through the oxidation of sulfhydryl groups, disulfide bonds, and double bonds in proteins, lipids and other cellular constituents (Kramer, 1997). While chlorine and irradiation have been used for a long time in the meat and poultry industry for disinfection, investigating and evaluating the use of PAA in those industries is a more recent focus. Some of the important advantages of using PAA include its fast-acting capability, rapid breakdown, and lack of harmful residues, as proven by several studies (Environmental Protection Agency, 2012).

Recent studies focused on the strong antimicrobial effect of PAA include a study done by (Kalchayanand et al., 2024) which demonstrated that spray treating beef trimmings and subprimals with four different PAA concentrations ranging from 130 ppm to 400 ppm significantly reduced *E. coli* O157:H7 and *Salmonella* on the surfaces of the treated parts. Another study on the control of Shiga toxin-producing *E. coli* (STEC) on hog carcasses showed that treatment by external wash with PAA at 400 ppm was effective, achieving a reduction of 3.4 log CFU/cm² (Pozuelo et al., 2021). In this same study, Pozuelo et al. (2021) revealed that the application of PAA at a 400 ppm concentration did not have any effect on the color of the treated raw pork products. In a study performed to evaluate the sensory characteristics of ground beef produced from beef trimmings treated with PAA, Pohlman et al. (2009) indicated that the redness of the ground beef using 0.02% PAA was comparable with their control ground beef from untreated trimmings. PAA is an effective antimicrobial used in the poultry and meat industry against foodborne pathogens (such as STEC and *Salmonella*), with exposure time, concentration, and presence of organic matter being important factors to consider for obtaining optimal microbiological control (Kalchayanand et al., 2016). The effect of temperature is another consideration especially in the meat industry where

temperatures might vary according to the stages of processing. Beltrame et al. (2012) confirmed that PAA was effective against *Salmonella choleraesuis*, *Staphylococcus aureus*, *E. coli*, and *Listeria monocytogenes* at different concentrations and exposure times at a temperature of 10°C, but those results were not replicable at 45°C where PAA is claimed to decompose and lose its efficiency at such a higher temperature (Beltrame et al., 2012). The pH of the treated food can also impact the antimicrobial's effectiveness, where a lower pH was found to have a complementary effect, further enhancing the diffusion of the applied organic acid solution (Rossi et al., 2023). Kroft et al. (2024) found that PAA washes at concentrations of 50, 200 and 500 ppm were more effective at 4°C than at 27°C for reducing *Salmonella* populations on raw chicken wings. In their study, treatment solution pH or treatment time (up to 10 s exposure) had no effect on *Salmonella* recovery.

2.5. Key Ground Beef Pathogens: Shiga Toxin-Producing *Escherichia coli* (STEC)

Characteristics and Prevalence

Escherichia coli (*E. coli*) belongs to the family of Enterobacteriaceae which include bacteria from different genera (including *Shigella*, *Salmonella*, *Proteus*, *Yersinia*, *Citrobacter*, *Klebsiella*, *Enterobacter*) that are characterized by similar properties such as shape, gram-staining, and motility (Borman et al., 1944; Farmer et al., 2010). Most *Enterobacteriaceae* are fermentative microorganisms able to ferment D-glucose and other sugars, producing gas (H₂) (Farmer et al., 2010). Since *E. coli* is a lactose fermenter, it also belongs to the group of “fecal coliforms” and has been used as a quantitative indicator of water quality due to its release in the environment by fecal deposition, contaminating water (Farmer et al., 2010; Razmi et al., 2023).

E. coli was named after the pediatrician Thomas Escherich who first discovered this bacterium while researching contaminated feces of ill children. Being an Enterobacteriaceae, it is a gram negative, non-sporulating, facultative anaerobic, rod-shaped bacterium that lives in the gastrointestinal tract of ruminant animals and humans (Sharma et al., 2016). Most *E. coli* strains do not cause illness and are referred to as the “good gut bacteria” that constitute a healthy intestinal tract (Centers for Disease Control and Prevention, 2024b). However, six *E. coli* pathotypes are associated with food-borne illnesses and are considered diarrheagenic pathogens and include: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), possibly Diffusely-Adherent *E. coli* (DAEC), Enteraggregative *E. coli* (EAEC), and Shiga toxin-producing *E. coli* (STEC) (Centers for Disease Control and Prevention, 2024d). Humans are the primary reservoirs for non-STEC pathotypes, while the intestinal tract of ruminant animals (mainly cattle) serves as the primary reservoir for STEC, both of which are characterized by severe gastrointestinal symptoms such as diarrhea, vomiting, fever, and stomach cramps (Centers for Disease Control and Prevention, 2024d).

STEC strains include pathogenic Enterohemorrhagic *E. coli* (EHEC) which cause severe human illness and are associated with bloody diarrhea and the development of Hemolytic Uremic Syndrome (HUS), a deadly disease that affects kidneys (Smith et al., 2014). 90% of individuals affected with STEC show symptoms of bloody diarrhea, while 5-15% of patients develop HUS (Smith et al., 2014). STEC consists of two types, O157 and non-O157, with non-O157 STEC causing more disease than STEC O157 in the United States (Smith et al., 2014). The most common non-O157 serogroups are O26, O111, O103, O121, O45, and O145, but they typically result in milder effects compared to O157 (Smith et al., 2014). Actually, the Centers for Disease Control and Prevention estimates that STEC O157 causes 97,000 illnesses, 3,270 hospitalizations, and 30

deaths each year in the United States, while STEC non-O157 causes an estimated 169,000 illnesses and 400 hospitalizations and no deaths (Centers for Disease Control and Prevention, 2024b). STEC serogroups are characterized by their production of one of two types of *stx* genes associated with the production of Shiga toxin, *stx1* and/or *stx2*, with the STEC O157:H7 (*E. coli* O157:H7) serogroup being known to produce *stx2* which is more commonly associated with HUS development (Hunt, 2010). Another virulence factor important in determining whether a STEC strain is part of the EHEC subgroup (with over 200 known serotypes) is the presence of an *eae* gene in the LEE chromosomal pathogenicity island that encodes for intimin, which is an outer membrane protein adhesin that facilitates bacterial attachment to the host intestinal cells (Newell & La Ragione, 2018; Pokharel & Dozois, 2023).

Being native organisms of the human gut, *E. coli* strains grow optimally at 35-37°C and at a neutral pH ranging from 6 to 8 (Farmer et al., 2010; Smith et al., 2014). However, *E. coli*, like other bacteria, trigger stress mechanisms to adapt to new environmental conditions such as the acid-resistance mechanisms which protect the cells against low pHs of 2 and to 2.5 (Smith et al., 2014). An important growth factor that influences the ability of bacteria to grow and nourish is the availability of iron in the environment. All STEC strains carry the catechol siderophore enterobactin as an iron chelator which helps in transporting iron, while STEC O157 is capable of acquiring iron from heme and hemoglobin for growth (Smith et al., 2014). This is one of the reasons why STEC O157 thrives in meat and meat products that contain heme iron essential for the bacteria's growth.

STEC Importance in the Meat Industry

The safety of animal protein-based foods is an important part of overall public health and cannot be neglected by meat and poultry processors or regulatory agencies. Meat processors must

focus their attention on controlling product contamination risks through implementing and continuously improving effective Hazard Analysis Critical Control Points (HACCP) programs that include validated antimicrobial pathogen elimination or control steps, along with stringent critical limits for hazards reasonably likely to occur in their specific product categories (U.S. Department of Agriculture Food Safety and Inspection Service, 2021a). These limits often mean “none detected” when using a regulatory-approved sampling and testing protocol. *E. coli* O157:H7 and six non-O157 STEC serogroups are considered “adulterants in raw non-intact beef and intact beef products intended for non-intact use” including ground beef as stated in the Federal Register due to their severe public health consequences (76 FR 58157) (U.S. Department of Agriculture Food Safety and Inspection Service, 2021a).

Reviewing U.S. foodborne infection trends from 1996-2022 generated from the CDC’s FoodNet pathogen surveillance sites (Centers for Disease Control and Prevention, 2023), the STEC infection rate (number of illnesses per 100,000 population) began to increase around 2012 from 2.1 to 6.26 (peaking) in 2019 and has fallen only slightly to 5.67 by 2022. A tri-agency group from the CDC, USDA-FSIS and FDA was first assembled in 2011 and recently collaborated to utilize their associated foodborne outbreak databases covering the time span of 1998-2022 to gain an accurate estimation of foodborne illnesses rates and sources of attribution in the United States (Interagency Food Safety Analytics Collaboration, 2024). They estimated that 9 million people become ill annually, with 56,000 being hospitalized and 1,300 dying because of known pathogens. Among seventeen food categories evaluated, beef caused the second highest number (21.4%) of all foodborne illnesses attributed to *E. coli* O157, substantially behind raw vegetable row crops (63.7%).

With seven serogroups of STEC being regulated as adulterants in raw beef manufacturing, meat processors invest substantial amounts of effort and resources into its control, including implementation of intervention processes throughout operations, targeted sanitation efforts, and notable volumes of pathogen specific testing of raw materials and finished products. Still, outbreaks and product recalls continue to occur, bringing significant costs and brand damage (Table 2.1).

Table 2.1 Number of meat product associated recalls due to STEC from 2019 to 2023 (meat includes beef, pork, chicken, fish, sheep, turkey, and other)

Year	Total Number of Recalls	Total Number of Pounds Recalled	Number of Recalls Related to STEC	Number of pounds recalled related to STEC	Percentage of Total Recalls Due to STEC
2019	124	20,427,455	7	298,500	5.6
2020	31	1,461,019	1	42,922	3.2
2021	47	15,501,273	2	300,096	4.3
2022	45	1,769,556	3	153,158	6.7
2023	65	4,005,384	5	72,858	7.7
Total	312	43,164,687	18	867,534	27.5

*This table was adapted from the U.S. Department of Agriculture Food Safety Inspection Services (USDA-FSIS) Summary of Recall Cases (U.S. Department of Agriculture Food Safety and Inspection Service, 2019b, 2020, 2021b, 2022, 2023).

2.6. Key Ground Beef Pathogens: *Salmonella*

Characteristics and Prevalence

Salmonella, belonging to the family of *Enterobacteriaceae*, is a facultative anaerobic, rod-shaped, non-sporulating gram-negative bacteria (Jajere, 2019; Roy et al., 2021). The genus *Salmonella* includes two species, *Salmonella enterica* and *Salmonella bongori*. *S. bongori* contains 22 serotypes but is not commonly studied since it is mainly associated with cold-blooded animals

(e.g., reptiles), while *S. enterica* constitutes six subspecies and is associated with warm-blooded animals (mammals and birds) (Lu et al., 2025). These subspecies include *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* with serovars of subsp. *enterica* causing 99% of all human *Salmonella* infections through colonization of the intestinal tract (Lu et al., 2025). With over 2,600 identified serotypes, *Salmonella* infections are categorized as typhoidal or non-typhoidal based on the disease they cause. While typhoidal *Salmonella* serovars, such as *S. Typhi* and *S. Paratyphi*, are human-specific and cause typhoid fever, non-typhoidal serovars, including *S. Typhimurium* and *S. Enteritidis*, can infect a wider range of hosts and typically cause gastroenteritis (Lu et al., 2025; B. X. Wang et al., 2023).

Salmonella enterica is considered a globally significant pathogen with major public health implications, causing millions of infections and thousands of deaths globally each year (Jajere, 2019; B. X. Wang et al., 2023). According to the CDC, *Salmonella* are a leading cause of foodborne illnesses, leading to hospitalizations and deaths in the United States and worldwide; non-typhoidal *Salmonella* being one of the top five pathogens that contribute to foodborne illnesses (Canning et al., 2023; Centers for Disease Control and Prevention, 2024a; Roy et al., 2021). Salmonellosis is the name attributed to *Salmonella* infections and is characterized by watery diarrhea, stomach cramps, headache, nausea, and vomiting, with symptoms that usually last 4 to 7 days (Centers for Disease Control and Prevention, 2024c). People get infected after consuming foods from different animal sources such as chicken, eggs, pork, seafood, beef, turkey, dairy and more. However, *Salmonellosis* can also occur after the consumption of contaminated plant-based foods such as fruits, vegetables, sprouts, flour and oils. An analysis of outbreak data from 1998 through 2022 estimated that 79.7% of foodborne *Salmonella* illnesses were linked to seven major food commodities, listed in order from highest to lowest (percentage of overall attribution):

chicken (19.7), fruits (14.6), seeded vegetables (12.0), pork (11.9), other produce (9.4), beef (6.9), and turkey (5.2) (Interagency Food Safety Analytics Collaboration, 2024).

Salmonella Importance in the Meat Industry

The CDC reported trending for *Salmonella* foodborne illnesses from the 1996-2022 FoodNet surveillance is rather disappointing relative to on-going efforts to reduce incidence rates in the U.S. (Centers for Disease Control and Prevention, 2023). According to their data, little progress has been made in lowering incidence (per 100,000 population), with rates hovering between 13.34 and 18.28 over this period.

The consumption of meat products such as chicken, turkey, pork, veal and beef poses a high risk for *Salmonella* contamination due to the favorable conditions these food products provide for the bacteria to survive and/or grow including essential nutrients, pH and water activity (Her et al., 2024). *Salmonella* has always been a significant concern in the poultry industry. Chicken and turkey are major sources of animal protein in the United States and have been associated with more than 24% of *Salmonellosis* cases in humans. Additionally, this pathogen has gained significant attention in the red meat industry due to its increased prevalence and poor industry control. *Salmonella* contamination is common in pork, beef, goat, and sheep meat where the intestinal colonization of animals is one major point of contamination during slaughter (Sarjit et al., 2019). In a study intended to describe *Salmonella* outbreaks linked to beef in the United States during 2012-2019, all *Salmonella* outbreaks linked to non-intact raw, intact raw, or ready-to-eat beef were reported showing 1103 illnesses, 254 hospitalizations and 2 deaths (Canning et al., 2023). 87% of the illnesses were caused by contaminated ground beef, which also caused 37% of the total hospitalizations (Canning et al., 2023). This indicates the importance of *Salmonella* contamination in beef, especially ground beef products. Indeed, beef was estimated to account for 6.9% of all

Salmonella foodborne illnesses between 1998-2022 (Interagency Food Safety Analytics Collaboration, 2024).

While STEC is considered an adulterant in non-intact raw beef, *Salmonella* is not currently, and the process of *Salmonella* pathogen sampling is considered a measure of process control. Initially, *Salmonella* regulation in food industries (specifically poultry and meat) relies on a *Salmonella* verification testing program set by the FSIS as part of the “Pathogen Reduction; Hazard Analysis and Critical Control Point” (PR-HACCP) final rule placed into effect in 1996 (61 FR 38805) aiming to reduce contamination (U.S. Department of Agriculture Food Safety and Inspection Service, 2024b; Williams et al., 2020). In 2019, the USDA-FSIS updated its *Salmonella* verification testing program for raw ground beef and beef manufacturing trimmings. The new standards establish performance criteria to reduce *Salmonella* contamination by strengthening the regulatory oversight. More rigorous sampling and testing includes increasing *Salmonella* sampling to once per week in beef manufacturing industries producing greater than 50,000 pounds of raw ground beef and beef manufacturing trimmings per day (U.S. Department of Agriculture Food Safety and Inspection Service, 2019a). More research on *Salmonella* in ground beef occurred in the past decade with a most recent project by the U.S. Department of Agriculture to develop a quantitative *Salmonella* baseline and characterize isolates from ground beef in the United States (U.S. Department of Agriculture-Agriculture Research Service (USDA-ARS), 2025). While all current prevalence-based performance standards have been proven effective in reducing *Salmonella* contaminated poultry and meat products, there has been no observable impact on human illness rates (U.S. Department of Agriculture Food Safety and Inspection Service, 2024b). There have been no significant reductions in human illnesses caused by *Salmonella* over the past 25 years which lead the National Advisory Committee on Microbiological Criteria for Food

(NACMCF) to recommend advanced control strategies to the USDA-FSIS (The National Advisory Committee on Microbiological Criteria in Foods, 2024). Hence, in 2024, the USDA-FSIS proposed an amended regulatory framework by incorporating stricter *Salmonella* regulations. The draft framework includes three main components: (1) requiring poultry establishments to identify *Salmonella* as a likely hazard and test incoming flocks before processing, (2) strengthening process control monitoring and FSIS verification efforts, and (3) enforcing a final product standard that defines certain levels or types of *Salmonella* contamination as adulteration (U.S. Department of Agriculture Food Safety Inspection Service, 2024). Even though the USDA-FSIS has recently stated that they have no specific plans currently to expand *Salmonella* standards to other raw meat products under the *Salmonella* Framework initiative for poultry (Government Accountability Office, 2025), similar guidelines for *Salmonella* contamination in raw pork and beef are expected by the industry (personal communications, 2025).

Complicating efforts to reduce or eliminate *Salmonella* from raw meat products is the fact that the pathogen can become internalized in the cells of meat animals and infect the lymph nodes of the animal without signs of illness (Food Safety Net Services, 2020). For raw, non-intact meats particularly, typical carcass and/or subprimal intervention strategies such as antimicrobial sprays and washes (addressing meat surface contamination) are limited for *Salmonella* that may be internalized.

2.7. Non-pathogenic *E. coli* Surrogates

Due to their rapid adaptation abilities and strong biofilm-forming capabilities, *E. coli* bacteria are the most studied microorganisms and their presence in nature continues to increase, highlighting the need for more effective management strategies to mitigate the negative impact of this pathogen on human health. New food technologies and antimicrobial strategies must be

thoroughly evaluated for effectiveness and supported by sufficient scientific evidence to establish critical operating parameters based on validated results. When evaluating the effectiveness of a new technology, the intended final application must be carefully considered. For instance, in this research, the application of nanobubble water in combination with peracetic acid is intended to serve as an antibiofilm and antimicrobial solution in meat processing facilities, and thus an in-plant validation study might be required by authorities to further ascertain the efficacy of this technology under real-time specific conditions found in the meat processing facility. Therefore, the use of non-pathogenic surrogates is required to perform such validation studies, given the practical limitations of using actual pathogens in USDA-inspected food processing establishments (Hu & Gurtler, 2017; Niebuhr et al., 2008). The U.S. Food and Drug Administration defined surrogate microorganisms as “non-pathogenic species and strains responding to a particular treatment in a manner equivalent to a pathogenic species and strains” (U.S. Food and Drug Administration, 2000). Several studies have used five USDA-approved non-pathogenic strains of “generic” or biotype I *E. coli* (Table 2.2) as surrogates for pathogenic STEC serogroups due to their similar behavior to meat processing antimicrobial treatments (Marshall et al., 2005; Pittman et al., 2012; Redemann et al., 2018; Wilder, 2016). For confirmation purposes, Keeling et al. (2009) found in a comparative study between the five *E. coli* surrogates and *E. coli* O157:H7 that in general, there was no significant difference between three surrogate strains and *E. coli* O157:H7 after refrigeration, fermentation, freezing and thermal inactivation. Additionally, several studies have justified the use of the five *E. coli* surrogates for *Salmonella* validation studies (Eblen et al., 2005; Niebuhr et al., 2008; Vaddu et al., 2021). This research will use the five *E. coli* surrogates listed in Table 2.2 in a laboratory validation study to investigate their interaction within a multi-species mixed biofilm derived from native microbial populations found in raw beef purge from

wholesale vacuum packaged products. Findings from this research will not only enhance our understanding of surrogate behavior in biofilms but also provide valuable data for predicting both pathogenic *E. coli* and *Salmonella* behavior and for future in-plant validation studies where the use of surrogates is essential.

Table 2.2 The five most commonly used USDA-approved non-pathogenic *Escherichia coli* surrogates

<i>E. coli</i> Strains	ATCC* Accession Number
P1	BAA-1427
P3	BAA-1428
P8	BAA-1429
P14	BAA-1430
P68	BAA-1431

*American Type Culture Collection (<https://www.atcc.org/>)

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Chapter 3 - Efficacy of Peracetic Acid Solutions Prepared Using Nanobubble Water for Controlling Statically Generated Mixed-Species Biofilms on Stainless Steel and Rubber

Abstract

Biofilms developed under static conditions can be problematic in meat processing, especially on equipment surfaces such as seals and crevices. This study evaluated the antimicrobial effects of air-based nanobubble (NW) or tap (TW) water containing peracetic acid (PAA) on mixed-species biofilm development on stainless steel (S) and Buna-N rubber (R) in a chilled static system with organic loading. Twelve treatment and two control solutions of 5 or 10% (v/v) beef purge with 0, 200, 400 ppm PAA in TW or NW were added to vessels containing SS and R coupons. Treatment vessels were inoculated with STEC surrogates and stored at 4°C statically for 10 days, with 20% of volume replenished with fresh solution and purge daily. Planktonic and biofilm populations were quantified on days 0, 3, 5 and 10. A mixed-model ANOVA was used to identify significant treatment effects with appropriate pairwise comparisons performed using Fisher's protected LSD ($n=335$, 3 replications, $\alpha=0.05$). No differences ($P > 0.05$) in APC or EB counts were observed comparing NW and TW treatments within PAA concentration across days. At 5% purge, R and S coupons showed no difference ($P = 0.52$) in APC biofilm growth; at 10% purge, R supported greater biofilm growth than S ($P = 0.008$). Organic level affected biofilm development in 200 ppm PAA solutions ($P < 0.0001$), reaching 4.5 log APC/coupon at 10% purge compared to 2.0 logs at 5% purge. At 400 ppm PPA, APC biofilm development was higher ($P = 0.043$) in TW solutions, but similar ($P=0.16$) in NW solutions between organic levels. PAA at 200 ppm inhibited biofilm growth for 5 days but allowed significant development between days 5 and

10 ($P=0.008$). PAA at ≥ 200 ppm inhibited biofilm development by native meat microflora on S and R surfaces under static, extended duration, refrigerated conditions encountered in meat processing, with a strong influence by the level of organic loading present.

3.1. Introduction

Bacterial biofilm formation has been a major food safety concern in different food industries, and importantly, also jeopardize product shelf-life. Bacteria in their planktonic, free-living form have the ability to attach to available surfaces, form aggregates of cells, and secrete an extracellular polymeric substance (EPS) that acts as a shield, protecting the embedded bacterial cells (Alvarez-Ordóñez et al., 2019; Rumbaugh & Sauer, 2020). Microbial resistance tends to be enhanced when bacteria are part of a biofilm community compared to bacteria in their planktonic form due to the beneficial properties that the EPS matrix provides; in fact, bacteria in a biofilm gain higher tolerance to unfavorable growth and environmental conditions, better nutrient acquisition and enhanced cell-to-cell communication (Flemming et al., 2016). Bacteria recognize common materials used in food processing environments such as wood, glass, stainless steel, polyethylene, rubber and others, as a suitable, ideal environments for their biofilm development (Carrascosa et al., 2021). The enhanced resistance that both spoilage and pathogenic bacteria gain from biofilm formation elevates the risk of microbial contamination in food processing facilities. This has led to several biofilm-associated foodborne outbreaks and increased costs to food processors due to equipment damage and product recalls (Alvarez-Ordóñez et al., 2019; Yuan et al., 2022). In addition, improper cleaning and sanitation have added to the complexity of the challenge in different environments.

Meat processing facilities are among the common food processing environments where biofilm development occurs. Ensuring meat safety is crucial, especially since meat is a valued component of the human diet and an important source of protein for many consumers. Meat processing environments can harbor biofilms due to the continual presence of organic residues, which promote and facilitate biofilm formation on equipment and other environmental surfaces,

posing a risk to the safety and quality of the final product. Dutra et al. (2018) demonstrated that food residues, particularly meat exudate, significantly enhanced the formation and microbial composition of *Escherichia coli* and *Staphylococcus aureus* biofilms on stainless steel surfaces, highlighting the critical influence of organic matter accumulation in simulated industrial conditions on biofilm formation (Dutra et al., 2018). Another study showed that the presence of natural organic and inorganic substrates found in beef processing facilities, represented by ground beef residues on stainless steel and high density polyethylene coupons, promoted the growth of *E. coli* O157H:7 biofilms compared to a slow biofilm growth in the absence of organics in sterilized growth media (Dourou et al., 2011). In addition to organic matter, the presence of food-associated microorganisms and native microflora contributes to mixed-species communities that promote the attachment and formation of biofilms, while allowing food-borne pathogens of lower biofilm-forming capabilities to contribute to biofilm formation, thereby increasing their resistance (Olanbiwoninu & Popoola, 2023; Yuan et al., 2022). In multi-species biofilms, synergistic relationships formed among bacteria can promote a better communication system, protecting and strengthening the bacterial community. Due to the natural ecology of a biofilm, which involves the formation of a complex community of bacteria, mixed-species biofilms are the most common form found in nature and in the food industry as the source of contamination (Flemming et al., 2016; Yuan et al., 2022).

Food processors must utilize a diligent cleaning and sanitation plan to control and eradicate biofilms. In order to control biofilm formation and bypass the sanitary design challenges of equipment (presence of hollow rollers and tubing, joints, welds and scrapers as examples) that create an obstacle for proper equipment cleaning, the standard cleaning process must be followed to prevent initial microbial attachment to surfaces, which is characterized as a weak attachment

that creates a thin film that can be easily removed at first (Cramer, 2013). The standard cleaning process involves six main steps: dry-cleaning to remove debris, a hot water rinse, detergent application including the use of oxidative agents and acids (such as peracetic acid), followed by a final warm water rinse before applying a sanitizer for a final sanitization step, followed by a proper inspection step to verify the effectiveness of this sanitation protocol (Cramer, 2013, pp. 105–106).

Once the initial thin bacterial film develops and forms thicker aggregates on surfaces, biofilms become harder to remove through conventional cleaning and sanitation practices. Some of the most common recently used physical methods include the use of ultrasound, ultraviolet irradiation, magnetic fields with magneto-materials, and cold-plasma treatments which all work on the same principle of an enhanced bacterial membrane penetration (Lu et al., 2022). The use of chemical sanitizers and disinfectants such as sodium hypochlorite, sodium hydroxide solutions, quaternary ammonia, hydrogen peroxide, and peracetic acid is a common sanitation practice in the food industry (Galié et al., 2018; Lu et al., 2022). Other innovative technologies attracting focus at present include the use of natural compounds and essential oils from plants, bacteriophages, bacteriocins and quorum sensing inhibitors (Galié et al., 2018). Use of industrial enzyme solutions containing proteolytic and/or polysaccharidases, in combination with buffers, surfactants and chelating agents, have shown efficacy in dispersing common food-associated biofilms on stainless steel surfaces (Lequette et al., 2010).

Because of the rigid structure of biofilms and the hardships faced by the food industry to eradicate them, food scientists continue to explore new strategies to control and eliminate biofilm growth, contributing to better product quality and safety. One of the more recent innovative approaches involves the application of nanobubbles (NBs), which are extremely fine gas bubbles with diameters below 500 nm that show remarkable characteristics in solution (Phan et al., 2020).

These tiny bubbles are generated in aqueous solutions and exhibit unique characteristics, such as long stability, high zeta potential, and high gas dissolution rate (Foudas et al., 2023). NBs are capable of producing highly reactive oxygen radicals (such as hydroxyl ions) that are also known as reactive oxygen species (ROS), allowing NBs to be used in advanced oxidation processes, such as water treatment and biofouling control (Phan et al., 2020). Moreover, they have shown efficacy in membrane defouling due to their enhanced effect on membrane permeability (Foudas et al., 2023).

Seeing the potential of NBs in solving challenging problems such as biofouling, research to effectively use NBs to advance biofilm control is on the rise. Shiroodi et al. (2021) demonstrated that pure oxygen NBs in deionized water inhibited the formation of biofilms on hard surfaces by preventing bacterial cell attachment, with an observed 3.4 log CFU/ cm² reduction in *E. coli* biofilms on stainless steel coupons. NBs are able to penetrate the EPS and target sessile bacteria embedded within biofilms and have shown enhanced synergistic performance when used with common disinfectants and sanitizers such as lactic acid, peracetic acid, and chlorine (Singh et al., 2021; Unger et al., 2023, 2024).

Disinfectants and sanitizers are types of antimicrobial agents that are commonly used in the food industry to control and inhibit the growth of undesirable microorganisms. Commonly used food plant sanitizers include chlorine-based compounds, quaternary ammonium compounds (QACs), iodophors, ozone, chlorine dioxide, and peracetic acid, where each is used with varying degrees of effectiveness against foodborne pathogens (Cramer, 2013, p. 159). Among these, peracetic acid, also known as peroxyacetic acid (PAA), has gained significant attention due to its wide spectrum of effectiveness against gram-negative and gram-positive bacteria, fast-acting capability, rapid breakdown, and lack of harmful residues (Environmental Protection Agency,

2012). Multiple studies have validated the efficacy of PAA in reducing bacterial contamination on food-contact surfaces and raw products, demonstrating its broad-spectrum activity against pathogens like *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* (Beltrame et al., 2012; Kalchayanand et al., 2016, 2024; Pozuelo et al., 2021; Rossi et al., 2023). Using PAA as an antimicrobial in the meat and poultry industry is a fairly recent approach, since chlorine and irradiation (e.g., ultraviolet light) have traditionally been applied for disinfection. Therefore, validating the effectiveness of PAA against biofilm formation in a meat processing environment requires further exploration. Seeing that both NBs and PAA individually have demonstrated efficacy in controlling and eradicating biofilms, a combination of both could offer an effective, and possibly synergistic, approach.

Despite the potential benefits of NBs and PAA, limited research exists on their effectiveness against important microorganisms (such as *E. coli*) involved in mixed-species biofilms developed under static conditions, with even fewer studies investigating biofilms on hard surfaces with organic matter accumulation at refrigerated conditions. Unger et al. (2023) investigated combinations of NBs derived using air, CO₂ or N₂ in combinations with common food-grade chemical sanitizers against *L. monocytogenes* statically grown biofilms (young or mature) in culture media on food-contact surface materials. They achieved higher *L. monocytogenes* reductions using NBs combined with 50-200 ppm chlorine compared to those combined with 20-40 ppm PAA; however, at 80 ppm PAA, reductions were similar to chlorine on stainless steel, polypropylene and silicone. Due to their rapid adaptation abilities and strong biofilm-forming capabilities, some strains of *E. coli* have been attributed to several foodborne illnesses related to product contamination in the U.S. with severe symptoms that can lead to Hemolytic Uremic Syndrome (HUS) and death (Smith et al., 2014). In particular, beef was

reported as the second most attributed food to *E. coli* O157 with an approximate 21.4% attribution rate in the year 2022, derived from an outbreak data analysis from 1998 through 2022 (Interagency Food Safety Analytics Collaboration, 2024). Therefore, *E. coli* O157 poses a harmful risk on consumers of beef products if contamination is observed and an even higher risk when involved in biofilm formation, leading to persistence in beef processing environments. The combination of NBs with PAA can be viewed as a new method in the reduction of static biofilm development by multi-species bacteria native to beef such as *Pseudomonas*, *Commamonas*, and *Lactobacillus* which can harbor pathogenic bacteria, like *E. coli* O157, increasing their virulence, resistance and biofilm-forming properties (Nan et al., 2024). Incorporating NBs has been suspected to improve the effectiveness and potency of antimicrobial agents against biofilms due to the increased surface area, better stability and production of hydroxyl radicals (Unger et al., 2024). The need for multi-species incorporation in biofilm studies is critical to better mimic actual beef processing environments and can be achieved by the addition of beef purge that leads to organic material buildup and natural microbial contamination in the experimental system. This study evaluated the efficacy of nanobubble water (NW) in combination with PAA at different concentrations (200 and 400 ppm) to control mixed-species biofilm formation, in combination with a cocktail of five *E. coli* surrogates, on stainless steel and rubber surfaces under static conditions, in the presence of beef purge at refrigerated temperatures (4°C) over an extended period of time (10 days).

3.2. Materials and Methods

Bacterial strains and inoculum preparation

Five strains of rifampicin-resistant non-pathogenic surrogate *Escherichia coli* [strains represented USDA-approved surrogate cultures from the American Type Culture Collection (ATCC) designated BAA-1427 P1, BAA-1428 P3, BAA-1429 P8, BAA-1430 P14, and BAA-

1431 P68, which were trained to be rifampicin resistant in the laboratory of Dr. John Luchansky at the USDA ARS Eastern Regional Research Center, Wyndmoor, PA] were selected in this study. The strains were stored at -80°C in cryogenic vials containing cryogenic beads. To prepare the inoculum, frozen beads of each culture were transferred individually to 10 mL of tryptic soy broth (TSB; BD Difco, Sparks, MD) and incubated at 35°C for 24 hours. Each strain was streaked for isolation on tryptic soy agar (TSA; BD Difco), incubated for 24 hours at 35°C, and then stored at 4°C. One isolated colony was transferred into another 10 mL of TSB and incubated at 35°C for 24 hours. One day prior to the start of the experiment (day -1), 0.1 mL from each culture tube was transferred into another 10 mL TSB tube and incubated at 35°C for 24 hours. After the second incubation, 5 mL from each TSB tube corresponding to each of the surrogates was transferred to a single 50 mL sterile conical tube, yielding 25 mL of a 5-strain cocktail culture to serve as the inoculation cocktail. The initial population level of each strain and of the mixed cocktail were verified by serially diluting, plating on EB 3M Petrifilm (Saint Paul, MN, USA) and enumerating after 18-24 h of incubation at 35°C. Overnight cultures reached 9.1, 9.0, 8.9, 9.1 and 9.0 Log₁₀ CFU/mL for ATCC BAA-1427 P1, BAA-1428 P3, BAA-1429 P8, BAA-1430 P14, and BAA-1431 P68, respectively, and 9.2 Log₁₀ CFU/mL for the cocktail.

Meat purge

Three different batches of meat purge collected from vacuum packaged beef subprimals were provided by Kansas State University's Meat Lab (Department of Animal Sciences and Industry, Weber Hall) and were used for each separate replication of the study. The Meat Lab procured the commercial subprimals from a national processor and opened the vacuum bags during normal cutting operations conducted by the laboratory to generate food service raw beef cuts under USDA FSIS inspection. The purge was used to imitate the typical organic matter containing fluids

that would be present in a normal beef processing facility. Upon receiving each batch of purge for each study replication, it was portioned into 40 mL conical tubes and stored at -20°C. The required volume of purge amount in conical tubes was thawed at 4°C one day prior to its use. The microbial populations of each meat purge batch as received were established by plating serial dilutions (in 0.1% peptone water) in duplicate on 3M Petrifilm (Saint Paul, MN, USA) for total aerobic plate counts (APC) and total Enterobacteriaceae (EB) counts. The average bacterial population levels found in the three different batches of meat purge used throughout this study were 7.53 and 4.04 Log₁₀ CFU/mL for APC and EB, respectively.

Coupon preparation

Buna-N rubber (RD128-BUNA) and stainless steel (RD128-304) coupons were purchased from BioSurface Technologies Corporation (Bozeman, MT, USA) and were prepared according to an adjusted protocol based on the standard method ASTM E3161-21 (ASTM, 2022). After use in each replication of the biofilm study, coupons were reconditioned by steam sterilizing at 121°C, 15 psi for 15 min. Following sterilization, they were vigorously scrubbed with a pipe cleaner brush by hand using a non-residue laboratory detergent to remove residual biofilm and organic material debris. The scrubbed coupons were then placed into clean conical tubes filled with the detergent water and were sonicated for 5 min using a Branson 3800 sonicator (Branson Ultrasonics Corporation, Danbury CT). After the initial sonication, the soapy water was replaced with tap water, and the coupons were sonicated again for 5 min. Then, the conical tubes were filled with deionized water, and the coupons underwent a final sonication step for 5 min to remove any remaining impurities. The coupons were then removed and exposed to UV light for 15 min inside a biosafety cabinet to achieve surface sterilization. Before use, cleaned coupons were placed into autoclave pouches and autoclaved at 121°C, 15 psi for 15 min.

Preparation of nanobubble water and tap water solutions

All batches of nanobubble water (NW) and tap water (TW) solutions needed throughout a full experimental replication were prepared one day prior to the start of the experiment and stored in large sterile glass bottles at 4°C to be used within the experiment over 10 days. A Moleaer XTB-25 nanobubble (NB) generator (Figure 3.1) (Moleaer Inc., Hawthorne, CA) was used to generate NBs in solution based on the principle of cavitation. The generator unit was comprised of a centrifugal pump, an air pressure gauge, an air connection, an air flow meter, a pump pressure gauge, a discharge flow valve and a starter (Moleaer Inc., 2020). Input water into the unit was standard municipal tap water (City of Manhattan, KS). NW generated by the unit was collected into a plastic 50-gal water holding reservoir. The air-based NBs were generated using food-grade compressed cylinders of air. According to the Moleaer XTB 25-50 Operation Manual, the average size of the gas bubbles generated into the resulting output water varies from 70 to 100 nm (Moleaer Inc., 2020), and this was confirmed during previous NB water studies (currently unpublished) in our laboratory utilizing a Malvern NanoSight LM10 nanoparticle analyzer (K-State Nanotechnology Innovation Center core laboratory under the direction of Tej Shrestha, Core Director; <https://nicks.ksu.edu/core-facilities/nicks-core/nanosight-microscope.html>).

The Moleaer system was first purged seven times at the beginning of each experimental replication to adequately flush out any debris or water from previous trials or laboratory water pipes before generating new nanobubbles. To initiate the production of the required TW and NW volumes for each replication, municipal TW with an approximate pH of 8.5, as delivered from the laboratory water supply connection, was used to fill the NB generator's 50-gal reservoir. TW samples were generated first by running the municipal water through the Moleaer unit with the cavitation turned off and collecting into the reservoir, subsequently dispensing this bulk water into

500 mL sterilized media bottles that were capped and stored at 4°C until needed. After draining the excess TW from the reservoir, NW was generated in a similar manner. The protocol entailed placing the Moleaer unit into a recirculation mode with municipal water running and opening the compressed air tank valve with the pressure set at a stable 120 psi. The system was allowed to run for 1 hour in this recirculation set-up to ensure saturation of the NW with a high concentration of NBs. After recirculating for 1 hour, the unit's valve was opened and the water diverted to fill the reservoir. Glass bottles were prepared and NW solutions were stored as described for the TW solutions.

Peracetic acid solutions and titration

The stored bulk TW or NW bottles were used to prepare initial (day 0) PAA treatment solutions, as well as to prepare new solutions daily for 20% replenishment requirements (section 3.2.7). Microtox 5® (The Vincit Group, Chattanooga, Tennessee) was the source of PAA used in these studies. This is a popular source of PAA across the U.S. meat and poultry industry. Three PAA concentrations were evaluated in this study: 0 ppm (control), and 200 ppm and 400 ppm $\pm$ 20 ppm. To prepare 200 ppm solutions, 570 microliters of Microtox 5® PAA were added to 250 mL of the appropriate water type (NW or TW). For preparing 400 ppm solutions, 1.15 mL of PAA were added to 250 mL of the appropriate water type. The PAA concentration was confirmed by titration using a peracetic acid test kit (Chem World, Kennesaw, GA). Using the bulk solutions now containing target concentrations of PAA, antimicrobial treatment solutions (250 mL; 4°C) were dispensed on day 0 of the experiment (according to section 3.2.6) into 100-mL sterile specimen cups (Thermo Fisher Scientific, Waltham, MA). Freshly prepared solutions were used for the daily replenishing of the vessels. pH of the prepared bulk solutions was measured using a

pH meter; an average pH of 3.43 was observed for 200 ppm solutions and 3.16 for 400 ppm solutions.

Experimental design

This study consisted of 12 experimental treatments and 2 control groups designed to evaluate static biofilm formation under different concentrations of PAA as an antimicrobial. The treatments included varying levels of beef purge to simulate organic buildup or what is commonly referred to in meat and poultry processing operations as “red water”: half with 10% purge (high organic load or "dirty system") and half with 5% purge (moderate organic load). The beef purge (i.e., juice collected from the subprimal bags) used in this study contained natural/native microbial populations that would contribute to the safety and quality of the commercial product. By using this purge as the source of nutrients and mixed-species inoculum for our treatments, biofilms developing on our test material coupons are representative of true meat processing scenarios.

The PAA concentrations used were 0 ppm, 200 ppm, or 400 ppm, provided using Microtox 5[®] food-grade sanitizer. The experimental design paired NW and TW at each target PAA concentration giving a total of 6 NW-based treatments and 6 TW-based treatments to compare. The treatments and controls were prepared in sterile specimen cups (vessels) with sealable lids. Each treatment vessel contained 4 stainless steel (S) coupons and 4 rubber (R) coupons, with one of each type designated for each specified sampling day. A volume of beef purge was added to each vessel on day 0 to yield either 5 or 10% purge levels (i.e., 3.75 mL or 7.5 mL, respectively). Biofilms were allowed to grow statically (no solution flow or agitation within the vessels) for 10 days under refrigerated conditions (4°C). Sampling was done on days 0, 3, 5, and 10.

All experimental treatments were inoculated on day 0 with 0.1 mL of the 5-strain surrogate *E. coli* inoculum cocktail, while the two control groups were not inoculated to allow

characterization of the native microbial populations contained in the purge within each treatment vessel. One control used 10% purge and the other 5% purge, both prepared with TW only and without PAA. After inoculation on day 0, the treatment and control vessels were allowed to sit in the refrigerator for 2 hours to allow possible bacterial attachment (i.e., loose attachment). After this period, the day 0 set of samples were analyzed to enumerate the concentration of planktonic bacteria in the vessel solutions and the level of attached biofilm populations on both coupon types. The sample vessels were returned to 4°C storage and similarly analyzed on days 3, 5, and 10 to monitor biofilm formation over time and assess the effects of the different treatments. Treatment vessels were replenished daily with new treatment solutions by removing 20% (v/v) of each solution (on days designated for microbiological analysis, the volume removed for preparing Petrifilm dilutions was included in the 20% replenishment calculation) and restoring to the initial volume by adding the appropriate ratio of fresh solution and fresh purge. This replenishment step was intended to mimic the manufacturing process of continuous antimicrobial solution and organic fluids being supplied during commercial beef processing. Table 3.1 lists sample IDs corresponding to the six treatment solutions and the control solutions used throughout the study.

Cell detachment and microbial analysis

On each sampling day (Days 0, 3, 5, and 10), both planktonic and firmly attached cells were analyzed for total aerobic plate counts (APC) and Enterobacteriaceae (EB) counts. For planktonic cells, 1 mL from each treatment vessel was transferred to 9 mL of Dey-Engley Neutralizing Broth (D/E Neutralizing Broth, BD Difco, Sparks, MD), then serially diluted using 9-mL tubes of 0.1% peptone water (BD Difco, Sparks, MD) and enumerated on APC and EB 3M Petrifilm (Saint Paul, MN, USA) plates. The bacterial population results for this analysis are referred to as planktonic free cells. Attached cells (biofilm populations) were analyzed according

to an adjusted ASTM E2871-19 protocol, the single tube method. One R and one SS coupon was removed from each treatment solution vessel using sterile forceps and was each placed in D/E Neutralizing broth (BD Difco, Sparks, MD) for 1 minute. Additionally, this step removed any non-attached or loosely attached cells from each coupon before analysis. After D/E neutralization, each coupon was transferred to a 50-mL sterile conical tube containing 25 mL of phosphate buffered saline solution (PBS; VWR, Solon, OH). A series of sequential sonication and vortexing followed, whereby, each conical tube was vortexed for 30 seconds, then sonicated for 30 seconds. This was repeated 3 times. The 25 mL of PBS in each conical tube containing the processed coupons was considered the 10^0 dilution, which was further serially diluted using 0.1% peptone water (BD Difco, Sparks, MD). Appropriate serial dilutions were plated on APC and EB 3M Petrifilm (Saint Paul, MN, USA) to establish the cell concentrations for each detached biofilm. All EB Petrifilms were enumerated after incubation at 35°C for 24 hours, while APC Petrifilms were enumerated after incubation at 35°C for 48 hours.

Statistical analysis

For planktonic bacterial cells (vessel solutions), CFU/mL values were converted to $\log_{10}$ CFU/mL. For attached bacterial cells (biofilm), CFU/mL values were converted to CFU/coupon and then to $\log_{10}$ CFU/coupon. When no CFUs were detected from the lowest dilution plate, half of the calculated detection limit was entered for purposes of analysis (i.e., 0.7 and 1.1 Log CFU per mL or per coupon for solutions and coupons, respectively). The log-transformed APC and EB counts were analyzed separately. Each resulting dataset was used to fit a linear mixed model via the Statistical Analysis Software (SAS version 9.4; Cary, NC) GLIMMIX Procedure with option DDFM = KR. Fixed effects were assessed using the type III tests and appropriate pair-wise

comparisons of least squares means (LSM) were conducted at the 0.05 significance level with no adjustment for multiplicity.

Table 3.1 Sample IDs for the Treatment and Control Solutions

Sample ID	Water Type	PAA Concentration (ppm)
<i>T1</i>	TW	0
<i>T2</i>	NW	0
<i>T3</i>	TW	200
<i>T4</i>	NW	200
<i>T5</i>	TW	400
<i>T6</i>	NW	400
<i>C*</i>	TW	0

Each treatment was applied at both specified purge levels (5% and 10%), resulting in a total of 12 experimental treatments and 2 control treatments. TW= Tap Water, PAA= Peracetic Acid, ppm= parts per million.

*Control samples differ from T1 in that they were not inoculated with the *E. coli* surrogates.

3.3. Results

Impact of antimicrobial treatment solutions on biofilm build-up on coupons

The effects of treatment solution, purge concentration, coupon type and time in days were evaluated against APC and EB biofilm growth, separately. Three out of the four main effects—treatment, purge, and day—significantly affected APC biofilm growth ($P < 0.05$), while coupon type showed no significant effect on APC biofilm growth ($P = 0.1559$). For EB growth, the main effects treatment, coupon type and day showed significant impact ($P < 0.05$), whereas purge had no significant effect ($P = 0.37$). Only two-way interactions significantly affected both APC and EB growth on coupons (Treatment * Purge, Purge * Coupon and Treatment * Day for APC growth, whereas Treatment * Coupon, Treatment * Day and Purge * Day for EB growth) ($P < 0.05$). No

3-way interactions of main effects significantly influenced APC or EB biofilm growth on coupons in this study. All main effects and interactions and their corresponding *p*-values are displayed in Table 3.2.

Table 3.2 P values of the main effects and the interactions of the main effects for the APC and EB biofilm growth on stainless steel or rubber coupons in 5% or 10% beef purge treatments solutions with 0, 200 or 400 ppm PAA in combination with TW or NW over a 10-day period at 4°C.

Effect	APC Pr > F	EB Pr > F
Treatment	<.0001*	<.0001*
Purge	<.0001*	0.37°°
Treatment * Purge	0.0004*	0.1841
Coupon	0.1559	0.0488*
Treatment * Coupon	0.2605	0.0371*
Purge * Coupon	0.0208*	0.7532
Day	<.0001*	0.0003*
Treatment * Day	0.0036*	0.0069*
Purge * Day	0.299	0.0041*

*Effects and interactions are significant ($P < 0.05$)

Aerobic Plate Count (APC) Growth

The observed APC growth on coupons of both material types in non-PAA solutions showed a significant increase over 10 days of storage at 4°C, with control samples bacterial populations increasing from 4.44 to 5.12 ($P = 0.04$), T1 samples (TW-0 PAA) from 4.65 to 5.68 ($P = 0.0028$), and T2 samples (NW-0 PAA) from 4.75 to 5.90 ($P = 0.0008$) ± 0.3 Log₁₀ CFU/coupon (Figure 3.1). No significant difference was observed between the three non-PAA solutions for APC growth across all 10 days including no observed influence of NW versus TW at 0 ppm PAA ($P > 0.05$). With respect to treatment solutions containing PAA, the efficacy of the treatments over 10 days

was assessed based on the results of a simple effect comparison of treatment by day interactions. Both 200 ppm PAA treatment solutions (T3 and T4), regardless of water type, showed variations in APC growth over time, but consistently demonstrated a significant inhibitory effect against APC build-up on coupons throughout the 10-day period when compared with control and treatment solutions without PAA. For further explanation, APC growth on coupons in 200 ppm PAA solutions showed a 2-log decrease compared to control coupons, and a 3-log difference compared to treatment solutions without PAA. 400 ppm PAA solutions in both TW and NW (T5 and T6, respectively) revealed no significant growth across all 10 days with an approximate 3.5-log difference when compared to control samples ($P < 0.001$). 400 ppm of PAA effectively inhibited APC biofilm build-up on coupons over a 10-day period. There was an observed significant difference between 200 ppm PAA solutions and 400 ppm PAA solutions in inhibiting APC growth on coupons ($P < 0.05$). 400 ppm PAA in both TW and NW solutions were able to inhibit a greater amount of APC build-up by an approximate difference of 1 to 1.6 logs between 200 ppm TW-and NW-based treatments after 10 days, respectively.

***Enterobacteriaceae* (EB) Growth**

The observed EB growth on coupons of both material types in non-PAA solutions that were inoculated with the STEC surrogate cocktail showed no significant change over the 10-day period at 4°C ($P > 0.05$) (Figure 3.2). Minor decreases in the EB populations on coupons submerged in treatment solutions without PAA were observed over the 10-day period; EB growth declined from 3.85 to $3.53 \pm 0.19 \text{ Log}_{10} \text{ CFU/coupon}$, and from 3.95 to $3.62 \pm 0.19 \text{ Log}_{10} \text{ CFU/coupon}$ in T1 (TW-0 PAA) and T2 (NW-0 PAA) treatments, respectively. Coupons in non-inoculated control sample solutions showed a natural presence of approximately 2 logs of EB population (from the added purge) on day 0 which increased to $2.65 \pm 0.19 \text{ Log}_{10} \text{ CFU/coupon}$ on

day 3 ($P = 0.0071$) but then remained stable until day 10. EB growth on non-inoculated control coupons was lower than EB growth on inoculated 0 PAA treatments T1 and T2 ($P < 0.0001$). NW alone did not show an impact on EB growth compared to TW alone across the 10 days ($P > 0.05$). Unlike APC biofilm growth on coupons in treatment solutions with PAA, both 200 ppm and 400 ppm PAA solutions showed a similar inhibitory effect on EB growth over 10 days, with no populations detected (level of detection $1.4 \text{ Log}_{10} \text{ CFU/coupon}$). 200 ppm PAA solutions in both TW and NW allowed a slight EB growth on coupons after 2 hours of refrigeration on day 0, detecting 1.87 and $1.90 \pm 0.19 \text{ Log}_{10} \text{ CFU/coupon}$, respectively, and then significantly decreased over the 10 days to 1.1 and $1.15 \pm 0.19 \text{ Log}_{10} \text{ CFU/coupon}$ ($P < 0.05$). With respect to 400 ppm PAA solutions, only the TW-based treatment allowed a slight EB growth on coupons after 2 hours of refrigeration on day 0 ($1.64 \pm 0.19 \text{ Log}_{10} \text{ CFU/coupon}$), which then decreased to below detectable levels by 10 days ($P = 0.0154$). In contrast, the NW-based solution at 400 ppm PAA maintained undetectable EB growth on coupons throughout the entire 10-day period. However, the log EB/coupon in T5 on day 0 was not considered significantly different from T6, as indicated by a p -value of 0.0652 . There was no significant impact of using NW in both 200 ppm and 400 ppm levels of PAA compared to TW on EB growth on coupons across the 10 days ($P > 0.05$). Both 200 ppm and 400 ppm treatment solutions regardless of water type showed a significant inhibitory effect on EB growth on both coupon types over the 10-day period when comparing with control and treatment solutions without PAA ($P < 0.0001$).

Organic Load Influence

The efficacy of each of the water type treatments containing PAA against APC biofilm growth, regardless of coupon type, was significantly impacted by the purge concentration ($P = 0.0004$). As observed in Figure 3.3, PAA at 200 ppm in T3 (NW) and T4 (TW) solutions showed

significantly higher APC growth at a 10% purge concentration than at 5%, with increases of 2.7 and 2.0 Log₁₀ CFU/coupon, respectively ($P < 0.0001$). However, at 400 ppm PAA, only TW-based samples showed a significant increase in APC growth at 10% purge concentration, with counts nearly 1 Log₁₀ CFU/coupon higher than at 5% purge ($P = 0.0439$), while NW-based solutions showed no significant difference ($P = 0.1602$). Additionally, purge concentration did not significantly impact EB growth on coupons ($P > 0.05$).

Surface Material Influence

Purge concentration had a significant impact on APC biofilm growth on S versus R coupons ($P = 0.0208$). At a 10% purge concentration, R coupons showed a higher affinity for biofilm development than S ($P = 0.0088$), while at a 5% purge concentration, APC growth did not differ between R and S ($P = 0.5219$). Additionally, control samples exhibited a higher EB attachment on R coupons compared to S coupons by 0.56 ± 0.16 Log₁₀ CFU/coupon, regardless of purge concentration ($P = 0.0004$). In general, R coupons show a higher affinity for biofilm development when compared to S coupons.

Impact of antimicrobial treatments on planktonic cells in solution

The effects of treatment solution, purge concentration, and time in days were evaluated against the growth of planktonic, free-living APC and EB populations, separately. For microbiological analyses of the solutions, the experimental limit of detection was 1.0 Log CFU/mL. The main effects of treatment and purge significantly affected APC growth in solutions ($P < 0.05$), whereas the interaction between treatment solutions and purge level showed a slightly higher p-value of 0.0517. Even though this interaction was only borderline significant ($P < 0.05$ for statistical significance), the comparison of Least Square Means (LSM) was still made to detect important comparisons among treatment solutions. Time in days did not show any significant

effect on APC growth ($P = 0.3184$). On the other hand, the main effects of treatment and day both revealed a significant impact on EB planktonic growth in solution ($P < 0.001$), whereas purge level had no significant effect ($P = 0.1277$). The interaction between treatment and day, as well as purge and day, significantly affected EB growth in solutions. Moreover, a three-way interaction among the main effects of treatment, purge and day showed a significant impact on planktonic EB growth ($P = 0.0092$). All main effects and interactions with their corresponding p -values are displayed in Table 3.3.

Table 3.3 P-values of the main effects and the interactions of the main effects for APC and EB planktonic bacterial growth in 5 or 10% beef purge treatment solutions with 0, 200 or 400 ppm PAA in combination with TW or NW over a 10-day period at 4°C.

Effect	APC	EB
	Pr > F	Pr > F
Treatment	<.0001*	<.0001*
Purge	0.0149*	0.1277
Treatment * Purge	0.0517	0.3084
Day	0.3184	<.0001*
Treatment * Day	0.3832	0.0014*
Purge * Day	0.6429	0.0117*
Treatment * Purge * Day	0.9878	0.0092*

*Effects and interactions are significant ($P < 0.05$)

Aerobic Plate Count (APC) Growth

Planktonic APC growth was considered significantly impacted by the purge concentration in treatment solutions with 200 ppm PAA in both NW and TW (T3 and T4, respectively) as observed in Figure 3.4. Purge concentration did not affect planktonic APC growth in the other treatments with 0 ppm PAA and 400 ppm PAA (C, T1, T2, T5, and T6 ($P > 0.05$)). The APC

population in T3 with 10% purge was $2.83 \pm 1.2 \text{ Log}_{10} \text{ CFU/mL}$ greater than APC population in T3 with 5% purge ($P = 0.0335$). In T4 with a 10% purge, the APC population was $5.06 \pm 1.2 \text{ Log}_{10} \text{ CFU/mL}$ higher than in T4 with a 5% purge ($P = 0.0009$), further confirming the significant influence that purge concentration has on planktonic APC growth. When comparing APC growth in 10% purge treatment solutions with 200 ppm PAA (T3 and T4) to the 10% purge control solution, planktonic APC populations were not significantly different ($P > 0.05$), whereas, in 5% purge, APC growth in T3 and T4 was significantly lower from the control solution ($P = 0.0013$ and $P = 0.0007$, respectively). Therefore, at higher organic levels, 200 ppm PAA was less effective than at lower organic levels for controlling planktonic APC growth. The inhibition effect of PAA in 10% purge solutions against planktonic APC bacterial populations depended on the concentration of PAA, since 400 ppm PAA results proved to be more efficient in controlling APC growth than 200 ppm PAA in both water types. In fact, this can be confirmed by comparing treatments where both TW- and NW-based solutions showed approximately 2-log differences between 200 ppm PAA and 400 ppm PAA, with higher counts found in lower PAA containing solutions. No effect of using NW versus TW on planktonic APC growth in solutions with all PAA levels was observed ($P > 0.05$).

Enterobacteriaceae (EB) Growth

Non-inoculated control samples in 5% and 10% beef purge solutions showed a natural EB growth of 2.9 and $2.7 \pm 0.54 \text{ Log}_{10} \text{ CFU/mL}$ on day 0 and remained nearly constant over the 10 storage days with no significant growth fluctuations ($P > 0.05$) (Figure 3.5). Treatment solutions of TW and NW without PAA (T1 and T2) showed a higher growth in planktonic EB populations when compared to control samples for both purge levels across days ($P < 0.05$). T1 and T2 exhibited a similar significant decline in planktonic EB populations from day 0 to day 3, with T1

(5% purge) at $5.8 \pm 0.54 \text{ Log}_{10} \text{ CFU/mL}$ and T2 (5% purge) at 5.7 ± 0.54 on day 0, both decreasing by approximately 2 logs on day 3 before remaining statistically unchanged through day 10 ($P > 0.05$). A similar trend was observed for the EB population in T1 and T2 solutions containing 10% purge. In 200 ppm PAA solutions (TW and NW), purge concentration significantly influenced the growth of planktonic EB on day 0, with T3 and T4 samples in 10% purge showing a 4.1 and $4.3 \pm 0.54 \text{ Log}_{10} \text{ CFU/mL}$, respectively. On the other hand, EB populations in 5% purge levels were below the detectable limit ($P < 0.0001$). After 3 days, planktonic EB populations in 200 ppm PAA solutions (TW and NW) dropped below detectable levels in both 5% and 10% purge. However, only NW-based solutions with 200 ppm PAA were able to maintain a controlled planktonic EB population through day 10, while TW-based solutions with 200 ppm PAA showed a significant increase of $1.35 \pm 0.5403 \text{ Log}_{10} \text{ CFU/mL}$ on day 10 ($P = 0.0191$). Therefore, 200 ppm PAA was more effective in NW for maintaining the stability of EB counts over 10 days, while in TW, PAA effectiveness lasted until 5 days before EB counts began to increase again. In 400 ppm PAA solutions, EB growth was below detectable levels from day 0 to day 10 in both NW and TW, as well as at both 5% and 10% purge.

3.4. Discussion

Biofilm formation on equipment and surfaces in food processing environments is considered a challenge which comes with the difficult task of eradication and control. Biofilm eradication greatly depends on the maturity of the biofilm developed, since the more mature the biofilm, the harder it is to completely remove it from surfaces as the EPS becomes thicker, enclosing embedded sessile bacteria that are harder to reach and kill by antimicrobials and sanitizers (Alvarez-Ordóñez et al., 2019; Wang et al., 2018). However, preventing biofilm formation on surfaces through the continuous supply of anti-biofilms and antimicrobials that can inhibit bacterial cells from attaching

to surfaces to initiate the biofilm formation process could be presumed to be an easier task. The initial attachment of planktonic cells in the first stage of biofilm development is a reversible step. According to several studies, bacterial cells can attach as fast as within 2 hours of contact with a surface and mature biofilm formation takes between 3-5 days depending on the bacterial species encountered as well as other factors such as temperature and surface characteristics (Ivers et al., 2024; Koti et al., 2024; Song et al., 2015; Wang et al., 2018).

In our study, the natural mixed-species microbial population found in the added meat purge showed an initial bacterial attachment on coupons submerged in control and treatment solutions without peracetic acid (PAA) for both APC and EB counts after 2 hours of undisturbed storage at 4°C. In fact, in Wang et al.'s (2018) study, meat-borne *Pseudomonas fluorescens*, a common psychotropic microorganism associated with meat spoilage, showed more than 4.5 logs attachment to the stainless-steel surfaces in just 5 hours of contact at 20°C from an initial 6 log inoculation. This indicates that more than 75% of *P. fluorescens* cells were capable of attaching to hard surfaces in such a short period of time. This correlates with our study where the beef purge contained an initial APC population of between 6.2 and 6.5 log CFU/mL in control samples with 5 and 10% meat purge added, and an initial average APC biofilm attachment of 4.4 logs was observed for control samples after 2 hours of storage at 4°C. Several psychrotrophic microorganisms are common in vacuum-packed beef such as *Pseudomonas*, *Commamonas*, and *Lactobacillus*, including *Carnobacterium* and *Raoultella* which can be present in the added beef purge (Nan et al., 2024). With respect to EB populations, control samples showed lower counts when compared to inoculated samples, by an approximate 2-log difference on day 0, which can be explained by the presence of the STEC surrogates which likely survived the cold solution temperature and contributed to the higher EB counts. Early development of biofilms on coupons can also be related

to the fact that bacterial cells do not need to be viable for adhesion to material surfaces to occur, meaning that even non-viable and/or non-culturable bacterial cells can still stick to the surfaces due to physicochemical interactions with components like extracellular and capsular polysaccharides and proteins. These attached components can help to attract and embed live bacterial cells into the biofilm on the fouled surfaces (Flemming, 2011). This phenomenon may explain the high biofilm counts encountered on the coupons early upon coupons being submerged in some of the treatment solutions with PAA on day 0, which then decrease and become more controlled with time.

STEC pathogens cannot grow at low temperatures since they are mesophilic in nature and in fact enter a metabolic slowdown or dormancy at such low temperatures ($\leq 6^{\circ}\text{C}$), regardless of pH (Walker et al., 2024). Walker et al. (2025) studied 48 strains of STEC (including serogroups of high significance in human health) inoculated into tryptic soy broth, with incubations at 5-11°C. The minimum growth varied among the strains evaluated, but no strain grew at $\leq 7.4^{\circ}\text{C}$ over a 60-day incubation period. These researchers noted two important considerations in the interpretation and use of their modeling data; 1) the evaluation of the growth of the strains occurred in an optimal nutrient broth environment with no external stresses like low pH (thus, in this context the minimum temperature for growth would be considered a conservative estimate), and 2) the cultures evaluated had not undergone any cold adaptation prior to being used in the temperature-controlled studies (thus, cold-shock proteins and other factors impacting membrane fluidity in cold conditions would not have been present, leading to perhaps a misrepresentation of the minimum growth temperature). Observations by Jones et al. (2004) support discussions provided by Walker et al. (2024) and Walker et al. (2025) but introduce further complexity when trying to prove and explain growth of *E. coli* cultures in chilled conditions. In experiments to deduce the effect of small

temperature shifts on growth rates of log-phase *E. coli* cultures during chilled storage, Jones et al. (2004) found that cultures incubated constantly at 2, 4 or 6°C, when allowed to briefly increase to 10°C for 35 minutes at different intervals (simulating temperature fluctuations that are common in retail storage of many foods), certain fluctuation frequencies led to more or less loss of viability rates. Interestingly, *E. coli* cells held at 2 and 4°C frequently appeared in an elongated form (filamentous) but were able to divide at 4°C when brief temperature increases occurred but could not when a constant 4°C temperature was held. Further, these researchers demonstrated that use of plating on laboratory media to enumerate the bacterial cell density provided much less recovery numbers compared to monitoring optical absorbance values. Jones et al. (2004) cautioned that understanding the microbiological safety risks of chilled foods that encounter slight temperature fluctuations during storage needs to be more thoroughly understood. Even though these studies were media-based and looked only at bacterial growth in a planktonic scenario, these observations concerning the physiological aspects of *E. coli* growth under chilled conditions warrant consideration as to the behavior of EB and APC species contained within our meat purge on rate of attachment and initiation of biofilms on our coupons, the sensitivity and accuracy of our Petrifilm-based enumeration protocol for quantifying planktonic and biofilm populations, and whether different biofilm development dynamics might have occurred if small temperature fluctuations throughout the storage period were allowed (as opposed to the constant 4°C storage temperature utilized). Relative to the discussion by Walker et al. (2025), results in our static biofilm study differ in that a predominance of the mixed-species bacterial populations derived from the chilled, vacuum-packaged beef and ultimately its purge, which was used to provide the organic substrate in our sample vessels, would be comprised of psychrotrophic spoilage flora that were in a cold-adapted physiological state as used to spike our treatment solutions.

Even though the STEC surrogates cannot grow at 4°C, their presence might have been viewed as a stress factor with respect to the native EB population and thus triggered the EB to respond to stress by increasing their growth or metabolic activity due to competition. Another explanation could be that the presence of more bacteria in an environment enhances quorum sensing where signaling molecules can more easily reach bacterial cells within the community, thus facilitating communication and ultimately resulting in a better biofilm development (Bremer et al., 2015; Flemming et al., 2016). The natural EB counts found in the purge that were able to multiply and grow at 4°C, although taxonomically uncharacterized in this study, could be assumed to belong to a certain group of meat spoilage bacteria that can grow at refrigerated temperatures such as from the genera of *Buttiauxella*, *Hafnia*, *Rahnella*, *Serratia* and *Yersinia* (Doulgeraki et al., 2011, 2012; Farmer et al., 2010; Säde et al., 2013). In fact, *Hafnia alvei* is a common meat spoilage coliform able to grow at temperatures as low as 2.6°C and was shown to produce Acyl Homoserine Lactones (AHLs) which are a type of quorum sensing molecule that helps in biofilm formation (Bruhn et al., 2004; Ramos-Vivas et al., 2022). Apart from performing a whole genome sequencing, a simple test for verification of the presence of *Hafnia alvei* could be a cellulose test since this bacterium is associated with the synthesis of cellulose in biofilms (Ramos-Vivas et al., 2022).

In general, efforts to prevent or control biofilm formation have predominantly focused on reducing and preventing initial bacterial attachment by altering the attachment surface, one of the key factors influencing biofilm formation (Narayana P. & Srihari P., 2020). Researchers have focused on surface modification approaches involving physical or chemical based processes such as surface coating with specialized polymers or surface treatment with cold plasma to alter surface chemistry and topography (Awad et al., 2018; Hage et al., 2022; Narayana P. & Srihari P., 2020).

Despite the prominence of these conventional preventive approaches, relatively few studies have studied the potential of nanobubble water (NW) in preventing bacterial cells from attaching to hard surfaces and forming biofilms at early stages. Actually, Le et al. (2024) were one of the few to study the effect of microbubbles in preventing static biofilm formation of *Staphylococcus aureus* on glass surfaces. However, the application of microbubbles was combined with an alteration by surface coating the glass to modify its characteristics, making their approach difficult to compare to our study. Some of the factors that differ from our study include the type of hard surface material, the use of ultrasound-generated microbubbles, the absence of antimicrobials, and the incorporation of surface modification which all have different effects on the overall study results. On the other hand, Zhang et al. (2019) studied the bacterial regrowth of *Pseudomonas aeruginosa* and *Bacillus* sp. in both planktonic and biofilm forms following treatment with peracetic acid (PAA) or free chlorine. Their study showed the inhibition effect of PAA against biofilm and planktonic regrowth in the presence of organic matter (they used growth media containing organic particles). Zhang et al. (2019) proved that PAA reacts with organic matter more slowly than free chlorine and undergoes slower self-decomposition in the presence of organic matter. As a result, PAA proved to be effective in preventing regrowth of bacterial cells in the presence of organic particles, which correlates with the results of our study where PAA was found to be effective in controlling biofilm and planktonic growth in the presence of heavy organic matter (up to 10% beef purge).

While only a few studies have explored the preventive approach of NBs in controlling biofilm formation, most existing research discovered the antimicrobial and antibiofilm effects of NBs alone and in combination with other antimicrobial technologies on eradicating or dispersing mature biofilms once they have developed (Sekhon et al., 2022; Shiroodi et al., 2021; Tiwari et

al., 2025; Unger et al., 2023). Our findings did not support the common discovery among studies showing an improved antimicrobial effect when using NW (Singh et al., 2021; Yi et al., 2022), but in fact PAA showed a similar inhibition effect when combined with both TW and NW. This can be explained by the fact that PAA solutions, whether TW- or NW-based, were added daily (at 20% v/v) as a replenishment which allowed a continuous supply of fresh antimicrobial. Moreover, PAA is known for its strong oxidative effect, oxidizing components such as sulfur bonds of proteins, enzymes and metabolites as well as disrupting cell wall permeability (Rossi et al., 2023). As a result, any potential differences between TW- and NW-based solutions were likely overshadowed by the daily replenishment with a strong antimicrobial and oxidizer, preventing a clear distinction in their effect. We can hypothesize that a more significant distinction of NW effect on PAA could have been observed if the biofilms were allowed to develop in such experimental conditions, followed by the antimicrobial treatment.

Few studies have focused on the effect of organic acids and antimicrobials, including PAA, on the background or natural microbiota, which is in our case the natural microorganisms found in the purge and detected through APC, while most studies report the effect of PAA using artificial inoculation with specific bacterial strains. The distinction between the use of NW versus TW became slightly more evident with the increase in organic content in the solutions, moving from 5% to 10% (v/v) beef purge between treatment solutions. The effect of PAA at 200 ppm concentration greatly depended on the organic load where both solution bases (TW and NW) showed higher biofilm and planktonic counts in treatment solutions with 10% purge. Even though bacterial counts were still significantly lower than treatment solutions without any antimicrobial, it is worth noting that 200 ppm of PAA was not completely effective in controlling microbial growth in a “dirty” system with 10% purge compared to a system with less organic matter

accumulation. The effectiveness of PAA significantly depends on the level of organic matter present which can impede its ability to penetrate and eradicate biofilms. A higher purge concentration is associated with a greater abundance of natural microflora and more organic matter that can serve as nutrient for bacterial cells, which certainly shows effects on biofilm formation. For example, *E. coli* O157:H7 biofilm formation was higher on stainless steel and HDPE coupons in the presence of ground beef, which represented the natural organic and inorganic substrates found in beef processing facilities, compared to growth in the presence of sterilized tryptic soy broth (Dourou et al., 2011). Moreover, the higher build-up of organic layers caused by the presence of meat exudate on stainless steel surfaces enhanced the attachment of cells to surfaces and strengthened biofilm formation which explains the difficulty in eradication and control by PAA at such a level (Dutra et al., 2018). The 200 ppm PAA concentration in NW appears to be a threshold, as PAA at 400 ppm proved much more effective for control. Notably, only the treatment solutions containing TW exhibited higher microbial counts in 10% beef purge solutions compared to 5%. However, in NW, the 400 ppm PAA was not affected by the higher organic load indicating that even if the system gets dirtier with higher organic matter build-up, the 400 ppm PAA in NW is able to maintain low static biofilm counts throughout 10 days of processing at 4°C. This finding can be explained by the unique properties of nanobubbles, which boost antimicrobial effectiveness in environments with high organic content. In particular, nanobubbles promote the production of ROS (such as hydroxyl ions)—a mechanism not found in conventional tap water solutions—thereby enhancing overall antimicrobial activity of the NW-based solutions. The generation of hydroxyl ions contributes to the degradation of organic matter, while the small size of nanobubbles facilitates enhanced penetration through both organic matter and the EPS of biofilms (Chaurasia, 2023; Ovissipour, 2021). The presence of OH⁻ ions play an important role since they are highly

reactive with organic and inorganic molecules present in the EPS of biofilms, as well as in the solution from organic matter accumulation such as DNA, carbohydrates, lipids, and proteins (Tvrdá & Benko, 2020). In Luo et al.'s (2024, 2025) two studies, they demonstrated the effect of air-based micro-nano bubbles (MNBs) application on water treatment to control microbial growth and obtain biological stability in water. An increased degradation of organic matter, effective bacterial reduction by 56.45%, as well as an inhibition of microbial growth such as *E. coli* in water treated with MNBs were observed and associated with the production of high levels of OH^- by the bubbles, which is related to bubble size and zeta potential (Luo et al., 2024, 2025). Therefore, it is likely that NW in our treatment solutions reduced organic matter accumulation in the specimen solutions, resulting in similar biofilm and planktonic cell growth outcomes at both 5% and 10%.

Although we did not perform a statistical comparison between planktonic bacterial levels and biofilm formation, a descriptive evaluation indicated no apparent relation between a decrease in planktonic cells and an increased biofilm development as one would assume when planktonic cells join a biofilm. This observation is consistent with findings from other studies. Actually, Dutra et al. (2018) demonstrated a positive correlation between planktonic cell density and biofilm accumulation in an environment which can be explained by the fact that biofilms promote precipitation of minerals (Flemming, 2011). An increased biofilm development leads to more EPS formation which includes polysaccharides and nutrients which can precipitate in the environment thus providing nutrients for viable planktonic cells to multiply and possible sublethally injured bacterial cells to slowly grow back, which explains the observed increased planktonic growth in treatment solutions without PAA showing biofilm development.

Our findings also show that rubber demonstrated a slightly higher biofilm development in control samples when compared to stainless steel. Stainless steel (S304) and Buna-N rubber being

very common hard surfaces in food processing environments are abundantly studied for biofilm formation. As compared to S, R has a rougher topography which provides bacterial attachment with greater protection against shear forces, facilitating the transition from reversible to irreversible adhesion and promoting biofilm formation by enhancing bacterial retention and an increased organic matter accumulation (Teughels et al., 2006). Rubber wears out faster than stainless steel, developing scratches and crevices that increase surface roughness (Hua & Zhu, 2024). These imperfections provide ideal sites for bacterial attachment, promoting biofilm formation and making sanitation more challenging.

3.5. Conclusion

Statically generated biofilms were employed in this study to replicate hard-to-reach contamination sites in meat processing facilities, where most often dynamic fluids do not reach to dislodge accumulated microbial cells. Overall, this study demonstrated that while PAA effectively suppressed biofilm development at both 200 and 400 ppm concentrations, the presence of organic matter had a substantial impact on outcomes, especially at higher levels (10%). NW at 400 ppm of PAA out-performed TW under elevated organic loads, suggesting that NW may offer enhanced disinfection and/or sanitation benefits in environments prone to higher organic accumulation. Moreover, PAA at 200 ppm provided effective control for up to five days at 4°C but allowed significant microbial rebound, thereafter, highlighting the need for reapplication strategies over extended processing times. In meat processing environments, managing organic matter buildup can be key in minimizing planktonic bacterial growth as well as biofilm formation on hard surfaces. Going forward (and addressed in Chapter 4), the incorporation of shear force to represent dynamic growth conditions can be an essential added factor to better reflect real-world dynamic flow conditions and evaluate how this factor can influence biofilm formation and control.

Additionally, systematically exploring different combinations of nanobubble water, disinfectants and surface materials under varied organic loads and at different durations and temperatures can allow better understanding of the influence of factors and may further optimize microbial control to enhance meat safety.

3.6. Figures

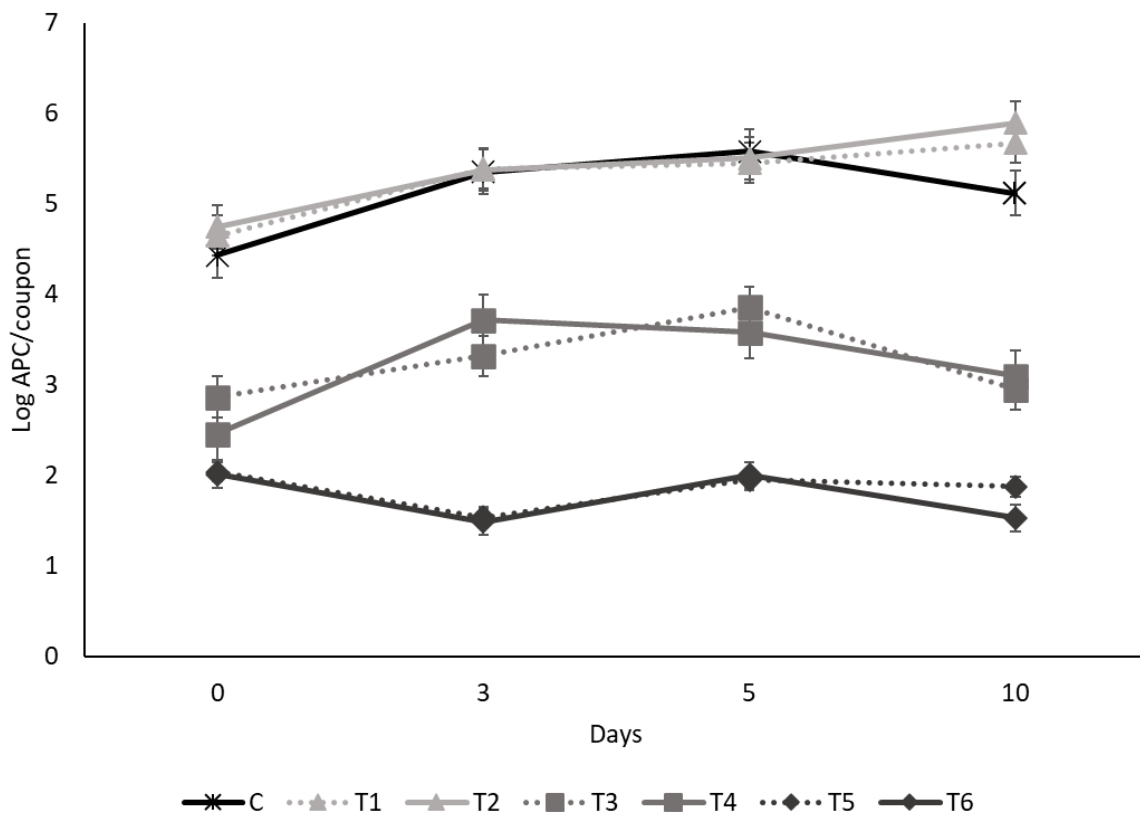


Figure 3.1 APC biofilm growth on coupons immersed in the six experimental treatments (T1-T6) and the control sample (C) represented by log CFU/coupon across the 10 experimental days at 4°C. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA). The limit of detection in Petrifilm assays was 1.4 Log CFU/coupon.

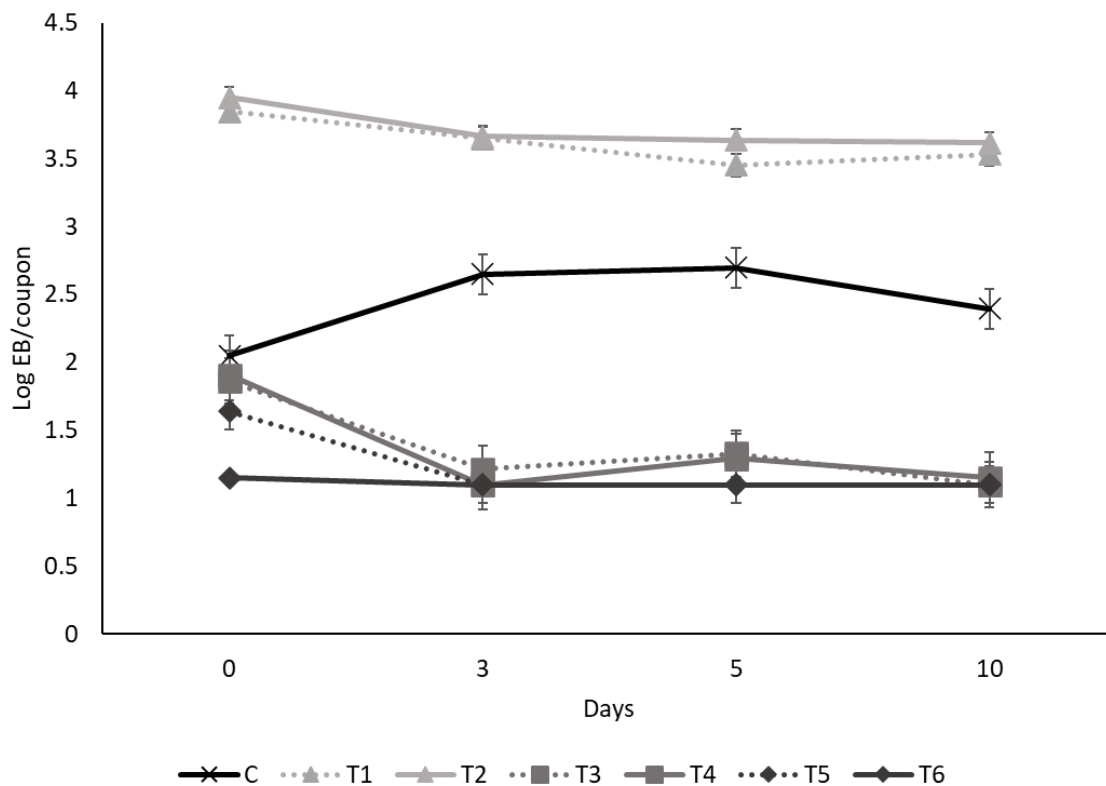


Figure 3.2 EB biofilm growth on coupons immersed in the six experimental treatments (T1-T6) and the control sample (C) represented by log CFU/Coupon across the 10 experimental days at 4°C. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA). The limit of detection in Petrifilm assays was 1.4 Log CFU/coupon.

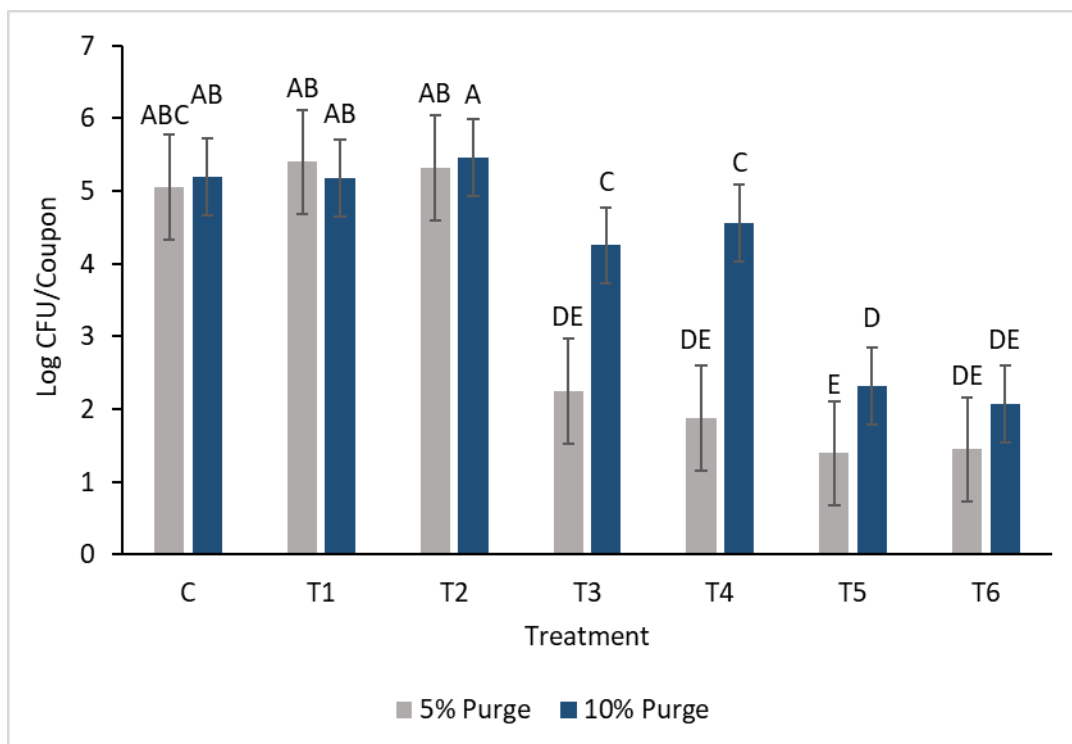


Figure 3.3 APC growth on coupons (log CFU/coupon) in treatment solutions comparing 5 and 10% purge concentrations. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA). Bars (LSM $\pm$ SE) with different letters are significantly different ($P < 0.05$) for APC growth over 10 days at 4°C. The limit of detection in Petrifilm assays was 1.4 Log CFU/coupon.

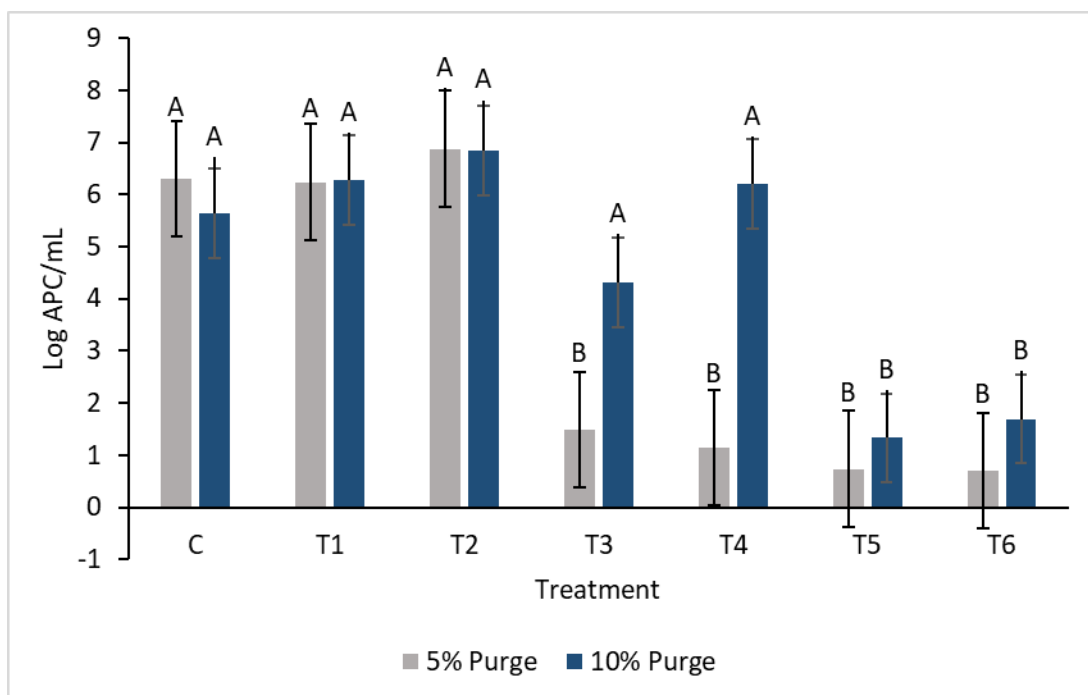


Figure 3.4 Planktonic APC growth (Log CFU/mL) in the different treatment solutions with PAA (0, 200 and 400 ppm) in varying levels of purge (5% vs 10%). (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA). Bars (LSM $\pm$ SE) with different letters are significantly different ($P < 0.05$) for APC growth over 10 days at 4°C. The limit of detection in Petrifilm assays was 1.0 Log CFU/coupon.

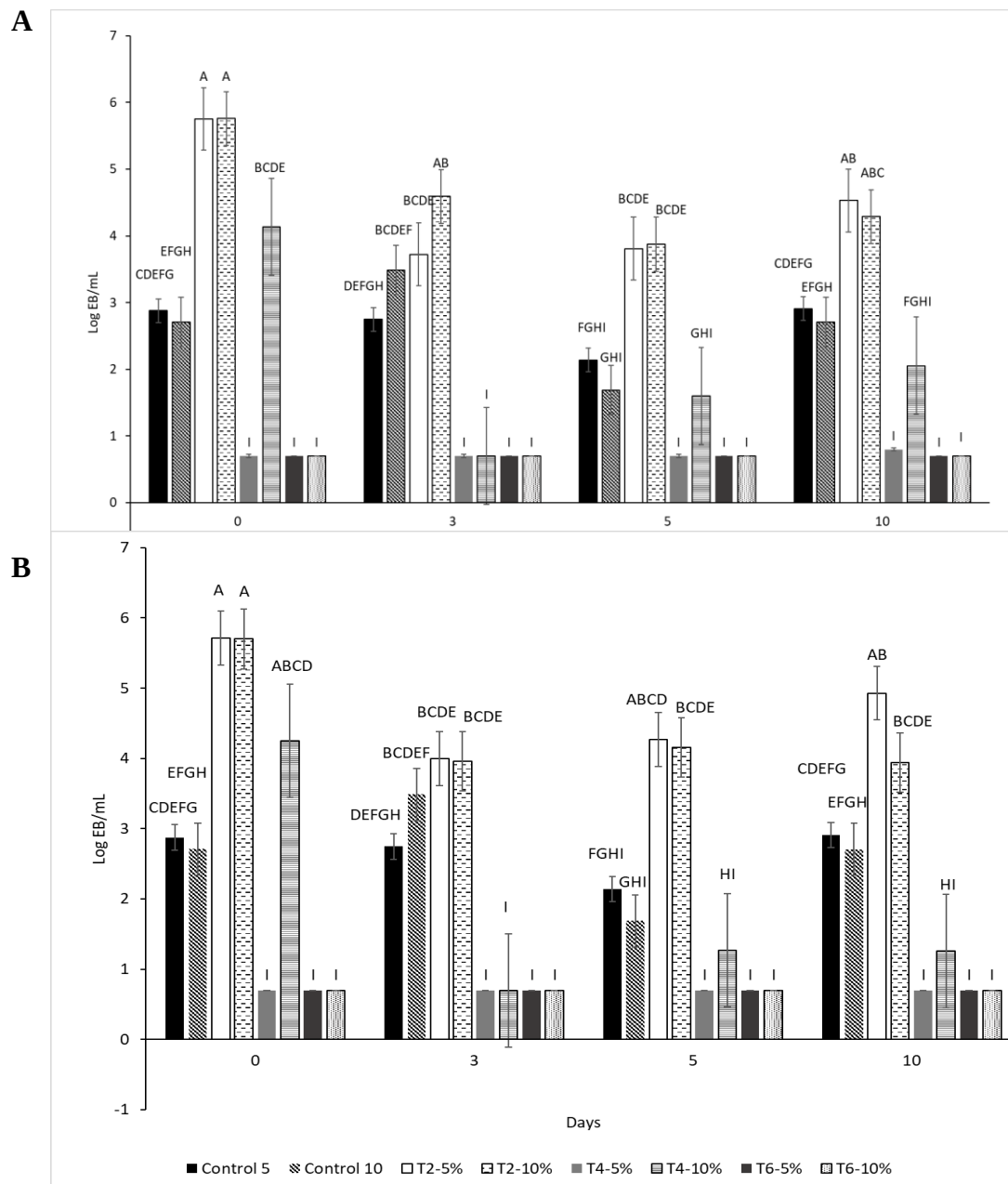


Figure 3.5 Planktonic EB growth (Log CFU/mL) in the different tap water-based (A) and nanobubble water-based (B) treatment solutions and with peracetic acid (0, 200 and 400 ppm) in varying levels of purge (5% vs 10%) across days. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA)

& C: non-inoculated control with TW-0 ppm PAA). Bars (LSM $\pm$ SE) with different letters are significantly different ($P < 0.05$) for APC growth over 10 days at 4°C. The limit of detection in Petrifilm assays was 1.0 Log CFU/coupon.

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Chapter 4 - Evaluation of the Antimicrobial Efficacy of Nanobubble Water with Peracetic Acid for Mixed-Species Dynamic Biofilm Control in Ground Beef Processing

Abstract

Biofilms pose challenges in food processing due to resilience against disinfectants and capacity to harbor pathogens. The aim of this study was to evaluate the antimicrobial effects of air-based nanobubble water (NW) or tap water (TW) containing peracetic acid (PAA) on mixed-species biofilm development on stainless steel (SS) and Buna-N rubber (R) coupons in the presence of organic loading in a dynamic system. CDC biofilm reactors filled with NW or TW infused with 0, 200 and 400 ppm PAA plus 10% (v/v) non-sterilized beef purge were inoculated with USDA-approved *E. coli* surrogates. Planktonic (unattached) bacterial counts in reactor fluid and biofilm development on coupons were assessed over 5 days at 4°C. Reactor fluid (20%) was replenished daily with fresh NW or TW containing new purge. APC and EB populations were quantified on days 0, 3 and 5 by neutralizing, sonicating, vortexing, and plating (3M Petrifilm), along with monitoring reactor pH. A mixed-model ANOVA was used to identify significant treatment effects with appropriate pair-wise comparisons performed using Fisher's protected LSD ($n=378$, 3 replications, $\alpha=0.05$). Biofilms increased ($P < 0.05$) to >5 log CFU/coupon by day 5 at 0 ppm PAA; NW alone (0 ppm PAA) reduced this growth by 0.7 log ($P = 0.02$) compared to TW alone. When treatment waters contained PAA (200 and 400 ppm), day 5 APC and EB biofilm development was notably reduced versus controls ($P < 0.0001$). At 400 ppm PAA, all coupon counts were undetectable (LOD=1.1 log CFU/coupon) on all sampling days. By days 3 and 5, APC biofilm levels were 1.4 and 1.2 log cycles higher, applying 200 ppm versus 400 ppm PAA,

respectively ($P < 0.05$). R coupons supported greater biofilm development than S coupons ($P < 0.0001$). Over the 5-day period, pH of bioreactor solutions increased from 5.8 to 6.7 at 0 ppm PAA, while pH for 200 ppm and 400 ppm PAA infused treatments remained at 4.6 and 4.4, respectively. PAA at ≥ 200 ppm in meat processing systems with moderately heavy organic loading can maintain biofilm control over extended processing periods where dynamic development conditions predominate.

4.1. Introduction

In food processing environments, bacterial contamination is ubiquitous and cross-contamination can easily occur. The presence of food residues, nutrients and other processing ingredients provides a conducive environment for bacterial growth on both food contact and non-food contact surfaces. Certain bacterial species find ways to survive and adapt to changing environmental conditions such as extreme temperatures, acidity, or low water activity, persisting in processing environments and causing food product contamination, especially in the absence of effective sanitation and antimicrobial measures. Bacteria can form biofilms on surface of materials where planktonic cells change from their free-living form to develop an attached microbial community of embedded bacterial cells and secrete an extracellular polymeric substance (EPS) which forms a protective layer (Elafify et al., 2024; Rumbaugh & Sauer, 2020). Biofilms form on all kinds of surfaces in food processing plants including rubber, wood, stainless steel, glass, polyethylene and others (Carrascosa et al., 2021).

In the meat industry, the presence of organic residues promotes and facilitates biofilm formation on machines and equipment in the processing environment, posing a risk to the microbiological safety and quality of final products. Several meat-associated bacteria have the ability to grow and persist in the form of biofilms including spoilage microorganisms, such as *Pseudomonas*, *Commamonas*, and *Lactobacillus* and pathogenic microorganisms such as *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*. A study performed to assess the effect of food residues, particularly meat exudate, showed enhanced biofilm formation by *Escherichia coli* and *Staphylococcus aureus* on stainless steel surfaces, emphasizing the important influence of organic matter build-up in simulated industrial settings on biofilm development (Dutra et al., 2018). Also, the growth of *E. coli* O157:H7 biofilms on stainless steel and high density polyethylene (HDPE)

coupons was strongly enhanced by the presence of natural organic and inorganic substrates found in beef processing facilities (i.e. ground beef residues) compared to a slower biofilm growth in the absence of organic matter (Dourou et al., 2011).

The food industry continues to face challenges in controlling and eradicating biofilms, particularly once they reach maturity and persist in the environment. Both spoilage and pathogenic bacteria belonging to a biofilm gain enhanced resistance which contributed to an increased risk of microbial contamination in food processing facilities (Yuan et al., 2022). Due to their increased resistance to conventional cleaning and disinfection methods, the complete removal of biofilms is more complicated. Several biofilm-associated foodborne outbreaks have had significant consequences on public health and have resulted in increased costs to the food industry due to equipment damage and product recalls (Alvarez-Ordóñez et al., 2019; Yuan et al., 2022). While preventing biofilm formation can be generally considered more feasible than eliminating stronger mature biofilms, the process remains complex and is not always guaranteed. The presence of several bacterial species in one biofilm is more common than single-species biofilms in nature; these mixed species biofilms reveal higher resistance to disinfectants, making the eradication process even more challenging (Elafify et al., 2024). In fact, multi-species biofilms allow synergistic relationships among bacteria which can promote a better communication system that allows further protection for the embedded bacterial community. Due to the natural ecology of a biofilm, mixed-species biofilms are the most common form found in nature and in the food industry as one main source of contamination (Flemming et al., 2016; Yuan et al., 2022). In addition, the components of the EPS help in forming the biofilm's rigid architecture through a continuous dynamic process where clustered bacterial cells form microcolonies and secreted EPS molecules fill the empty spaces between those cells (Flemming et al., 2016). Studying the dynamic

nature of biofilm formation has intrigued several scientists since the presence of shear force and rotation has been proven to help in forming more rigid biofilm structures (Wang et al., 2022). Wang et al. (2018) demonstrated that *P. fluorescens*, a common meat spoilage microorganism, showed biofilm formation under dynamic conditions resulted in a mature biofilm with a thicker layer of EPS that exhibited more resistance against common disinfectants such as sodium hypochlorite and chlorine dioxide. Moreover, Huijiboom et al. (2024) demonstrated that *L. plantarum* biofilms grown in dynamic conditions exhibited greater resistance to disinfectants such as peracetic acid. This highlights the need to study biofilms and their resistance to antimicrobials in dynamic environments, as they may more accurately represent the more resilient and resistant biofilms encountered in the food industry.

Food processing facilities must continue to implement rigorous cleaning and sanitation plans to effectively control and eliminate the risk of biofilm development. With the challenge of equipment designs that sometimes include hollow rollers, tubing, joints, welds and scrapers, proper cleaning becomes more challenging. Conventional cleaning methods and antimicrobial treatments in the food industry follow a standardized cleaning process which aims to prevent the initial microbial attachment that leads to biofilm formation (Cramer, 2013). At this early stage, bacterial attachment is weak and more easily removable from the thin film they might have formed. Six main steps are part of the standard cleaning process: (1) dry-cleaning to remove debris, (2) a hot water rinse, (3) detergent application including the use of oxidative agents and acids (such as peracetic acid), (4) a final warm water rinse, (5) sanitizer application, and (6) a proper final inspection for protocol verification purposes (Cramer, 2013, pp. 105–106). However, once bacteria establish thicker biofilm layers, the removal process becomes significantly harder using conventional cleaning and sanitation methods. Research has advanced the field of biofilm removal

where the use of advanced physical techniques such as ultrasound, ultraviolet irradiation, magnetic fields, and cold-plasma treatments have been studied and used in food processing plants for enhanced membrane penetration and biofilm disruption (Lu et al., 2022). Other chemical treatments include the application of quaternary ammonia, antimicrobials, and compounds such as sodium hypochlorite, sodium hydroxide, hydrogen peroxide, and peracetic acid (Galié et al., 2018; Lu et al., 2022). Nowadays, other innovative approaches are gaining attention, including natural compounds, essential oils, bacteriophages, bacteriocins, and quorum sensing inhibitors, which offer promising biofilm control strategies with potentially lower environmental impact (Galié et al., 2018).

Another recent technology that has been proven by several studies to have an impact on biofilm eradication is the application of nanobubbles (NBs). NBs are tiny gas bubbles with diameters below 500 nm that have unique characteristics in solution such as long stability, high zeta potential, and high gas dissolution rate (Foudas et al., 2023; Phan et al., 2020). NBs have been used in several applications such as cleaning, surface coating, water reservoir aeration, carbon foot-print reduction, as well as in biofouling and flotation processes (Chaurasia, 2023; E. Singh et al., 2024; Wu et al., 2008; Xiao et al., 2020; Zhu et al., 2016). Their antimicrobial properties correlate with their ability to produce highly reactive oxygen radicals (such as hydroxyl ions) known as reactive oxygen species (ROS) which have improved advanced oxidation processes, such as water treatment and biofouling control (Phan et al., 2020). Due to the potential NBs demonstrate against biofouling and membrane defouling, researchers began looking at the antibiofilm properties of NBs. Pure oxygen NBs produced by the Moleaer NB generator in deionized water were effective against *E. coli* biofilm formation on stainless steel at room temperature ($22 \pm 2^\circ\text{C}$), achieving 3.4, 3.5, and 4.6 log CFU/cm² reductions after 2, 5, and 10 min

treatments, respectively (Shiroodi et al., 2021). Moreover, NBs have been found to enhance the efficacy of antimicrobials that are commonly used alone to reduce microbial loads or tackle biofilm formation (Burfoot et al., 2017; Shiroodi et al., 2021; Ushida et al., 2017). The ROS produced by NBs enhance the antimicrobial and antibiofilm properties of peracetic acid, with greater log reductions observed in fresh and aged *L. monocytogenes* biofilms on polypropylene and stainless-steel surfaces compared with antimicrobial solutions that did not contain NBs (Unger et al., 2023).

Commonly used sanitizers include chlorine-based compounds, quaternary ammonium compounds (QACs), iodophors, ozone, chlorine dioxide, and peracetic acid, where each is used with varying degrees of effectiveness against foodborne pathogens (Cramer, 2013, p. 159). Among these, peracetic acid, also known as peroxyacetic acid (PAA), is commonly used due to its wide spectrum of effectiveness against gram-negative and gram-positive bacteria, fast-acting capability, rapid breakdown, and lack of harmful residues (Environmental Protection Agency, 2012). PAA has been validated against bacterial contamination on both food-contact surfaces and raw food products and has demonstrated its ability to reduce pathogens like *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* (Beltrame et al., 2012; Kalchayanand et al., 2016, 2024; Pozuelo et al., 2021; Rossi et al., 2023). The use of PAA as an antimicrobial in the meat and poultry industry is replacing some applications that historically used chlorine-based sanitizers in sanitation programs. Further validation studies and research would be beneficial to characterize and quantify the effects of PAA applications specifically towards biofilms. Since both NBs and PAA show potential for advancing biofilm control and eradication, combining them could provide an innovative and effective approach. While taking into consideration the effect of mixed-species biofilms, natural microbial contamination, organic residues and dynamic flow environments of biofilm development in meat processing environments, particularly beef, this study mimics natural

biofilm development on hard surfaces in beef processing environments over an extended processing duration. The purpose of this study was to evaluate the efficacy of combining nanobubble water (NW) with PAA at varying concentrations (200 and 400 ppm) in controlling mixed-species biofilm formation, in combination with a cocktail of five USDA-approved *E. coli* surrogates, on rubber and stainless-steel surfaces in the presence of beef purge (organic matter) at beef processing temperature (4°C) over a period of 5 days, with a daily replenishment of treatment solutions and purge to simulate continuous organic load accumulation.

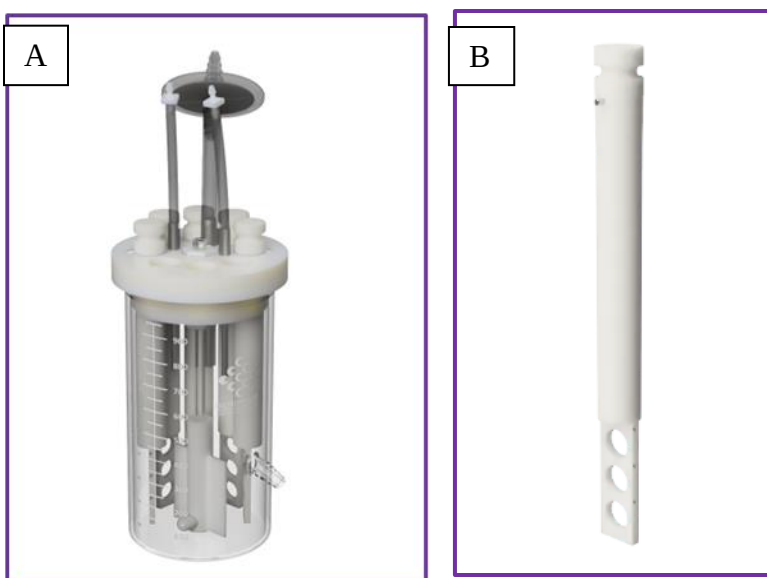
4.2. Materials and Methods

Experimental system overview

Six experimental solutions were analyzed in this study performed in three separate replications. A Centers for Disease and Control (CDC) biofilm reactor (BioSurface Technologies, Bozeman, Montana) (Photograph 1A) was set up and maintained at 4°C on a stir plate running at 90 rpm for 5 days, creating a dynamic environment for biofilm formation. The reactor was run as a batch phase rather than a continuous phase where a volume of 315 mL of chilled nanobubble water (NW) or tap water (TW) combined with peracetic acid (PAA) at three different concentrations (0, 200 and 400 ppm) filled the bioreactor vessel for each treatment solution at one time with no continuous media supply. Beef purge (35 mL collected from wholesale subprimal bags purchased from a national supplier) was added to all treatment solutions to result in a 10% (v/v) concentration, bringing the total treatment solution volume in the bioreactor vessel to 350 mL. The purge, containing natural (undefined) microbial populations affiliated with raw vacuum-packaged beef, contributed to the organic build-up as well as the formation of mixed-culture biofilms in the reactor system. Therefore, the six different combinations of treatment solutions

were as follows: NW-0 ppm PAA, TW-0 ppm PAA, NW-200 ppm PAA, TW-200 ppm PAA, NW-400 ppm PAA, and TW-400 ppm PAA, referred to as T1 to T6 in the respective order.

All six treatment solutions were inoculated with a cocktail of five strains of non-pathogenic surrogate *Escherichia coli*. A seventh bioreactor, filled with TW and 0 ppm PAA was non-inoculated with the surrogates and served as the control, demonstrating biofilm growth only from the natural microbial population present in the beef purge, without any antimicrobial treatment or possible NW effect. Each bioreactor contained eight vertically positioned rods holding three coupons per rod (Photograph 1B), totaling 24 coupons per bioreactor. Equal quantities of coupons of each surface material used [stainless steel (RD128-304) and Buna-N rubber (RD128-BUNA)] were placed in a single bioreactor for analysis. Three coupons (triplicates) of each type were analyzed on each of the sampling days 0, 3, and 5. Day 0 samples were analyzed after 2 hours of chilled storage to promote possible early bacterial attachment. On a daily basis, bioreactors were replenished with new solutions by removing 20% (v/v) of each initial solution and restoring the initial volume by adding the appropriate combination of fresh solutions and purge. The intent of this replenishment step was to mimic the manufacturing process of continuous antimicrobial solution supply defined in a proprietary, novel ground beef manufacturing system affiliated with this research that operates continuously over a 5-day manufacturing period.



Photograph 1: (A) Standard CDC Biofilm Reactor®; (B) Polypropylene coupon holder (holds 3 coupons each), 8 included with a CDC Biofilm Reactor. Source: BioSurface Technologies Corp.

Culture preparation

Stock cultures stored at -80°C of five strains of rifampicin-resistant non-pathogenic surrogate *Escherichia coli* [strains represented USDA-approved surrogate cultures from the American Type Culture Collection (ATCC) designated BAA-1427 P1, BAA-1428 P3, BAA-1429 P8, BAA-1430 P14, and BAA-1431 P68, which were trained to be rifampicin resistant in the laboratory of Dr. John Luchansky at the USDA ARS Eastern Regional Research Center, Wyndmoor, PA] were selected in this study. Frozen beads of each culture were transferred individually to 10 mL of tryptic soy broth (TSB; BD Difco, Sparks, MD) and incubated at 35°C for 24 hours. Each strain was streaked for isolation on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD) and then one isolated colony was transferred into another 10 mL of TSB and incubated at 35°C for 24 hours. One day prior to the start of the experiment (day -1), 0.1 mL from each culture tube was transferred into another 10 mL TSB tube and incubated at 35°C for 24 hours. After the second incubation, a 25 mL cocktail was prepared from equal amounts of the five surrogate *E. coli*

strains, serving as the inoculation cocktail. The initial cocktail bacterial population was verified by serially diluting, plating on EB 3M Petrifilm (Saint Paul, MN, USA) and enumerating after 24 h of incubation at 35 °C. Overnight cultures reached an average of 8.7 log CFU/mL for the inoculum cocktail. Each bioreactor was inoculated with 0.5 mL of this cocktail on day 0 of the experiment (with the exception of the control bioreactor).

Bioreactor and coupon preparation

Both coupon types, Buna-N rubber (R; RD128-BUNA) and stainless steel (S; RD128-304), as well as the CDC biofilm reactors were purchased from BioSurface Technologies Corporation (Bozeman, MT, USA) and were prepared according to the standard method ASTM E3161-21 (ASTM, 2022) with some minor adjustments. Coupons were autoclaved at 121°C, 15 psi for 15 min then scrubbed with a pipe cleaner brush by hand using a non-residue laboratory detergent before sonicating in clean conical tubes filled with the detergent water for 5 minutes using a Branson 3800 sonicator (Branson Ultrasonics Corporation, Danbury CT). Coupons were then rinsed and sonicated for another 5 min in tap water before a final rinse and sonication in deionized water for 5 min. Finally, the coupons were removed and exposed to UV light for 15 min in a biosafety cabinet, after which they were ready for use. The bioreactors were also autoclaved, rigorously scrubbed with soapy water, then rinsed with tap water and left to air-dry before use. Both cleaning processes were done between each treatment across experimental replications for equipment reuse. Bioreactors were then assembled within the biosafety cabinet and coupons were aseptically inserted into the rods. The CDC bioreactor was used in a batch set-up (i.e., solutions and/or nutrients were not continuously circulated from an external reservoir), therefore aluminum foil was used to cover unused reactor openings, including the beaker outlet, the upper in-flow port and the syringe filter tip on the top tube. The fully assembled bioreactor was autoclaved at

121°C, 15 psi for 15 min, cooled at room temperature for 15 min, then filled with the appropriate 4°C pre-chilled test solution and purge amount. Bioreactors filled with treatment solutions were inoculated before being placed on the stirrer at 90 rpm set up in a walk-in cooler with monitored temperature at 4°C.

Beef purge

Beef purge (approximately 500 mL) was provided by Kansas State University's Meat Lab (Department of Animal Sciences and Industry, Weber Hall). The purge was collected from vacuum packaged beef subprimals and was intended to mimic the organic build-up in a normal beef processing facility. The beef purge was portioned into 50-mL conical tubes, stored at -20°C and thawed overnight at 4°C one day prior to its use. The Meat Lab procured the commercial subprimals from a national processor and opened the vacuum bags during normal cutting operations conducted by the laboratory to generate food service raw beef cuts under USDA FSIS inspection. The microbial population of each meat purge batch was analyzed upon collection and portioning by serially diluting 1 mL in 0.1% peptone water and then plating in duplicate on 3M Petrifilm (Saint Paul, MN, USA) for both total aerobic plate counts (APC) and Enterobacteriaceae (EB) counts. The average bacterial population found in the three different batches of meat purge used throughout this study was 7.38 log CFU/mL for APC and 4.38 log CFU/mL for EB.

Nanobubble water and tap water preparation and storage

A Moleaer XTB-25 nanobubble (NB) generator (Moleaer Inc., Hawthorne, CA) was used to generate NBs in solution based on the principle of cavitation. The generator unit was comprised of a centrifugal pump, an air pressure gauge, an air connection, an air flow meter, a pump pressure gauge, a discharge flow valve and a starter (Moleaer Inc., 2020). Input water into the unit was standard municipal tap water (City of Manhattan, KS). NB generated by the unit was collected

into a plastic 50-gal water holding reservoir. The air-based NBs were generated using food-grade compressed cylinders of air (Matheson, Basking Ridge, NJ) According to the Moleaer XTB 25-50 Operation Manual, the average size of the gas bubbles produced by the unit varies from 70 to 100 nm (Moleaer Inc., 2020), and this was confirmed during previous NB water studies (currently unpublished) in our laboratory utilizing a Malvern NanoSight LM10 nanoparticle analyzer (K-State Nanotechnology Innovation Center core laboratory under the direction of Tej Shrestha, Core Director; <https://nicks.ksu.edu/core-facilities/nicks-core/nanosight-microscope.html>). The system was first purged seven times at the beginning of each experimental repetition to adequately flush out any remaining debris or water from previous trials before generating new NBs. At first, TW samples were collected by running municipal water with an approximate unadjusted pH of 8.5 through the Moleaer unit with the cavitation turned off. TW samples were collected from the reservoir and stored in 500 mL sterilized capped glass bottles. The gas valve was then opened and the pressure regulator set to deliver gas at a stable 120 psi. The system was allowed to run in a recirculation mode for 1 hour to ensure solution saturation with NBs, at which time a valve was opened to divert the NW into the 50-gal collection reservoir. Glass bottles were filled as described for the TW solutions. All water solutions needed for one full experimental repetition were prepared one day prior to the start of the experiment and stored in sterile glass capped bottles at 4°C to be used within the experiment over the 5-day period.

Peracetic acid solutions and titration

PAA solutions were prepared on day 0 of the experiment to initially fill up the bioreactors as well as on every day of the experiment for daily bioreactor replenishment. The stored TW or NW bottles were used for the antimicrobial solutions which were prepared at two different concentrations: 200 ppm and 400 ppm $\pm$ 20 ppm by adding 570 microliters and 1.15 mL to 250

mL of water, respectively. Microtox 5[®] (The Vincit Group, Chattanooga, Tennessee), a popular source of PAA across the U.S. meat and poultry industry, was used as the source of PAA. PAA concentrations were confirmed by titration using a peracetic acid test kit (Chem World, Kennesaw, GA) and the pH of each solution was measured using a pH meter. Table 4.1 shows the average pH and titration of each prepared antimicrobial solution. Using the bulk solutions now containing target concentrations of PAA, 315 mL of each antimicrobial treatment solution were dispensed on day 0 of the experiment into the bioreactor (in addition to the 35 mL of beef purge). This solution plus purge mixture would be referred to as “red water” in lay meat processor language.

Table 4.1 Average Titration Results and Corresponding Average pH Values of the Antimicrobial Solutions Prepared Across the 5-Day Period.

TREATMENT SOLUTION	WATER TYPE	TARGET PAA CONCENTRATION (PPM)	AVERAGE TITRATION (PPM)	AVERAGE PH
T1	TW	0	0	8.25
T2	NW	0	0	8.24
T3	TW	200	201	4.11
T4	NW	200	197	4.02
T5	TW	400	395	3.83
T6	NW	400	393	3.86

(NW: Nanobubble Water, TW: Tap Water)

Microbial analysis: recovery and enumeration of bacterial cells

On each sampling day (days 0, 3, and 5), one rod containing 3 S coupons and one rod containing 3 R coupons were aseptically pulled out of a bioreactor to analyze for total aerobic plate counts (APC) and Enterobacteriaceae (EB) counts of cells firmly attached to coupon material. An

adjusted ASTM E2871-19 protocol was used to recover and enumerate biofilm cells. Coupons were carefully removed from the rods using sterile forceps and were each placed in D/E Neutralizing broth (BD Difco, Sparks, MD) for 1 minute of neutralization. Upon removal from the neutralizing broth, each coupon was allowed to drip for 10 s to remove excess liquid and loosely and/or non-attached cells from coupon before placing it into a 50-mL sterile conical tube containing 25 mL of Phosphate Buffered Saline solution (Phosphate Buffer Saline, VWR, Solon, OH). Conical tubes containing the coupons were vortexed for 30 s and then sonicated for 30 s using a Branson 3800 sonicator; this process was repeated three times. The conical tubes containing the coupons and 25 mL of buffer were considered the first dilution and were serially diluted in 9 mL tubes of 0.1% peptone water (BD Difco, Sparks, MD). Dilutions were then plated on APC and EB 3M Petrifilm (Saint Paul, MN, USA). In addition to coupon analysis for attached bacterial cells, the bioreactor's solution was also analyzed at each sampling to establish free-living microbial populations in each solution. Planktonic cells were enumerated by transferring 1 mL of each bioreactor's solution to 9 mL of Dey-Engley Neutralizing Broth (D/E Neutralizing Broth, BD Difco, Sparks, MD) and subsequently diluting into 0.1% peptone water (BD Difco, Sparks, MD). Enumeration was also performed using APC and EB 3M Petrifilm (Saint Paul, MN, USA) plates. All EB Petrifilms were enumerated after incubation at 35°C for 24 hours, while APC Petrifilms were enumerated after incubation at 35°C for 48 hours.

pH and protein analysis

The pH of an aliquot of each bioreactor's solution was taken using a M530 Pinnacle pH meter (Corning Inc., Corning, NY) daily to assess changes throughout the 5-day duration of the experiment across all treatment solutions. From days, 35 mL of the control (TW plus purge but no surrogate inoculation) bioreactor solution was transferred to a sterile 50-mL conical tube and

frozen at -20°C for total protein analysis. After the completion of all three repetitions, all stored control solution samples were thawed and analyzed for protein using the Dilution-Free Rapid Gold BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA) (Smith et al., 1985). The bioreactor solutions were diluted 1:10 with TW to ensure the measured absorbances fell within the linear range of the standard curve. Therefore, to determine the actual protein content of the original samples, the readings obtained from the assay were multiplied by 10. The primary purpose for monitoring pH in each solution type over storage, along with using the control bioreactor solution to determine the level of total protein build-up in the solution over 5 days of microbiological growth along with daily addition of a volume of fresh purge (in the 20% v/v replenishment step), was to begin to correlate these rapid measurements with our ability to control biofilm development in the aforementioned continuously recirculating proprietary manufacturing process. It will likely be possible to continuously monitor these parameters during extended processing to allow for process control optimization.

Statistical analysis

For this experiment, the pH values of all test solutions were analyzed as a repeated measures design. For planktonic bacterial cells (from solutions), CFU/mL values were transformed to $\log_{10}$ CFU/mL. For attached bacterial cells (biofilm), CFU/mL values were converted to CFU/coupon and then to $\log_{10}$ CFU/coupon. The log APC counts and log EB counts taken from solutions were analyzed separately assuming a split plot design. The log APC counts, and the log EB counts observed on the coupons, were analyzed separately assuming they were collected in a split-split plot design. For each dataset, a linear mixed model was fit via the GLIMMIX Procedure in SAS/STAT software (SAS Institute Inc., Cary, NC) using the option DDFM=KR. Type III tests were used to identify all significant fixed effects at the 0.05 significance level. Appropriate pair-

wise comparisons of least squares means (LSM) were then conducted, each at the 0.05 significance level, with no adjustment for multiplicity.

4.3. Results

Impact of antimicrobial treatment solutions on biofilm build-up on coupons

One of the main objectives of this study was to evaluate the potential of peracetic acid (PAA) in combination with nanobubble water (NW) in inhibiting biofilm growth on both stainless-steel (S) and rubber (R) surfaces in a dynamic environment with a constant shear force for an extended period of 5 days under chilled conditions. Biofilm growth was assessed by enumerating bacterial cells on Petrifilm after sonicating and vortexing samples to detach the potentially attached cells from coupons. The three locations of coupons on one single rod of the bioreactor were taken into consideration when analyzing the data where results were separated based on the location of the coupon the rod [upper (A), middle (B), or lower(C)]. However, there was no significant difference in biofilm growth on coupons based on location for both APC and EB growth ($P = 0.7085$ and $P = 0.3435$, respectively). Therefore, results were analyzed against the three main effects: treatment solution, coupon type and time in days at 4°C. All three main effects showed significance effect against both APC and EB biofilm growth ($P < 0.0001$). The interactions between treatment and coupon type, treatment and day, as well as coupon type and day significantly impacted APC growth ($P < 0.05$). For EB growth, the interactions between treatment and coupon type as well as treatment and day both had significant effects ($P < 0.0001$). All main effects and interactions and their corresponding p -values are displayed in Table 4.2.

Table 4.2 P-values of the main effects and the interactions of the main effects for the APC and EB biofilm growth on stainless steel or rubber coupons treatment solutions with 0, 200 or 400 ppm PAA in combination with TW or NW over a 5-day period kept at 4°C.

Effect	APC Pr > F	EB Pr > F
Treatment	<.0001*	<.0001*
Location	0.7085	0.3435
Treatment * Location	0.6285	0.8697
Coupon	<.0001*	<.0001*
Treatment * Coupon	0.0005*	<.0001*
Day	<.0001*	<.0001*
Treatment * Day	<.0001*	<.0001*
Coupon * Day	0.0007*	0.3455
Trt * Coupon * Day	0.0847	0.2853

*Effects and interactions are significant ($P < 0.05$)

Aerobic Plate Count (APC) Biofilm Growth

APC, representing the total aerobic plate count, is one of the most common tests in microbiological quality control. It is used as a general indicator of the number of viable aerobic microorganisms, including many spoilage microorganisms and some pathogenic bacteria. APC may serve as an assessment tool to verify sanitary quality or may suggest inadequate practices, thus reflecting non-compliance with Good Manufacturing Practices (GMPs). In this study, APC (Figure 4.1) was used to assess biofilm growth by general psychrotrophic spoilage microorganisms originating from raw beef purge on coupons. Control coupon samples (TW + purge, no PAA and no surrogate inoculation) showed a significant increase in APC biofilm growth from 3.65 to $5.87 \pm 0.20 \log_{10}$ CFU/coupon from day 0 to day 5 ($P < 0.0001$) under the chilled dynamic conditions evaluated. Similarly, coupon samples from treatment solutions without PAA in both TW and NW (T1 and T2) both showed significant APC growth that followed a similar increasing trend through

day 5. Coupons in T1 demonstrated an increase from 3.88 to $6.16 \pm 0.20 \log_{10}$ CFU/coupon from day 0 to day 5 ($P < 0.0001$), while coupons in T2 showed an increase from 4.47 to $5.48 \pm 0.20 \log_{10}$ CFU/coupon from day 0 to day 5 ($P < 0.0001$). Samples in NW-0 ppm PAA showed the lowest APC biofilm growth on day 5 among the three non-PAA treatment solutions, being 0.7 logs less than APC growth on coupons in TW-0 ppm PAA ($P = 0.0269$). NW showed a significant difference in APC biofilm accumulation on coupons after 5 days of storage at 4°C. With respect to treatment solutions containing PAA, APC biofilm growth on coupons in both 200 ppm and 400 ppm PAA solutions exhibited similar starting points with counts being below the level of detection (LOD = $1.4 \log_{10}$ CFU/coupon), meaning that PAA at 200 and 400 levels was effective in inhibiting APC biofilm growth after two hours of storage at 4°C in a dynamic environment. However, the growth trend then differs between treatment solutions with 200 ppm PAA which showed significant growth after 3 days compared to treatment solutions containing 400 ppm PAA which showed no observed growth across the whole 5 days. 400 ppm PAA was able to inhibit APC biofilm growth over the 5-day period, showing 4.5 and 4.7 logs lower in TW-400 ppm and NW-400 ppm PAA solutions, respectively compared to the control. 200 ppm PAA allowed a significant APC growth from 1.25 to $2.66 \pm 0.20 \log_{10}$ CFU/coupon from day 0 to day 3 ($P < 0.0001$) in TW and a significant increase from 1.32 to $2.43 \pm 0.20 \log_{10}$ CFU/coupon from day 0 to day 3 in NW ($P < 0.0001$). After day 3, coupons in 200 ppm PAA solutions (both TW and NW) showed no significant changes, proving that major biofilm development occurred during the first 3 days of storage. However, slight APC biofilm population decreases occurred in both treatment solutions on day 5, but still a significant biofilm accumulation occurred overall with a 1.26 and $0.84 \pm 0.15 \log_{10}$ CFU/coupon growth on T3 and T4 coupons, respectively, from day 0 to day 5 ($P < 0.001$). Biofilm accumulation on coupons in 200 ppm PAA samples remained significantly lower

than in control samples by approximately 3.4 logs in TW-200 ppm PAA and 3.7 logs in NW-200 ppm PAA, respectively. Over the 5-day period, in TW, 200 ppm PAA yielded biofilm counts 1.2 logs higher than 400 ppm PAA ($P = 0.0004$), and in NW, 200 ppm PAA was 1.06 logs higher ($P = 0.0012$)—demonstrating that 400 ppm PAA was more effective overall. There was no significant impact observed for using NW instead of TW at both 200 ppm and 400 ppm PAA levels, where both showed similar inhibiting effects ($P > 0.05$).

***Enterobacteriaceae* (EB) Biofilm Growth**

EB counts (Figure 4.2) were used to assess the possible growth of the inoculated *E. coli* surrogates as well as other spoilage microorganisms belonging to the family of Enterobacteriaceae associated with meat and meat products found naturally in the added purge. On day 0, after 2 hours of storage at 4°C, EB did not significantly grow biofilm on coupons in the non-inoculated control sample showing an average of $1.34 \pm 0.16 \log_{10}$ CFU/coupon, which did not significantly differ from EB growth on coupons in the four treatment solutions with PAA at both 200 and 400 ppm levels (T3, T4, T5, and T6). In fact, 200 ppm and 400 ppm PAA solutions in both TW and NW showed a similar inhibitory effect on EB growth throughout the 5 days, with counts declining below the level of detection (LOD = $1.4 \log_{10}$ CFU/coupon). However, the control sample significantly increased by approximately 0.4 logs on day 3 and continued to increase to $2.82 \pm 0.16 \log_{10}$ CFU/coupon on day 5 ($P < 0.0001$). This EB growth showed that biofilm accumulation on control treatment coupons occurred after 3 days and continued to increase due to the natural EB population in the beef purge. On day 5, EB growth of control samples was significantly higher from EB counts of the PAA-treated samples by approximately 1.7 logs ($P < 0.0001$). However, control samples showed significantly lower counts from the inoculated treatment solutions without PAA in both TW and NW (T1 and T2, respectively) throughout the 5 days. The presence of the

added *E. coli* surrogates and the absence of the antimicrobial PAA in solutions T1 and T2 affected EB growth in a way that resulted in higher EB counts than control samples. On day 0, an initial attachment of EB was observed after 2 hours of refrigeration showing approximately 3 log CFU/coupon. EB counts on coupons in non-PAA solutions T1 and T2 continued to gradually increase throughout day 3 and day 5 by a total of 0.8 logs each to reach approximately 3.77 and $3.879 \pm 0.16 \log_{10}$ CFU/coupon on day 5 in T1 and T2, respectively ($P < 0.0001$). NW alone did not show an impact on EB growth compared to TW alone across the 5 days as both solutions projected similar increasing trends ($P > 0.05$).

Surface Material Influence on Dynamic Biofilms

Hard surface material had a significant impact on both APC and EB biofilm populations recovered from coupons in solutions where biofilm growth was observed (Figures 4.3 and 4.4). In general, rubber (R) coupons showed less than 1 log higher APC and EB biofilm growth than stainless steel (S) but was still considered a significant difference. This indicated that R coupons showed higher affinity for biofilm development under these specific experimental conditions. Relative to S coupons, control R coupons showed 0.43 log CFU/coupon higher APC ($P = 0.0004$) and 0.25 logs higher EB counts ($P = 0.0008$). R coupons in treatment solutions without PAA in TW and NW showed higher APC counts by 0.63 and 0.57 logs, respectively, and higher EB counts by 0.5 and 0.4 logs, respectively, compared to S coupons ($P < 0.0001$). In treatment solutions with 200 ppm PAA, only APC exhibited differences between R and S, while EB counts were the same due to the insignificant EB growth in PAA containing solutions ($P > 0.05$). R coupons in TW-200 ppm PAA solution showed 0.30 log APC higher than S coupons ($P = 0.0120$), while R coupons in NW-200 ppm PAA showed 0.4 log APC higher than S coupons ($P = 0.0008$). Therefore, biofilm

development was higher on R surfaces compared to S over the 5-d chilled storage conditions under dynamic growth conditions.

Impact of antimicrobial treatments on planktonic cells in solution

For planktonic bacterial cells, the effects of treatment solutions and time in days were evaluated against the growth of both APC and EB populations, separately. The main individual effects of treatment and day significantly affected APC growth in solutions ($P < 0.0001$ and $P = 0.0006$, respectively), whereas the interaction between treatment and day was not statistically significant but trended toward significance ($P = 0.0719$). Nevertheless, because examining how treatments behave over time was a primary objective in this study, we considered the interaction comparisons to be pre-planned and therefore examined the least square means (LSM) for treatment*day. On the other hand, the main effects of treatment and day as well as their interaction all revealed a significant impact on EB planktonic growth in solution ($P < 0.05$).

Aerobic Plate Count (APC) Growth in Treatment Solutions

Planktonic APC growth (Figure 4.5) was affected by the addition of PAA to treatment solutions in both NW and TW at the two concentrations, 200 and 400 ppm. When comparing treatment solutions containing PAA (T3, T4, T5 and T6) to both the control solution (C) and the treatment solutions without PAA (T1 and T2), a significant difference in the growth of APC cells was observed from day 0 to day 5 ($P < 0.0001$). No significant difference was observed between the use of TW or NW alone (T1 vs T2) against APC planktonic bacterial growth in solutions across days ($P > 0.05$). Similarly, using NW instead of TW with 200 ppm and 400 ppm PAA did not enhance the antimicrobial properties of PAA against APC planktonic growth across the days ($P > 0.05$). Both solutions using TW and NW with PAA concentrations exhibited similar antimicrobial properties. Planktonic APC growth after two hours of refrigeration on day 0 was 1.7 ± 0.48 log

CFU/mL for both TW solutions with 200 ppm and 400 ppm, indicating no significant difference on day 0 between using 200 and 400 ppm of PAA ($P > 0.05$). However, in NW solutions, 200 ppm showed higher growth of the APC population (2.13 ± 0.48 log CFU/mL) compared to 400 ppm NW solution on day 0 (0.7 ± 0.48 log CFU/mL), indicating a significant benefit in using higher PAA concentrations in this case ($P = 0.039$). Over time, a trend emerged in which significant differences in APC growth were observed between 200 ppm and 400 ppm PAA solutions in both water types. Specifically, greater growth occurred in the 200 ppm PAA solutions compared to the 400 ppm PAA solutions on days 3 and 5 with a difference of 2.78 and 3.51 ± 0.67 log CFU/mL, respectively ($P = 0.0002$ and $P < 0.0001$, respectively for days 3 and 5). This indicated that higher PAA concentrations provide better control of planktonic APC cells as exposure duration increased at 4°C under chilled dynamic conditions.

***Enterobacteriaceae* (EB) Growth in Treatment Solutions**

The control sample, which was not inoculated with the cocktail of *E. coli* surrogates, showed a natural EB growth of 4.17 ± 0.27 log₁₀ CFU/mL on day 0 (Figure 4.6), originating from the 10% v/v beef purge added. This natural EB growth remained constant after 3 days with 20% (v/v) daily purge and solution replenishment, but then slightly increased by approximately 1.2-log from day 3 to day 5 ($P = 0.0002$). For treatment solutions without PAA in both TW- and NW-based solutions (T1 and T2), EB growth on day 0 was approximately 5.0 and 5.7 logs, respectively with no further significant changes, either increases or decreases, observed over the 5-day period ($P > 0.05$). EB growth in the control solution was significantly lower than in the inoculated samples T1 and T2 on days 0 and 3 ($P < 0.05$), but by day 5, no significant differences were observed among the three samples due to the increase in EB cell count in the control sample by day 5. There was no significant impact of using NW instead of TW with all three PAA concentrations (0, 200

and 400 ppm) on planktonic EB growth in solutions across days ($P > 0.05$). Both 200 and 400 ppm PAA were effective against controlling the growth of planktonic EB populations from day 0 to day 5, when compared with treatment solutions without PAA ($P < 0.0001$). No significant difference was observed between using 200 and 400 ppm of PAA in treatment solutions, where planktonic EB growth was maintained below detectable levels for T3, T4, T5 and T6 across the 5 days ($P > 0.05$).

pH and protein analysis of treatment solutions

The pH of the treatment solutions plus purge in the bioreactor was measured daily before replenishment of the bioreactors with 20% (v/v) of new respective solutions and purge. This analysis of the organically loaded solutions was done to monitor any significant pH fluctuations over time, assuming that biofilm build-up on coupons, organic matter accumulation and bacterial proliferation would collectively influence the pH of treatment solutions. The two main effects of treatment solution and time in days showed significant effects on pH variations throughout the study ($P < 0.0001$). There were no significant differences in pH activity or fluctuations among the control sample, TW-0 ppm PAA (T1) and NW-0 ppm PAA (T2) throughout the experimental days, where they all showed a similar trend ($P > 0.05$). As observed in Figure 4.7, in all three PAA-free solutions, pH remained stable between 5.7 and 6 from day 0 through day 3, respectively, with no significant fluctuations ($P > 0.05$). This stability was then interrupted with a significant increase in pH from day 3 to day 4 in all three non-PAA solutions. Specifically, the control sample solution pH increased from 6.05 ± 0.158 to 6.52 ± 0.10 ($P = 0.0012$), pH of T1 increased from 5.77 ± 0.158 to 6.48 ± 0.099 ($P < 0.0001$), and pH of T2 rose from 6.05 ± 0.158 to 6.55 ± 0.10 ($P = 0.0006$). The pH of the three samples then continued to slightly increase to reach an average of 6.62 ± 0.072 on day 5.

As for treatment solutions containing PAA (T3, T4, T5 and T6), solution pH values remained rather stable across days ($P > 0.05$), likely due to the oxidation effect of the added PAA against biofilm growth and organic matter build up in solution. pH in PAA-containing treatment solutions was significantly lower than all treatment solutions without PAA ($P < 0.0001$). Moreover, there were no observable differences between TW-based solutions and NW-based solutions for each of the PAA levels (200 and 400 ppm). The 200 ppm solutions had an average pH of 4.61 while 400 ppm solutions had an average pH of 4.35 across days, showing no significant difference between the two different PAA levels.

The protein content of the control solution was measured from samples taken on each of the 6 total experimental days for all three experimental replications in order to correlate the protein build-up in untreated solutions without antimicrobial with the pH. Figure 4.8 shows the protein build-up as well as the pH change over chilled storage time. Protein concentration was approximately 6.2 mg/mL on day 0 and then gradually decreased to approximately 5.2 mg/mL on day 2. This decrease can be associated with the daily replenishment of the bioreactor's solution where 20% of the total solution volume was removed and replaced by the same volume of fresh solution made up of 90% tap water and 10% beef purge. The protein build-up that occurred overnight in the first two days was probably lost during the replenishment procedure. However, the protein concentration increased after day 2 and continued to steadily increase from day 3 (at 5.21 mg/mL) through day 5 (reaching 6.30 mg/mL). This change correlated with the observed pH variation in the control bioreactor's solution alongside the protein content. During the first three days, the solution's pH remained stable at around 5.8. From day 3 to day 5, however, it rose to approximately 6.6, likely due to an increase in organic compounds—some of which are proteins—contributing to a more alkaline environment.

4.4. Discussion

Microbial biofilm formation is a complex process involving cell-to-cell communication to achieve rigid, well-structured biofilm complexes. In nature, microorganisms form biofilms that include multiple species. Research involving mixed meat-associated species, while jointly analyzing the efficacy of peracetic acid (PAA), on the natural post-treatment microbiota of raw meat products is lacking in the literature (Rossi et al., 2023). Initially, single-species biofilm formation was more commonly investigated, however more recently, studies on dual and triple species are becoming more common as scientists are interested in analyzing potential relationships among microorganisms in biofilms. Using the CDC bioreactor in our study, we were able to observe mixed-species biofilm development within a dynamic chilled environment on stainless steel (S) and rubber (R) coupons submerged in solutions containing tap water (TW) or nanobubble water (NW) including a relatively high presence of beef purge organic content (10% v/v). This set of environmental conditions represents rather common commercial meat manufacturing scenarios.

The use of PAA at 200 ppm and 400 ppm clearly demonstrated a beneficial effect in terms of overall system microbiological and biofilm control, as significant log differences were observed when PAA was included in the solutions. Attached bacterial cells in dynamically developed biofilms collected from S and R coupons were enumerated on both EB and APC Petrifilm to represent the Enterobacteriaceae and total aerobic populations, respectively, originating from the added beef purge. Psychrotrophic microorganisms that have been commonly associated with vacuum-packed beef include *Pseudomonas*, *Commamonas*, and *Lactobacillus*, *Carnobacterium* and *Raoultella*, which can grow at 4°C (Nan et al., 2024). *Pseudomonas* species are widely spread throughout meat processing environments and are associated with meat spoilage (Giaouris et al., 2014), primarily aerobically packaged products. Several studies have confirmed the strong biofilm

forming properties of *Pseudomonas* and their possible synergistic interactions with some foodborne pathogens (Abdallah et al., 2015; Haddad et al., 2021; Pant et al., 2025; H. Wang et al., 2018). Therefore, it is very likely that some of the psychrotrophic microorganisms evaluated on APC Petrifilms in our study belong to the species of *Pseudomonas*, although taxonomic identification was not pursued. Initial bacterial attachment on S and R surfaces was observed 2 hours after refrigeration for both APC and EB populations in treatment solutions without PAA, which was similarly observed in another study that indicated a fast initial attachment of meat-borne *Pseudomonas fluorescens* to stainless steel (Wang et al., 2018), as well as another study showing a 2-hour initial attachment of *Salmonella* biofilm on nylon, wood, and HDPE (Ivers et al., 2024a).

The control coupons (submerged in TW with no PAA and no surrogate inoculation) did not show significant biofilm development by EB populations at the initial 2-hour attachment phase at 4°C under dynamic solution parameters compared to the other inoculated treatment solutions without PAA (T1 and T2) that did show initial EB biofilm attachment (approximately 3 logs). Moreover, EB biofilm growth was, in general, greater on coupons in the treatment solutions inoculated with the cocktail of the five USDA-approved non-pathogenic *Escherichia coli* strains, reaching around 3.7 logs compared to 2.8 logs on control coupons on day 5. One possible interpretation could be related to the absence of the *E. coli* surrogates in the control bioreactor. STEC, which these surrogates are often used to mimic, cannot grow at low temperatures and in fact enters a metabolic slowdown or dormancy (Walker et al., 2024). Regardless, some of the *E. coli* bacteria might have survived the first few hours in the chilled solutions and their presence in inoculated solutions of TW or NW without PAA might have enhanced the biofilm forming capabilities of EB bacterial cells present. Furthermore, it is important not to neglect consideration

of bacteria that may transition to the viable but not culturable (VBNC) state under stress (decreased temperature) which are considered a subpopulation of cells that cannot be cultured on media but are still metabolically present in the environment, especially since a considerable number of cells in a biofilm is in a resting, dormant or VBNC state (Flemming, 2011; J. Liu et al., 2023). Eventually, EB biofilm growth increased on non-inoculated control coupons as well (reaching $2.82 \pm 0.162 \log_{10}$ CFU/coupon on day 5), demonstrating that some EB species can grow at low temperatures but would require more time to form biofilms since we observe a steep increase from day 3 to day 5. Meat spoilage bacteria belonging to the family of EB that can grow at refrigerated temperatures include bacteria from the genera *Buttiauxella*, *Hafnia*, *Rahnella*, *Serratia* and *Yersinia* (Doulgeraki et al., 2011, 2012; Farmer et al., 2010; Sade et al., 2013). The presence of the *E. coli* surrogate bacteria may have facilitated and accelerated the initial attachment of EB cells. Surviving *E. coli* bacterial cells can contribute to biofilm formation through synergistic relationships with other bacteria, while dormant cells may support the process through alternative mechanisms. Actually, several studies have shown that synergistic relationships between different bacterial species can help in bacterial attachment to surfaces and overall biofilm formation. For example, Lapointe et al. (2019) demonstrated that the presence of *Pseudomonas fluorescens* enhanced the initial attachment of *Lactobacillus plantarum* and *Leuconostoc pseudomesenteroides* by $1 \log_{10}$ CFU/cm² in the CDC bioreactor in the presence of meat slurry at 20°C as they simulated meat processing conditions. Another study also demonstrated that *Pseudomonas fluorescens* offered a protective effect to *L. monocytogenes*, promoting its dynamic biofilm growth in a CDC bioreactor even under harsh conditions, such as simulated desiccation and exposure to a sanitizer, compared to when *L. monocytogenes* was present alone (Haddad et al., 2021).

Furthermore, Flemming (2011) pointed out the importance of not neglecting the effect of inactive and/or dead bacteria, which in our case were either killed by PAA or possibly became dormant at low temperature (i.e., *E. coli*) and thus remained capable to attach to coupon surfaces. Bacterial cells do not need to be viable for adhesion to occur. Even if the cells cannot multiply, they can still adhere to surfaces and assist in integrating live cells into the biofilm (Flemming, 2011). This phenomenon could also explain the APC biofilm growth on coupons submerged in 200 ppm PAA treatment solutions which increased from day 0 to day 3, followed by a subsequent decrease and stabilization over time. It is likely that 200 ppm PAA was not entirely sufficient to eliminate all the microorganisms present in the solution. Some of the bacteria that died likely contributed to surface adhesion and biofilm formation, while others that were still viable, which did not immediately develop biofilms within the initial 2 hours, gained stress resistance and were able to form biofilms, reaching approximately 1.4 logs in TW and 1.1 logs in NW. In contrast, at the higher 400 ppm PAA, no surviving viable microorganisms were able to form biofilms. With respect to planktonic bacterial cells, both levels of PAA (200 and 400 ppm) were effective in controlling EB free cells, preventing their growth in solutions. However, 200 ppm PAA was not as effective in preventing the APC planktonic growth which can be explained by the fact that APC concentration in the initial purge was higher than the EB concentration (7.38 log CFU/mL for APC versus 4.38 log CFU/mL for EB).

The two studies conducted by Haddad et al. (2021) and Lapointe et al. (2019) used similar simulated meat processing conditions (SMPC) as in the current study, however, they used autoclaved (sterilized) meat purge. Therefore, the purge contributed organic material loading to the system being evaluated but the natural microbial community found in the meat slurry was not accounted for as a contributor to the overall biofilm forming capabilities of specific bacteria. The

presence of APC populations from the added beef purge in our study impacted the biofilm forming abilities of some EB species in a substantial way. A study that evaluated the microbial dynamics of collected surface bacteria from meat processing conveyor belts revealed the prevalence of multiple bacterial species that interacted with *Salmonella* Typhimurium and *E. coli* O157:H7's biofilm forming abilities and prevalence in the biofilm (Yang et al., 2023). Indeed, the impact of natural mixed-species microflora is very significant, regardless of whether a microorganism is identified as a biofilm former or non-former in a single species culture, as its successful integration into complex multi-species biofilms strongly depends on the presence of certain other resident bacteria (Yang et al., 2023). This highlights the critical need to consider the entire microbial community rather than focusing on individual strains when developing biofilm control strategies.

Importantly, this study highlights the antibiofilm and antimicrobial potential of nanobubbles (NBs), specifically biofilms developed under dynamic conditions at 4°C. The total APC biofilm count on coupons in NW without PAA was less than those in TW alone by 0.7 logs after 5 days ($P = 0.0269$), indicating a NB effect on biofilm control in the presence of high organic loading. This log difference in biofilm formation between regular TW and air-based NW can be explained by the unique properties of NBs in solution. First, the zeta potential proves that NBs are negatively charged in solution, which makes them good surfactants preventing organic material (negatively charged particles) adhesion to surfaces (Shiroodi et al., 2021). Another explanation can be attributed to the production of reactive oxygen species (ROS) such as hydroxyl ions ($\text{OH}^\cdot$) in the solution which can react with organic and inorganic molecules of the EPS and in the added beef purge (Chaurasia, 2023; Tvrdá & Benko, 2020). The presence of ROS in solutions containing NBs have been demonstrated by multiple studies (Atkinson et al., 2019; Liu et al., 2018; Liu et al., 2016a; Liu et al., 2016b). NBs have high internal pressure, allowing them to release more surface

energy when they explode, thus triggering free electrons to the excited state, which facilitates the conversion of O₂ molecules in solutions into ROS (Javed et al., 2023). Liu, Oshita, Makino, et al. (2016) proved the production of ROS by air and oxygen NBs using a fluorescent reagent, 3'-p-(aminophenyl) fluorescein (APF) that reacts with ROS. ROS are considered very unstable free radicals since they possess free unpaired electrons, making them electrophilic and able to react by accepting or donating electrons with other molecules of high electron density, such as compounds with nitrogen atoms including proteins (Tvrdá & Benko, 2020). OH⁻ ions have the strong ability to reduce disulfide bonds due to their highly reactive nature, and thus, break down organic matter.

Several studies have demonstrated the antibiofilm effect of NBs alone and in combination with other antimicrobial technologies. Shiroodi et al. (2021) demonstrated the efficacy of NBs in removing *E. coli* O157:H7, *Vibrio parahaemolyticus*, and *Listeria innocua* biofilms on plastic and S coupons with the effect of exposure time. *E. coli* biofilms on S coupons exhibited 3.4, 3.5, and 4.6 log CFU/cm² reductions after 2, 5, and 10 minutes of NBs treatment, respectively, while plastic coupons showed 2.5, 2.3, and 3.4 log CFU/cm² reductions, respectively (Shiroodi et al., 2021). Our study focused on a preventive approach to reduce biofilm development, whereas Shiroodi et al. concentrated on treating mature, fully developed biofilms. However, they further investigated the anti-adhesion properties of NBs by treating coupons with NBs before inoculating with *L. innocua* and then incubating at 37°C to observe the effect (Shiroodi et al., 2021). They confirmed that NB decreased the adhesion of bacterial cells to surfaces, observing 3.8 log CFU/cm² on NB-treated S coupons versus 4.2 log CFU/cm² on untreated coupons (Shiroodi et al., 2021). Their 0.4 log difference can be compared to our 0.7 log difference between coupons in NW and coupons in TW. However, key differences between our study and theirs include the presence of organic matter, which contributes to a more natural microflora in our study, and the different

environmental dynamics (including a large temperature difference) in which biofilms were developed. While Shiroodi et al.'s study utilized a static environment, our study employed a dynamic setting. Another study by Unger et al. (2024) also attributed the enhanced efficiency of antimicrobial solutions (PAA and free Cl_2) against fresh (3 days) and aged (3 months) *E. coli* biofilms on different surfaces including polypropylene, silicone, and stainless-steel to the production of ROS in solutions with NBs, also referred to as ultra-fine bubbles. In another study, they demonstrated that the gas used to produce NBs had an effect on the antimicrobial and antibiofilm properties of the solutions (Unger et al., 2023). NBs produced with CO_2 led to the production of carbonic acid, which helped PAA penetrate the membrane of microbial cells and thus was the most effective among gases used (air and N_2) against *L. monocytogenes* biofilm (Unger et al., 2023). This was similarly observed Singh et al.'s (2021) study where CO_2 NBs significantly increased the potency of PAA and free Cl_2 antimicrobial solutions against *E. coli* and *L. monocytogenes*. On the other hand, when using air or N_2 , NBs did not show any additional impact in reducing bacterial loads in solutions (Singh et al., 2021), which was similarly observed in our planktonic bacterial cells. Therefore, there is a possibility that an enhanced efficacy of PAA may have been observed against planktonic and biofilm growth with the use of NW instead of TW, if a gas other than air was used in our study to produce NBs. However, it is important to note that the studies mentioned have produced biofilms in a static environment at higher temperatures and did not account for shear force incorporation, which might lead to different results. All in all, since biofilms offer additional protection to embedded bacterial cells, sometimes the combination of one or more antimicrobial intervention is key to achieving better overall control. In biofilm research, scientists have found that increasing the concentration of antimicrobials and/or combining other effective intervention techniques can lead to a more effective outcome (Mendez et al., 2022).

Therefore, as the combination of applying NBs and PAA can serve as an overall more effective strategy than applying PAA alone or NW alone in this study, Mendez et al. (2022) similarly discovered greater log reductions of mature *L. monocytogenes* biofilm (grown at 30°C using a CDC bioreactor) on S coupons when exposed to chemical sanitizers such as PAA in combination with UV light, highlighting the advantage of using combined treatments over single treatments.

Using the CDC bioreactor to assess biofilm growth provides the advantage of replicating dynamic biofilm formation under real-world conditions. This is particularly relevant in closed machine circuits of processing equipment which involve fluid flow, where shear force and specific fluid motion significantly influence biofilm development. Huijboom et al. (2024) compared the development of biofilms formed by a lactic acid bacterial species, *Lactiplantibacillus plantarum*, in static versus dynamic conditions at 30°C and showed that flow enhances biofilm growth and resistance to eradication methods. Movement and flow increase nutrient availability, enabling non-motile cells, that would not normally engage in biofilm formation, to colonize surfaces, thus leading to higher cell numbers in a biofilm (Huijboom et al., 2024). Moreover, these researchers demonstrated that *L. plantarum* biofilms grown in dynamic conditions exhibited greater resistance to disinfectants such as peracetic acid. In our study, the 200 ppm PAA solutions showed some countable biofilm growth on coupons due to the rigid and stable initial biofilm structure which can serve as a strong base for further bacterial attachment. In fact, the dynamic environment in the bioreactor creates more stable biofilms since high shear force can select for EPS with stronger cohesive forces, washing away polymers that cannot adhere firmly to the matrix, creating a strong biofilm (Flemming, 2011). A study by Pant et al. (2025) showed that biofilms formed on S coupons in a CDC bioreactor (dynamic environment at 30°C) resulted in higher cell counts, more EPS formation, as well as higher tolerance to antimicrobial sodium hypochlorite than biofilms formed

in 48-well plates (static environment) which showed less rigidity. Using epifluorescence microscopy, they also demonstrated that the triple-species biofilms formed by *P. fluorescens*, *S. aureus*, and *L. monocytogenes* in the continuous system showed formation of fluid channels which enhanced the supply of nutrients to embedded bacterial cells, thus allowing more growth. Not only does flow have a significant effect on biofilm attachment, rigidity and resistance against antimicrobials, but also other conditions such as biofilm maturity and surface hydrophobicity play a key role in biofilm development and resistance (Ivers et al., 2024b). In fact, as we have observed in our study, the type of surface material interfered with the potential biofilm growth on coupons with R surfaces showing greater biofilm growth than S surfaces. Rubber is known to degrade faster than stainless steel, allowing more crevices and scratches that alter the textural surface characteristics of the material, increasing surface roughness (Hua & Zhu, 2024). The alterations in surface topography, whether caused by material wearing or by other factors like cleanliness, should be evaluated as they can offer favorable sites for bacterial attachment (Ivers et al., 2024b).

pH is an essential parameter as it directly relates to microbial activity. In fact, pH is one of the most critical extrinsic factors influencing bacterial growth with different microorganisms responding uniquely to changes in acidity or alkalinity of their environment (Gaspar et al., 2021). Any shifts in pH can have significant impacts on bacterial growth and solution stability. In our study, we monitored pH over time to investigate whether it could serve as a real-time predictor of system sanitation from both the standpoints of generic bacterial population levels in circulating solutions or affiliated equipment biofilms and on the overall protein/organic level accumulation in the system over time. Figure 4.7 illustrates that the pH in treatment solutions without PAA remained substantially higher pH (5.7-6.6) throughout the whole 6 days of the experiment (from day 0 to day 5). PAA is a strong antimicrobial that can inactivate vegetative bacterial cells,

endospores, yeast, and mold spores, therefore explaining the lower planktonic and biofilm counts in treatment solutions containing PAA (Organic Materials Review Institute, 2000). Results showed that acidity ranged between a high of 4.61 for 200 ppm solutions and a low of 4.35 for 400 ppm solutions as observed in Figure 4.7, which correlates with Kalchayanand et al.'s (2024) study where the pH results of their PAA solutions were approximately 4.5 and 4.0 for 200 ppm and 400 ppm, respectively. The antimicrobial effect of PAA largely depends on its oxidizing mechanism of action which allows the denaturation of protein, disruption of organic matter, as well as oxidization of sulfhydryl and sulfur bond proteins, enzymes and metabolites (Rossi et al., 2023). Therefore, the low biofilm growth and subsequently low EPS presence, and the decreased organic matter presence in the PAA-containing system, contributed to overall bioreactor solution pH stability at lower acidic pH values (pH 4 range). Treatment solutions containing no PAA showed a significant increase in pH after 4 days. Due to the absence of PAA, as well as the increased biofilm and planktonic bacterial counts in treatment solutions without PAA, the system presumably contains more organic matter from the accumulation of the added purge as well as the increased EPS formation which can leak proteins, carbohydrates, lipids, and other organic molecules which all contribute to a more basic pH (Bremer et al., 2015). In fact, mature biofilm formation takes between 3-5 days with the peak of biofilm maturity typically occurring after 96 hours, which aligns with the sudden increase in pH observed in our study from day 3 to day 4 (Ivers, et al., 2024a; Koti et al., 2024; Song et al., 2015; H. Wang et al., 2018). This increase in protein accumulation in the control solution correlated with the pH increase observed at this timepoint. Protein content in the solution represents some of the organic matter that could be present from the beef purge added as well as from the EPS formation, all contributing to a more basic pH.

4.5. Conclusion

Several factors must be considered in biofilm development such as surface characteristics, organic matter presence, shear stresses, environmental conditions including temperature and pH, as well as the bacterial species and natural microbiota involved. The incorporation of shear stress using the CDC bioreactor and the addition of beef purge to simulate meat processing settings with natural microflora and organic matter contributed to a clearer understanding of dynamic biofilm development and its control using NW containing PAA. Prioritizing the adoption of a preventive approach in food safety is critical, especially in biofilm development in food processing environments. The application of NW in a dynamic environment demonstrated a 0.7 log reduction in biofilm growth on coupons compared to a system without NBs, showing promising uses for NBs in preventing biofilm growth. The addition of PAA at 200 and 400 ppm concentrations is an effective strategy to mitigate the risk of biofilm formation on equipment surfaces, specifically those made of stainless steel and rubber. In order to validate microbial control, both planktonic and sessile populations must be tracked to ensure consistent safety and quality standards. The accumulation of organic build-up and residues is an important component that can be overlooked by processors and scientists when studying the control of pathogens in real-life meat processing facilities. This research contributes to new innovative approaches for controlling biofilm formation in meat processing environments, while considering low processing temperatures, organic matter accumulation, natural microorganisms and extended processing durations.

Certain limitations are associated with this study and can serve as potential future research ideas to strengthen the findings and contribute to the existing knowledge base. First, a whole genome sequencing analysis of the developed biofilms such as 16S rRNA sequencing would

provide more details about the specific species present in the added meat purge and can help in understanding inter-bacterial interactions during different phases of biofilm development. Secondly, inoculation with an important pathogen in the meat industry such as STEC or *Salmonella* using precise enumeration methods could help in understanding the impact of the psychrotrophic mixed-culture biofilms on pathogens in single, dual or triplex species/strain biofilms. Third, future studies should explore the effect of NW with PAA on pre-existing developed and mature mixed-species biofilms in order to depict the difference between the inhibition and eradication action of this antimicrobial solution. Fourth, previous research has used confocal imaging to differentiate between dead and live bacterial cells in the biofilm complex. Subsequently, 3D confocal imaging would allow a better understanding of the NW-PAA solution efficacy on bacterial cells according to time where images at different time points (such as 48, 72 and 96 hours) can show the predominance of dead versus viable bacteria and possibly showing a resurgence of live bacteria within biofilms (including deducing the impact of VNBC bacterial cells). Finally, the role of nanobubbles in initial biofilm cell attachment to hard surfaces mechanistically, and the physiological impact nanobubbles have on early- versus late-stage microbial communities that comprise meat-based biofilms, should be explored in greater detail, including a comparison of nanobubbles generated with CO₂ and/or O₂. Such work would need to scientifically characterize the resultant product quality (i.e., color, shelf life, fat oxidation) from each type of treatment.

4.6. Figures

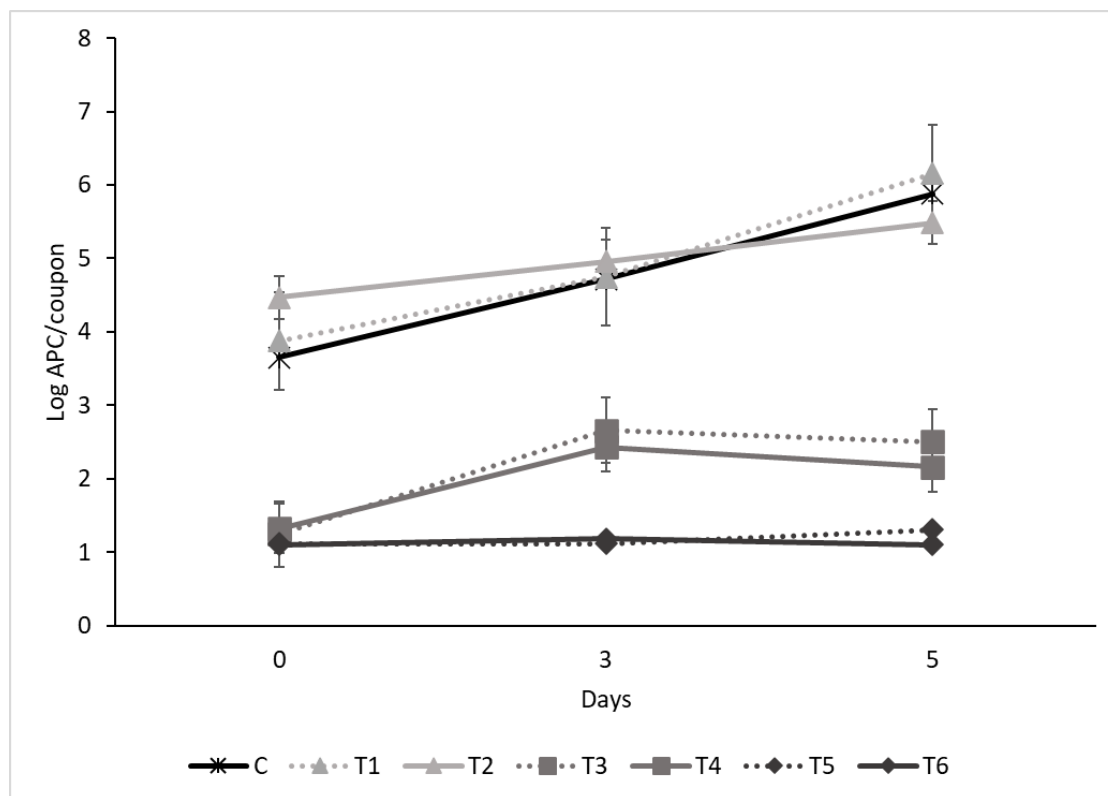


Figure 4.1 Aerobic Plate Count (APC) biofilm growth on stainless steel and rubber coupons immersed in the six experimental treatments (T1-T6) and the control sample (C) under chilled dynamic conditions over 5 experimental days. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA); where TW: Tap water. NW: Nanobubble water and PAA: Peracetic Acid.

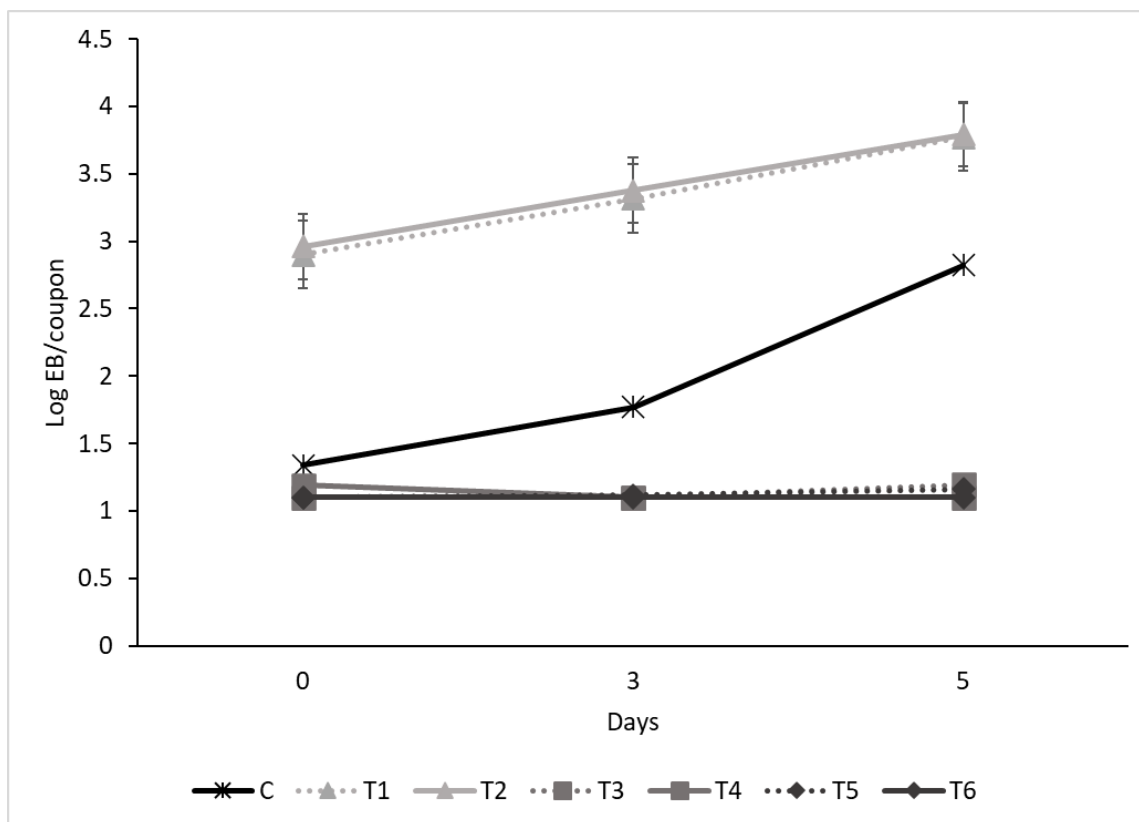


Figure 4.2 Enterobacteriaceae (EB) biofilm growth on stainless steel and rubber coupons immersed in the six experimental treatments (T1-T6) and the control sample (C) under chilled dynamic conditions over 5 experimental days. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA); where TW: Tap water. NW: Nanobubble water and PAA: Peracetic Acid.

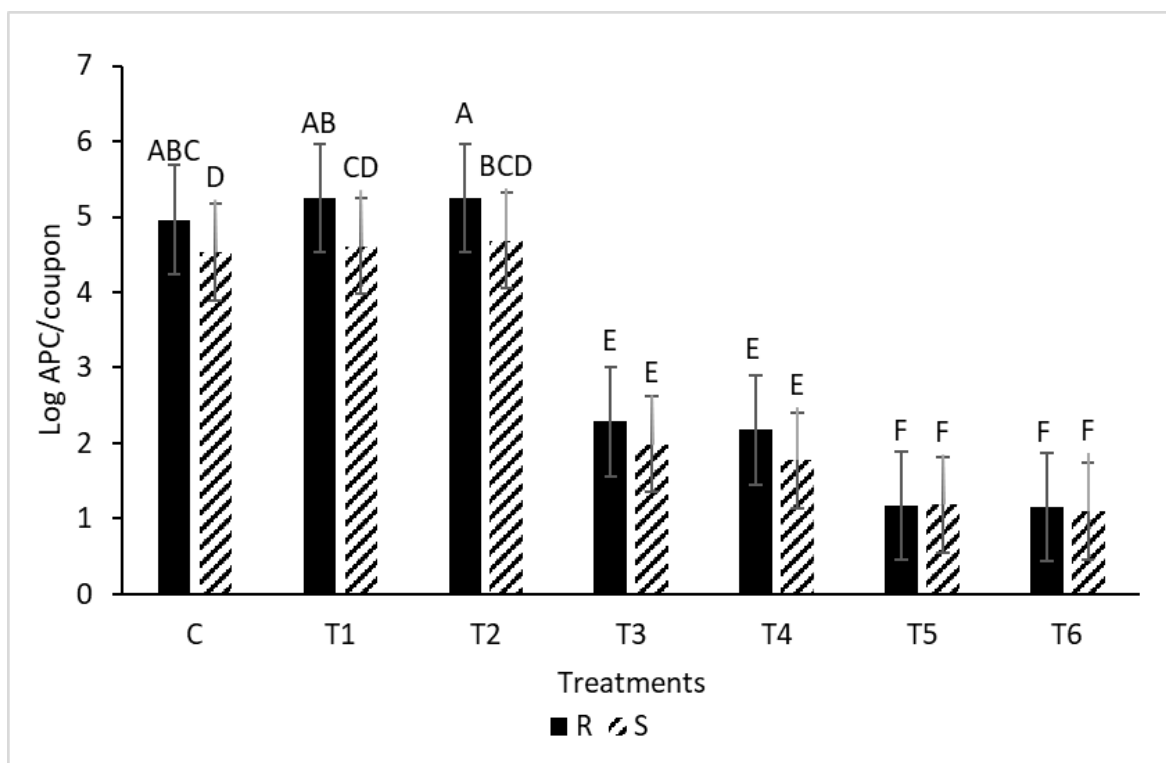


Figure 4.3 Comparing Aerobic Plate Count (APC) biofilm growth on S (stainless steel) and R (rubber) coupons immersed in six experimental treatments (T1-T6) and a control sample (C) under chilled dynamic conditions. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA); where TW: Tap water. NW: Nanobubble water and PAA: Peracetic Acid. Bars (LSM $\pm$ SE) with different letters are significantly different ($P < 0.05$) for APC growth.

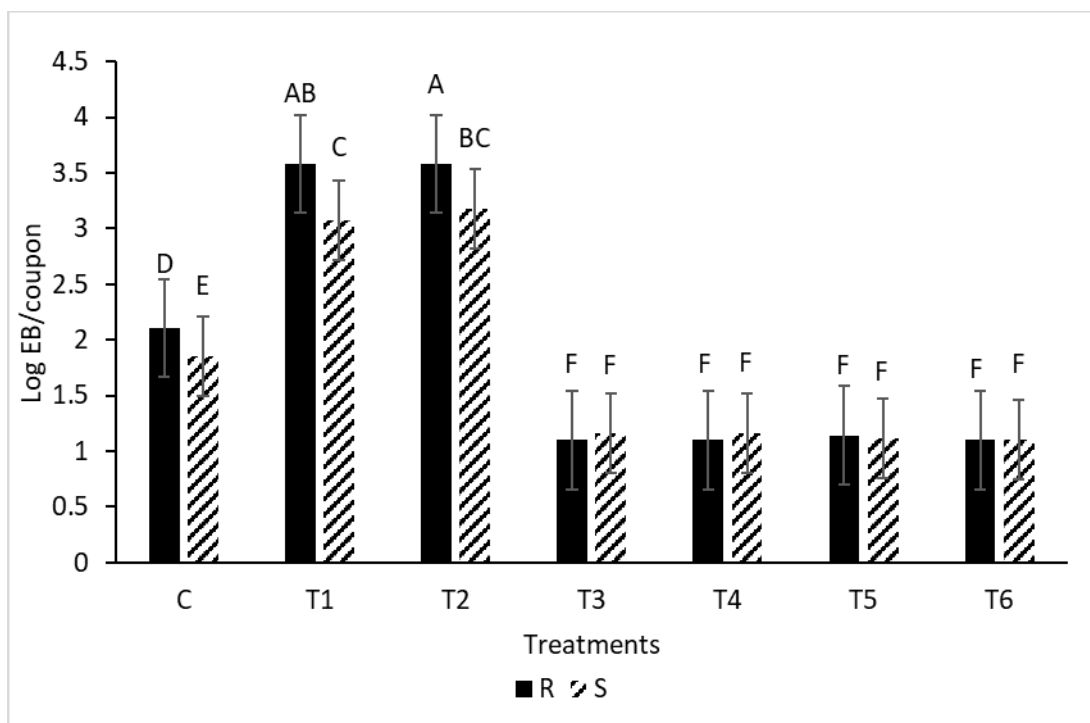


Figure 4.4 Comparing Enterobacteriaceae (EB) biofilm growth on S (stainless steel) and R (rubber) coupons immersed in six experimental treatments (T1-T6) and a control sample (C) under chilled dynamic conditions. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA); where TW: Tap water, NW: Nanobubble water and PAA: Peracetic Acid. Bars (LSM $\pm$ SE) with different letters are significantly different ($P < 0.05$) for EB growth.

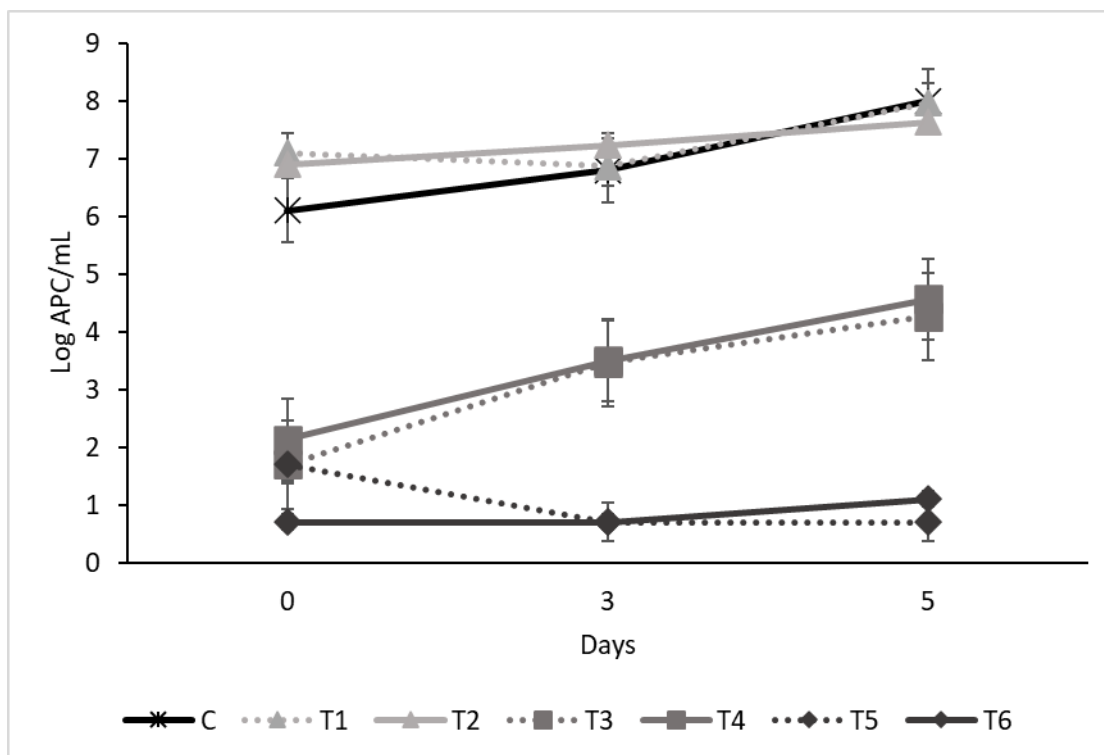


Figure 4.5 Aerobic Plate Count (APC) planktonic growth in six experimental treatment solutions (T1-T6) and a control sample (C) under chilled dynamic conditions over 5 experimental days. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA); where TW: Tap water. NW: Nanobubble water and PAA: Peracetic Acid.

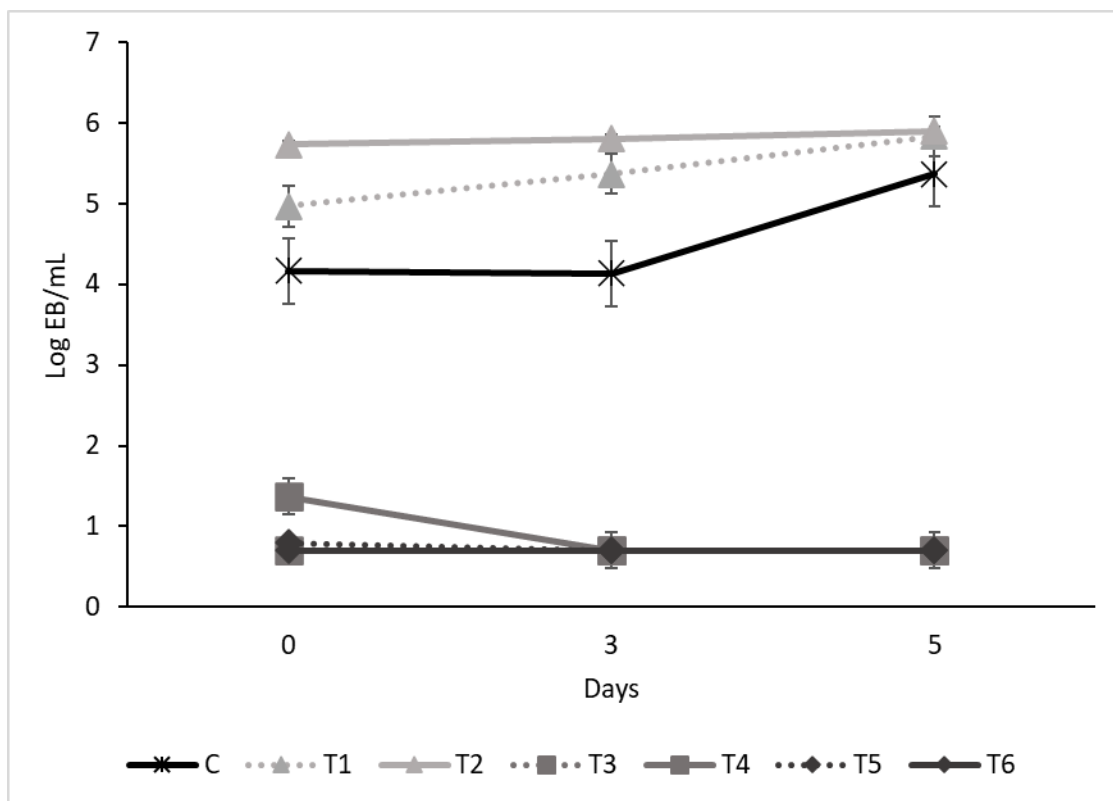


Figure 4.6 Enterobacteriaceae (EB) planktonic growth in six experimental treatment solutions (T1-T6) and a control sample (C) under chilled dynamic conditions over 5 experimental days. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA); where TW: Tap water. NW: Nanobubble water and PAA: Peracetic Acid.

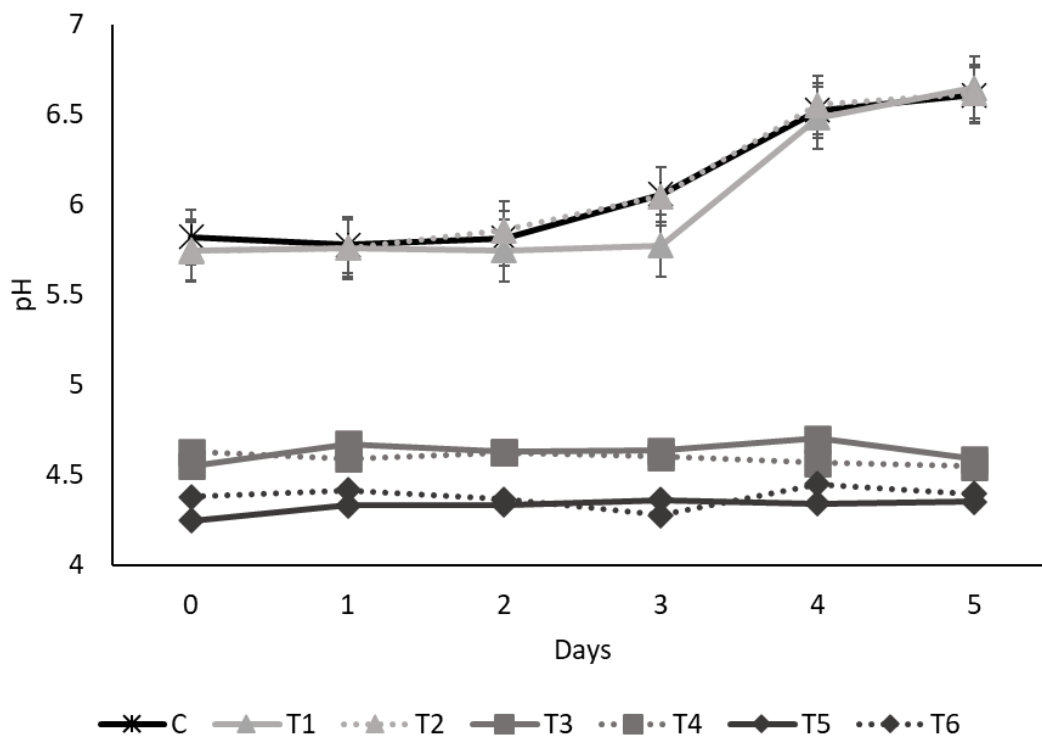


Figure 4.7 pH variations in CDC bioreactor treatment solutions across 5 days at 4°C under dynamic biofilm growth conditions. (T1: TW-0 ppm PAA, T2: NW-0 ppm PAA, T3: TW-200 ppm PAA, T4: NW-200 ppm PAA, T5: TW-400 ppm PAA, T6: NW-400 ppm PAA & C: non-inoculated control with TW-0 ppm PAA); where TW: Tap water. NW: Nanobubble water and PAA: Peracetic Acid.

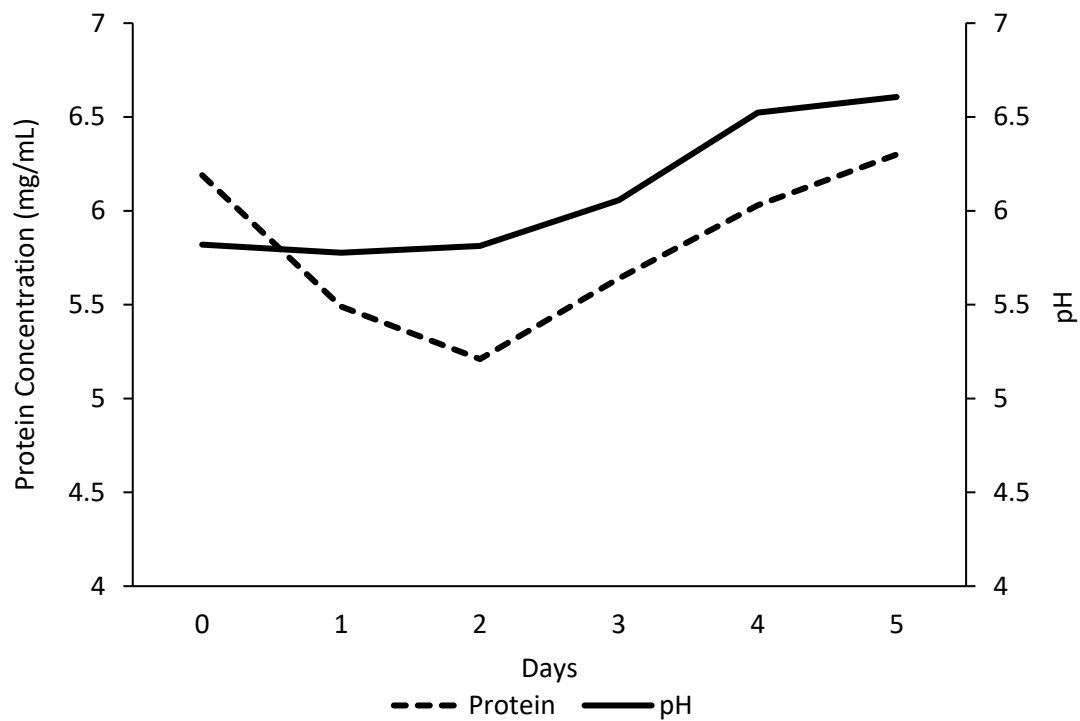


Figure 4.8 Protein concentration (mg/mL) measured in the control bioreactor solution over the experimental period of 5 days at 4°C under dynamic biofilm growth conditions.

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Chapter 5 - Overall Conclusion

Ground beef is a staple food in the United States, as well as other countries all over the world. The production of safe ground beef is a critical public health priority that requires governing by specific regulations. The Food Safety and Inspection Service (FSIS) is the main public health agency in the U.S. Department of Agriculture (USDA) responsible for ensuring that the specific regulations for the production of safe meat products around the nation are being effectively applied. In order for companies to obtain USDA approval for meat processing methods and sell their products in the marketplace, they must demonstrate compliance with USDA guidelines and provide scientific evidence that prove their process meets microbial safety standards and ensures a safe final product through validation of antimicrobial pathogen elimination or control steps (U.S. Department of Agriculture Food Safety and Inspection Service, 2021). Since 1996, *E. coli* O157:H7 has been declared an adulterant in ground beef and thus meat processing facilities are required to implement and follow stringent pathogen reduction hazard analysis and critical control point (HACCP) program in their processing (U.S. Department of Agriculture Food Safety and Inspection Service, 2021). The presence of untreated biofilms in food processing facilities can increase the risk of harboring virulent foodborne pathogens, such as *E. coli* O157:H7, since biofilms can serve as niches that attract, embed and protect bacteria, leading to stronger resilience. Therefore, this can lead to decreased microbial shelf-life and elevated risks of microbial persistence and harmful biological hazards in the final products, as well as increased end-product recalls and possible foodborne outbreaks (Alvarez-Ordóñez et al., 2019).

This research tackled the control of biofilms in beef processing settings, contributing to advancing preventive control measures that aim to reduce the risk throughout the processing operation and not only by final product testing. The combination of air-based nanobubble water

(NW) with the commonly used antimicrobial peracetic acid (PAA) proved to be an effective control strategy against mixed-species biofilms developed in refrigerated conditions with moderate-to-high organic loads typical of commercial raw beef manufacturing. Findings from our studies demonstrate that this antimicrobial combination can provide a better native-culture biofilm control than using regular municipal water comparably infused with PAA by achieving an overall greater microbial reduction in biofilm populations. Although no direct statistical comparison was conducted between the two experiments described in chapters 3 and 4, an overall descriptive comparison suggests that biofilms in dynamic environments achieve greater growth and resilience against antimicrobials than those in static environments, aligning with findings from previously reported research. Moreover, microbial loading and organic matter presence in the environment greatly influence microbial biofilm growth, with higher organic accumulations in beef processing fluid systems requiring higher PAA concentrations. Biofilm growth from native beef purge bacterial populations showed higher affinity for attachment onto rubber surfaces compared to stainless steel, indicating the importance of material type in biofilm attachment and initiation. The accumulation of organic build-up and residue is an important component that should be considered by processors and scientists when studying the control of pathogens in real-life meat processing facilities.

In addition to the general body of knowledge that this research provides to meat and poultry processors, this research was conducted in support of continued development and commercialization of a proprietary novel ground beef manufacturing system. A significant advantage of the antimicrobial control strategy stemming from this research is that both components of the antimicrobial solution being utilized, air-based nanobubbles and USDA-approved concentrations of PAA, are “natural” components proven safe and (now) effective for

this specific application, with no harmful residues (environmental or food-related) being generated. Overall, commitment to food safety and product quality in ground beef production is essential and therefore, to ensure a safe, well-controlled antimicrobial efficacy of the new patented process, production experts should do the following:

- Control organic buildup in the system's recirculating processing fluid (called "red water") by routinely monitoring the purge concentration over extended processing periods to maintain a relatively low organics level (i.e., 5 % v/v or less). As done in our Chapter 4 investigation, total protein content determinations could be a useful tool in defining when operational hygiene measures need to be taken.
- Limit the proprietary recirculation system operation (containing NW plus 200 ppm or greater PAA) to a maximum of 5 continuous days, after which time a complete equipment shutdown and thorough cleaning should be performed incorporating physical scrubbing of equipment surfaces and components.
- Maintain PAA concentration at 200 ppm for effective biofilm control in moderate to low organic load conditions during extended chilled processing. Ensure timely replenishment of the novel system's recirculation fluid with approximately 20% fresh NW plus PAA solutions (at least daily).
- Regularly inspect rubber equipment components (high-affinity materials for biofilm formation) for replacement and cleaning as needed.
- Sample and monitor biofilm growth on both stainless steel and rubber surfaces to adjust cleaning frequency and antimicrobial needs. Similarly inspect and manage other equipment and environmental materials (e.g., plastics, neoprene, silicone), as they can easily develop

biofilms, even though the current studies were confined to stainless steel and Buna N rubber.

- Verify microbial control by tracking both planktonic and sessile populations in key processing components, adjusting protocols to ensure consistent safety standards.
- Over a period of time (months to years), correlate routine microbiological testing results (APC, EB or coliform) of finished ground beef generated by the proprietary system to corresponding population counts observed in recirculation fluid (NW+PAA) during extended processing operations.

By following these recommendations, the risk of biofilm accumulation, and consequently, the risk of dangerous pathogens persisting can be minimized. Additionally, shelf-life of raw ground beef from the system is likely to be notably enhanced.

The successful laboratory-based studies reported in this thesis for control of meat-based biofilms and planktonic microbial populations in processing water, as with any research, should be confirmed under actual large-scale commercial operation of the proprietary ground beef manufacturing system. In such studies, multi-day extended operation of the system utilizing different lots of procured boneless beef manufacturing trimmings, and accounting for variations in the natural processing conditions that would exist within a commercial facility, should be considered. Further, no actual ground beef was manufactured in the current study (previous K-State studies have investigated NW+PAA against non-pathogenic surrogate organisms, along with *Salmonella* and Shiga toxin-producing *E. coli* on raw beef tissues), and therefore, commercial-level ground beef shelf-life evaluations should be conducted (focusing on raw color, fat oxidation, and microbial populations) to ensure that the resultant products are of acceptable marketing quality.

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