Efficacy and Comparison of ANK Neutral Anolyte and 77X for Pathogen Control on Pre harvest Citrus Fruit and Their Suitability as Hard Surface Sanitizers

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Abstract

Fresh citrus fruit and all surfaces that come into contact with them during harvesting, processing, and storage pose serious food safety risks because they become contaminated with pathogenic bacteria. This study tested ANK Neutral Anolyte and 77X for their ability to kill bacteria and stop biofilm formation on hard plastic crate surfaces and pre-harvest citrus fruit against *E. coli* ATCC 25922, *S. aureus* 6538, and *P. aeruginosa* 9027. The products' performance was assessed by comparing them with commercial sanitizers to determine their effectiveness as disinfectants for hard surfaces used in fruit production. Disc diffusion assays, minimum inhibitory concentration (MIC) analysis, and 96-well biofilm metabolic (XTT-menadione) assays were used to determine the antimicrobial activity of the treatment. The results showed that 77X exhibited strong antibacterial activity, with activity increasing with higher concentrations across all tested microorganisms (13.33 ± 0.58 mm, 19 ± 0.58 mm, and 14.33 ± 0.58 mm against *E. coli*, *S. aureus*, and *P. aeruginosa,* respectively). The product displayed broad-spectrum effectiveness, which is proven through its lower minimum inhibitory concentration values and its ability to create measurable inhibition zones. ANK Neutral Anolyte showed minimal antibacterial activity in agar diffusion tests, but it produced inhibitory effects through MIC determination and direct contact biofilm tests, which demonstrate that its antimicrobial efficiency depends on both concentration and time and environmental factors. Both agents reduced existing biofilms' metabolic processes, but their effectiveness increased with longer contact times and better concentration levels. 77X showed matching results to standard sanitizers while ANK Neutral Anolyte needed more precise optimization processes before reaching equivalent performance levels. This study proved that ANK Neutral Anolyte and 77X act as effective sanitizers for both plastic crates and pre-harvest citrus fruit. The study found that 77X demonstrated superior and more consistent antimicrobial performance.

Keywords: 77X; Anolyte; Biofilm; Citrus; Sanitizer

Contents

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1. Introduction

The worldwide fruit industry serves as an essential provider of fresh, wholesome produce which people across the globe can access. The supply chain for fruit requires effective methods to maintain microbial safety, which proves to be a significant obstacle. Fresh fruits are often consumed raw without cooking or further processing, which increases the risk of foodborne illness if contamination occurs during harvesting, handling, transportation, or storage (Campos et al., 2020; Patra et al., 2022). Microbial contamination can originate from multiple sources, including soil, which comes into contact with irrigation water and workers who handle equipment and food contact surfaces during processing and packaging procedures. Pathogenic microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* are the contaminating agents in fresh produce systems and pose potential health risks to consumers (Mahunu et al., 2024). The supply chain requires food safety because companies need to maintain proper sanitation and pathogen control measures throughout their operations.

Microbial contamination can occur when food contact surfaces used during fruit harvesting, packaging, and transportation activities become contaminated. Many fruit handling systems operate with reusable materials, which include plastic crates, conveyor belts and stainless-steel tables as their primary components. If these surfaces are inadequately cleaned or sanitized, they can harbor pathogenic microorganisms that may subsequently contaminate fresh produce (Srisamran et al., 2022). The primary obstacle which food processing facilities face regarding their microbial contamination problem resides in the ability of bacteria to establish biofilms. Biofilms are structured microbial organisms which exist within a self-produced extracellular matrix that enables cells to stick to different surfaces.

Previous research demonstrates that *E. coli* bacteria can establish biofilms on common food processing surfaces which include stainless steel and plastic materials (Richiardi et al., 2023). Biofilms gain protection from their extracellular polymeric matrix, which enables bacterial cells to survive environmental challenges and resist antimicrobial treatments. The biofilm bacteria continue to survive on food contact surfaces because standard cleaning methods do not eliminate them. Fruits that encounter the contaminated surfaces during handling or processing or storage create a risk for cross-contamination (Campos et al., 2020).

Escherichia. coli serves as the primary fecal contamination indicator microorganism among the various microbial indicators which food safety authorities use for their monitoring activities. This bacterium exists naturally within human and animal intestines, so its discovery on food or food contact surfaces indicates unsafe hygiene practices and potential fecal contamination (Gemeda et al., 2025). The monitoring of *E. coli* contamination establishes essential data which enables the assessment of microbiological safety in food processing facilities.

Sanitizers function as critical components which safeguard fruit production operations from microbial contamination hazards throughout processing systems. The application of chemical disinfectants serves to diminish microbial populations present on both fruit surfaces and food contact materials. The fruit industry has relied on chlorine-based sanitizers for disinfection because these agents produce strong antimicrobial effects at an economical price (Zaman et al., 2024). Microorganisms that live inside biofilms demonstrate increased resistance against traditional sanitizing agents, which reduces their efficiency at eliminating these organisms (Bland et al., 2022).

The search for different sanitizing methods has become more vital because the existing methods face serious limitations. Conventional sanitizers lose their effectiveness when organic matter is present. The chemical residues from sanitizers create safety risks for both the food surfaces and the surrounding environment and workers. The study examined three sanitizing agents, specifically electrolyzed water, organic acids, and quaternary ammonium compounds, to evaluate their effectiveness in controlling the growth of the microorganisms which can contaminate food processing areas. The study found that electrolyzed water possesses antimicrobial properties which scientists consider to be environmentally safe. The solutions of electrolyzed water produce reactive oxygen and chlorine species which can kill microorganisms while their active components break down into safe waste materials after their operational period (Rebezov et al., 2022).

The success rate of sanitizing agents shows variations which result from the specific microbial species present, the particular surface types, the sanitizer concentration, and the duration of contact time. The research requires additional studies to investigate how different sanitizers perform against pathogens that form biofilms and usually exist in fruit processing facilities.

The current research study tests how effective ANK Neutral Anolyte and an organic acid-based 77X are in killing biofilm-forming pathogens, which include *S. aureus*, *P. aeruginosa* and *E. coli* on plastic surfaces and citrus fruit. The study assessed their effectiveness by comparing them with standard commercial sanitizers to determine their effectiveness as sanitizers for controlling pathogens on hard surfaces in the fruit industry.

2. Literature review

The fruit industry operates with the goal of delivering fresh produce to customers around the world for every season of the year. The protection of fruits against microbiological threats presents an ongoing problem, which becomes especially difficult during the stages of harvesting, transportation, and storage. The handling of fresh fruits requires precise methods to stop microbial contamination because people usually eat these fruits without any cooking or processing. Pathogenic microorganisms such as *E. coli*, *Listeria monocytogenes*, and *S. aureus* can stick to fruit surfaces or remain on food-contact surfaces, which include plastic crates and human hands and stainless-steel equipment that processors and transporters use (Hua & Zhu 2024).

The control of pathogens become difficult because they form biofilms, on surfaces of contacted food. Biofilms function as organized systems of microorganisms which exist in a state of protection through their extracellular matrix. All biofilm-forming bacterial species show higher resistance against both environmental stressors including disinfectants, which makes it more challenging to eliminate them through standard cleaning methods (Fernandes et al. 2024). The use of conventional sanitizing agents such as chlorine-based solutions does not guarantee success in eliminating microorganisms that are linked to biofilm formation (Hamilton et al. 2025).

2.1 Fruit Industry

The global fruit industry generates billions of dollars each year and produces over one billion tons of fruit to meet rising international demand for fresh produce. It supports economic growth and food security in many fruit-exporting countries, including New Zealand (Patra et al., 2022). The industry requires optimization of production processes because it has surpassed existing capacity, leading to operational challenges during post-harvest activities, storage, and transportation that demand both efficiency and food safety. The risk of foodborne pathogens on fresh fruits persists, as bacteria can survive during handling and packaging (Patra et al., 2022). Fruit processing and transport facilities often utilise plastic crates and stainless-steel equipment for durability. However, these materials can become hazardous when used as storage for harmful microorganisms, especially if proper cleaning practices are not observed. These surfaces frequently come into direct contact with fruit, increasing the risk of cross-contamination. They pose a danger because pathogens such as *L. monocytogenes* and *E. coli* can form biofilms on these surfaces that resist standard disinfection methods (Rebezov et al., 2022). The risk of contamination rises during long-distance transport because reusable crates can enable microorganisms to survive and spread under storage conditions (Patra et al., 2022). Adequate sanitation procedures for food-contact surfaces are crucial to prevent microbial contamination and uphold quality standards for fresh produce. Recent research has focused on developing improved sanitization methods capable of destroying biofilms and eradicating microorganisms from surfaces that contact food (Kandemir et al., 2022). The new sanitation techniques aim to enhance pathogen control while still permitting the handling of fresh produce (Nirmal et al., 2023).

2.2 Transport Packaging

The fruit industry depends on three main types of plastic materials: polyethene (PE), polypropylene (PP), and polytetrafluoroethylene (PTFE) for its operations. The fruit packaging and handling systems utilise these materials because they offer durability and light weight while remaining cost-effective (Sarihan et al., 2022). Selecting packaging and handling materials requires careful consideration because their surface properties determine whether microbes adhere to and survive on them, potentially leading to contamination that affects fruit safety and quality. The surfaces of PE and PP, used for packaging fruit, can become contaminated with microbes when proper cleaning and sanitizing procedures are not followed (Agarwal et al., 2023). During processing and handling, fruits come into contact with stainless-steel surfaces on conveyors, slicers, and storage equipment. These plastic and metal surfaces can act as sites for pathogen transmission, as biofilms can develop on them, rendering standard cleaning methods ineffective (Fernandez et al., 2023).

The distribution process necessitates sanitary transport packaging to maintain fruit safety and quality. The risk of contamination during transportation arises from three main factors: frequent handling, extended storage, and contact with contaminated packaging materials (Sarihan Mungan & Aydin, 2022). Research indicates that packaging materials such as reusable plastic crates and containers used during the post-harvest period can harbour microbial populations that pose safety hazards to fruit (Al-Dairi et al., 2022). Damage to packaging materials during transport creates new surfaces that are easier for microbes to attach to and that facilitate microbial proliferation.

2.3 Pathogens and Indicator Organisms

The processing and handling operations of fruits include contact between the fruits and stainless-steel surfaces which exist on conveyors, slicers, and storage equipment. The plastic and metal surfaces serve as sites for pathogen transmission because biofilms can develop on these surfaces, which makes standard cleaning methods ineffective (Fernandez et al., 2023). The distribution process requires sanitary transport packaging because it protects the safety and quality of fruits. The contamination risk for fruits during transportation arises from three factors, which include frequent handling, extended storage, and contact with contaminated packaging materials (Sarihan Mungan & Aydin, 2022). A certain study shows that packaging materials like reusable plastic crates and containers used during the post-harvest period contain microbial populations that create safety hazards for fruit (Al-Dairi et al., 2022). The damage to packaging materials which occurs during transport leads to the creation of new surfaces which become easier for microbes to attach to and which help microbes to multiply. The most common pathogens that pose risks to the safety of fresh fruits and their consumers include:

2.3.1 *Escherichia coli*

The fruit industry faces substantial challenges from pathogenic *E. coli* especially the O157:H7 strain, a leading cause foodborne illness. The bacterium exists naturally in the intestines of humans and animals, and it enters the fruit production system through water irrigation and soil contamination and through contact with contaminated surfaces during harvesting and processing. Equipment used in the process, such as plastic crates and handling surfaces, can also lead to cross-contamination (Richiardi et al., 2023). Pathogenic *E. coli* infections produce symptoms including diarrhea and abdominal cramps, and vomiting, which can progress to severe cases that result in haemolytic uremic syndrome. *E. coli* serves as a standard indicator organism for detecting fecal contamination in both food and environmental samples. *E. coli* exists as a normal intestinal bacterium in humans and warm-blooded animals; therefore, finding them outside these host indicates fecal contamination, together with possible enteric pathogen contamination. The abundant presence of *E. coli* in feces provides an effective method to assess hygienic quality because standard microbiological methods allow for its easy detection, and its presence indicates potential health dangers (Odonkor & Ampofo, 2013). *E. coli* functions as a common surrogate organism used to measure microbial contamination in water testing, food inspections, and surface sanitation evaluations.

2.3.2 *Staphylococcus aureus*

The bacterium *S. aureus* functions as a common foodborne pathogen because it produces heat-stable enterotoxins that cause foodborne illnesses. The bacteria can remain active on various surfaces that contact food, which includes both fresh produce and stainless-steel equipment. The bacterium frequently exists on human skin and in nasal passages, which creates a risk for contamination that happens when people do not practice proper hygiene during the handling and processing of food. Consumption of food contaminated with *S. aureus* toxins may result in symptoms such as nausea, vomiting, abdominal cramps, and diarrhea (McFeters, 2024).

2.3.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa demonstrates the unique ability to create biofilms on all types of surfaces which extend to food-contact materials that are utilized in fruit processing facilities. The organism primarily acts as an opportunistic pathogen which targets people who have weakened immune systems, yet it still poses a risk of contaminating food-processing facilities through its ability to spread via infected water, equipment, and surfaces during the process of handling and storage (Campos et al., 2020).

2.3.4 *Listeria monocytogenes*

Listeria monocytogenes exists as a critical foodborne pathogen because its ability to reproduce at cold temperatures enables it to survive throughout refrigerated storage and distribution. Fruits may become contaminated during post-harvest handling, processing, or storage through contact with contaminated equipment or environments. *L. monocytogenes* infection results in listeriosis which is a severe disease that primarily targets pregnant women, newborns, elderly individuals and people who have compromised immune systems (Richiardi et al., 2023).

2.3.5 *Salmonella spp.*

Salmonella species constitute one of the leading causes of foodborne illnesses throughout the globe. The bacteria enter the fruit through its contact with infected irrigation water and soil and processing equipment which occurs during the harvesting and handling process. *Salmonella* infection causes patients to experience diarrhea along with fever and abdominal cramps and vomiting. (McFeters, 2024).

2.3.6 *Clostridium botulinum*

Clostridium botulinum is a bacterium that forms spores and produces botulinum toxin, which causes the disease known as botulism. While canned foods that have been improperly processed represent the primary source of contamination, fruit-based products become contaminated when spore survival and toxin production conditions exist, especially in low-acid environments or through improper storage practices.

2.4 Biofilms

Biofilms constitute a significant problem of microbial control, especially in food industries where sanitation is paramount to food safety. Bacterial biofilms represent consortia of microorganisms that are surrounded by an extra-cellular matrix which is self-produced and attached to the food contact surfaces, making it very difficult to clean by external means (Liu et al., 2024). Biofilms produced by *E. coli*, *S. aureus*, and *P. aeruginosa* can survive in food processing environments due to this property even though these areas are sanitized on a routine basis. The biofilm matrix restricts the diffusion of sanitizers, and this produces a protective environment in which bacterial colonies cannot be fully eliminated (Shree et al., 2023). Biofilms develop when bacteria clinging to a surface start to secrete an extracellular matrix which acts as a protective venerable enabling the bacteria to survive in a colony and become ever more resistant to cleaning agents and disinfectants (Figure 1).

Complete comprehension of biofilm organization and behavior is necessary for creating effective methods to combat bacteria that persist and develop resistance within the food industry (Zhao et al., 2023). The new biofilm disruption technologies that use dispersal enzymes and innovative antimicrobial solutions enable effective biofilm sanitation through their ability to dismantle biofilm structural components (Wang et al., 2023). The existing methods require further examination to reach their full operational potential within fruit processing and handling facilities. In this situation, high antimicrobial effectiveness sanitizers function as essential tools for controlling biofilm development. The fruit industry will benefit from sanitizers which enhance food-contact surface and equipment sanitation because they can disrupt biofilms to protect fresh produce from microbial contamination and decrease the chances of pathogen spread (Philipp et al., 2024).

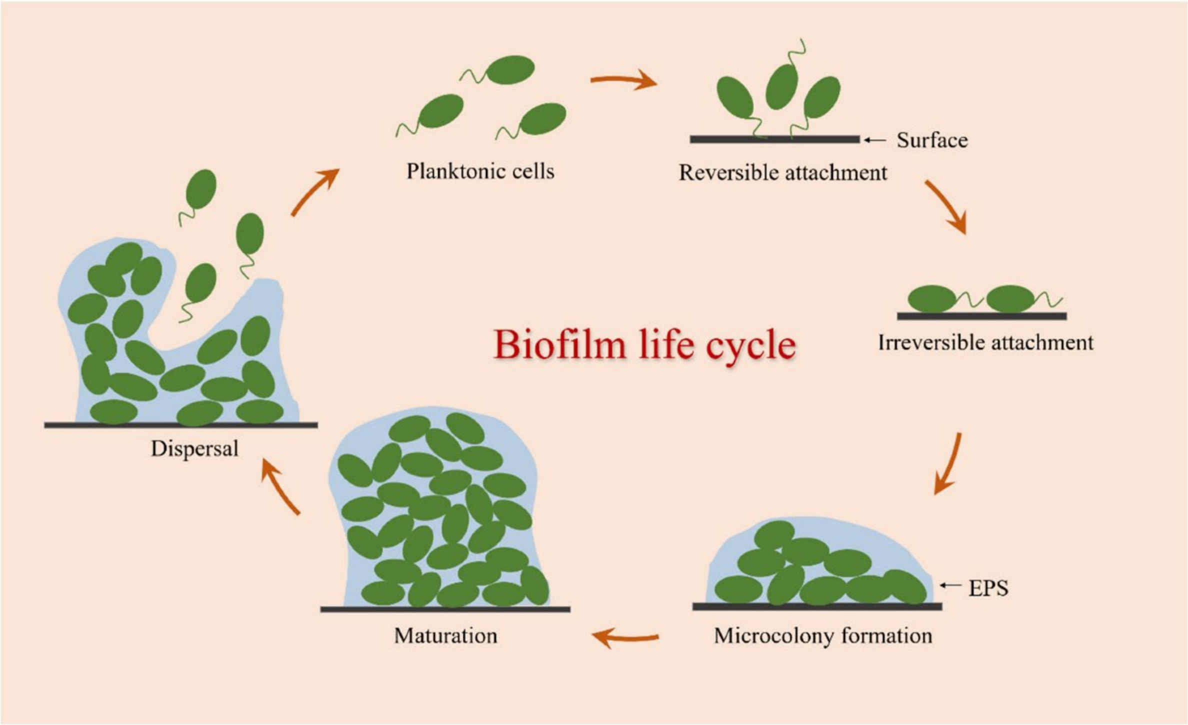

Figure 1. Biofilm Life Cycle. Note. Phases against Pathogenic Bacterial Biofilms and Biofilm-Based Infections: A Review (Reproduced from the Source: Sharma et al., 2024)

2.5 Sanitizers

2.5.1 Different Sanitizers used in the Fruit Industry

The fruit industry needs effective sanitization practices to prevent microbial contamination, which occurs during post-harvest handling and processing. Food production environments use different types of sanitizers which operate through unique mechanisms. The food processing industry has historically used chlorine-based sanitizers which include bleach solutions as common disinfectants. These sanitizers can kill many different types of microorganisms which makes them suitable for disinfecting surfaces. The presence of organic matter, such as fruit residues, soil, and plant material, will decrease their antimicrobial effectiveness (Hu et al., 2022).

2.5.2 History of Sanitizers in the Fruit Industry

The fruit industry has employed sanitizers since the necessity of more efficient and environmentally friendly methods has become more evident. Original sanitizers were based on chlorine compounds, iodine compounds, and alcohol-based sanitizers. Nevertheless, this was not the case when it came to newer and more efficient sanitizing agents, due to the problem of antimicrobial resistance (AMR) and the formation of resistant biofilms on surfaces such as plastic crates and stainless steel (Bland et al., 2022).

2.5.3 Current Sanitizers Used in the Fruit Industry

Currently, several different sanitizers are available in the fruit industry, with each having its weaknesses and strengths:

- Chlorine Compounds: Chlorine-based sanitizers prove effective for controlling numerous microorganisms, which have become standard disinfectants throughout food processing facilities. The presence of organic materials, which include fruit residues and soil, together with plant debris, will cause chlorine to lose its ability to kill germs.
- Iodine Compounds: Iodine-based sanitizers function as broad-spectrum disinfectants which possess low harmful effects on human health. The product will create stains on equipment and surfaces, which will require extended contact time before it can effectively kill all germs present.
- Alcohols and Proxygenes: These sanitizers provide two main benefits because they function as environmentally friendly disinfectants which kill a wide range of germs. The quick evaporation of alcohol-based sanitizers creates a challenge because their antimicrobial properties become ineffective after a short time.
- Quaternary Ammonium Compounds (Quats): The sanitizers in this category provide two main advantages because they do not create corrosion damage, but they can kill multiple bacterial strains. The presence of organic materials will hinder their ability to interact with microbial cell membranes (Bland et al., 2022).

The search for sustainable solutions began because traditional sanitizers which food processors used, experienced efficiency problems when dealing with biofilm control and complex environmental conditions.

2.6 Electrolysed Water: A Modern Solution

Electrolysed water serves as a potential disinfectant alternative because its production process requires electrical current to transform salt solutions into hypochlorous acid, which functions as a powerful antimicrobial disinfectant. The ANK Neutral Anolyte electrolysed water solution has demonstrated effective pathogen control against both *L. monocytogenes* and *Salmonella* species (Kim et al., 2003). The electrolysis of water produces chemical byproducts, which break down into safe materials that leave behind minimal chemical traces after use (Hu et al. 2022). The sanitizers provide better environmental protection because they produce smaller amounts of harmful by-products during their operation, which allows their use in fresh produce sanitation. The presence of organic matter, which includes fruit residues and plant debris, causes electrolysed water to reduce its ability to protect against harmful microorganisms. The equipment used for processing becomes more vulnerable to damage when exposed to solutions that contain elevated levels of free chlorine. The fruit industry can benefit from electrolysed water technology because it delivers effective pathogen reduction and biofilm disruption while protecting the environment (Bland et al. 2022).

2.7 Organic Acids and 77X: Focus on Biofilm Control

Researchers examined different options like organic acids and 77X, which serves as a short-chain fatty acid-based sanitizer. The substances function through their ability to break open bacterial cell walls, which enables them to kill harmful bacteria, specifically *S. aureus* and *P. aeruginosa*. According to Klein et al. (2022), 77X presents a safer and more environmentally friendly choice for sanitizing fruit because it operates with diminished environmental effects while being biodegradable and non-harmful when compared to standard chemical sanitizers.

The application of organic acid-based sanitizers in real-world situations continues to face multiple limitations. The researchers found that it becomes challenging to identify which operational conditions produce the best outcome because they require specific details about concentration levels, contact durations, and temperature settings needed for peak antimicrobial performance (Bhatt et al., 2024). Organic acid-based sanitizers demonstrate restricted capacity to eliminate existing biofilms from surfaces that come into contact with food. The researchers need to conduct additional studies to determine the most effective application procedures of sanitizing agents, which they will use to assess how well these sanitizers protect fruit processing facilities.

2.8 Sanitizers Used in the Fruit Industry: Traditional vs. Emerging Alternatives

Fruit industry sanitizing methods need to become safer and more environmentally friendly because people now require better protection from dangerous pathogens and environmental pollution. Sanitizers that use chlorine-based compounds still maintain their popularity, but researchers found their disinfection power decreases when microorganisms exist in biofilms or organic materials block sanitizing functions (Hu et al. 2022; Bland et al. 2022). The excessive application of chlorine-based sanitizers creates dangerous substances that harm fruit quality and endanger customer protection.

The industry is investigating new sanitization methods through research. ANK Neutral Anolyte produces hypochlorous acid through its electrolyzed water solution which effectively kills *L. monocytogenes* and *Salmonella* spp. pathogens while creating non-toxic waste products (Hu et al. 2022). The 77X sanitizer which comes from short-chain fatty acid functions as a bactericidal agent that breaks down bacterial cell membranes and scientists have confirmed its ability to break down naturally and its positive effect on the environment (Klein et al. 2022). The sanitizers demonstrate effective (sanitizing properties however additional research must assess their actual performance during real production environments while making sure they can effectively manage biofilm-related pathogens. 77X operates as a sanitizer which uses short-chain fatty acids to destroy bacterial cell membranes while the product remains environmentally safe through its biodegradable properties (Hu et al., 2022).

2.8.1 Alternative Sanitizers: ANK Neutral Anolyte and 77X

The ANK Neutral Anolyte is an electrolysed water solution of hypochlorous acid that has proven to be effective against a wide spectrum of pathogens, such as *Salmonella* and *L. monocytogenes* (Fabrizio & Cutter, 2003; Riešutė et al., 2022). Its strength is that it is environmentally friendly because it decomposes into non-toxic compounds after use and thus represents a harmless option when it comes to sanitizing post-harvested fruits (Saxena, 2024). In the same way, the 77X product, which includes a mixture of short-chain fatty acids and alcohols, has proved to be effective in killing bacteria such as *S. aureus* and *P. aeruginosa*

(Klein et al., 2022). By offering high antimicrobial properties and leaving no harmful residues, such sanitizers are a good alternative to the traditional chemicals that are harmful to the environment. Since the fruit industry remains focused on food safety, more effective sanitizing agents will be essential in minimising the pathogen contamination and maintaining consumer health (Bhatt et al., 2024).

2.8.2 Classes of Sanitizers

Sanitizers that are utilised in food processing and fresh produce industries can be categorised into various classes depending on their composition. Some of the main classes of sanitizers that are frequently utilised in controlling microbes in food processing industries include:

- Alcohols
- Chlorine Compounds
- Iodine Compounds
- Quaternary Ammonium Compounds (Quats)
- Peroxygens (Oxidising Agents)
- Phenolics
- Aldehydes
- Biguanides
- Acids
- Alkalis
- Heavy Metals
- Natural/Biobased Sanitizers

Researchers have studied different chemical and natural disinfectants to control microbial growth in water treatment facilities and food processing plants. The disinfectants available for use include aldehydes and quaternary ammonium compounds as well as chlorine-based compounds, alkalis, and heavy metals, because these substances provide strong antimicrobial protection together with broad-spectrum killing capabilities. The investigation of different methods for disinfection arose because people developed safety concerns about the dangerous health effects, environmental hazards, equipment damage and toxic byproducts that result from current disinfection methods. The scientific community has started to focus on hydrogen peroxide and peracetic acid as replacement oxidizing agents, while new technologies such as electrolyzed water and UV-based advanced oxidation processes gather scientific interest. The rising demand for environmentally sustainable food processing methods has prompted researchers to investigate organic acids, plant-derived compounds and other biobased sanitizers as environmentally safe disinfectant alternatives. The major sanitizing agent categories which academic research has identified in the literature are shown in Table 1, together with their corresponding references that demonstrate their ability to fight against microorganisms.

Table 1: Characteristics, Advantages, and Disadvantages of Sanitizer Classes

Sanitizer Class	Characteristics	Advantages	Disadvantages	References
Alcohols	Typically includes ethanol and isopropanol. Kills by denaturing proteins.	Fast-acting, effective at killing a broad range of microorganisms.	Evaporates quickly, may not leave residual protection, can be flammable.	McDonnell, and Russell (1999)
Chlorine Compounds	Includes bleach and hypochlorite solutions. Works by oxidising and disrupting cell walls.	Widely available, inexpensive, and effective at high concentrations.	Can leave harmful residues, may cause corrosion, less effective in the presence of organic matter.	Huang et al., (2022)
Iodine Compounds	Includes iodine tinctures and iodophors. Effective against bacteria and viruses.	Broad-spectrum, low toxicity when used properly.	Stains surfaces, can be corrosive, may require longer contact times.	Rutala & Weber, (2019)
Quaternary Ammonium	Includes compounds like	Low toxicity, effective against	Can leave residues,	Gerba (2015)

Compounds (Quats)	benzalkonium chloride. Works by disrupting microbial cell membranes.	a wide range of microbes, non-corrosive.	effectiveness may decrease in the presence of organic matter or hard water.	
Peroxygens (Oxidising Agents)	Includes hydrogen peroxide. Kills by oxidation, breaking down microbial structures.	Powerful disinfectant, breaks down into non-toxic byproducts (water and oxygen).	Can be corrosive to some surfaces, requires careful handling.	Block (2001)
Phenolics	Includes phenol and its derivatives. Denatures proteins and disrupts cell walls.	Effective against a broad range of pathogens, including fungi and bacteria.	Toxic at high concentrations, unpleasant odour, may irritate the skin.	McDonnell, and Russell (1999)
Aldehydes	Includes formaldehyde and glutaraldehyde. Cross-links proteins and nucleic acids.	Very effective against a wide range of pathogens.	Toxic and irritating, potential carcinogen, can be hazardous to handle.	McDonnell, and Russell (1999)
Biguanides	Includes chlorhexidine. Kills by disrupting microbial cell membranes.	Effective against bacteria and some viruses, non-toxic in low concentrations.	Limited effectiveness against certain fungi and spores, may cause skin irritation.	Rutala & Weber, (2019)

Acids	Includes organic acids like acetic acid (vinegar). Works by lowering pH, disrupting microbial function.	Naturally occurring, safe for food contact, effective against certain pathogens like *E. coli*.	Limited spectrum of activity, effectiveness may be reduced in high pH environments.	Gerba (2015)
Alkalis	Includes sodium hydroxide and potassium hydroxide. Alkaline substances that disrupt microbial membranes.	Effective at breaking down biofilms, high alkalinity can break down proteins.	Corrosive, can damage surfaces, requires careful handling.	Marriott et al., (2018)
Heavy Metals	Includes copper and silver compounds. Disrupts enzyme activity and cellular processes.	Effective against a wide range of microbes, especially fungi and bacteria.	Toxicity concerns, accumulation in the environment, potential to cause resistance.	Lemire et al., (2013)
Natural/Biobased Sanitizers	Derived from natural sources like plant extracts. May contain essential oils.	Environmentally friendly, biodegradable, and often non-toxic.	May have limited effectiveness, often require longer contact times, potential for allergic reactions.	Gyawali, & Ibrahim, (2014)

2.9 Electrolysed Water in the Food Industry and Organic Acids in Fruits

Research conducted by Ren et al. (2025) demonstrated that Slightly Acidic Electrolysed Water (SAEW) could eliminate *L. monocytogenes* biofilms on surfaces, which further confirms the product's potential as an acceptable alternative to traditional disinfectants. The study established that SAEW in conjunction with ultrasonic treatment was especially useful in the disruption of biofilm, one of the most persistent problems in the food sector, particularly with regard to plastic surfaces where fruits are kept. Ultrasound can be used together with SAEW to produce an effect of synergy that amplifies its ability to kill microbes (Kong et al., 2023). In addition, the applicability of SAEW is not limited to the sanitation of fruits; it can also be applied in meat processing and even water treatment, which means that it is a multi-purpose sanitizing agent (Zhang et al., 2022).

Organic acids play a crucial role in the taste, preservation, and the nutritional value of fruits. For instance, in the ripening of such fruits as peaches, the variety of organic acids is one of the key factors that influence the flavour profile and quality of the product. The comparative network analysis by Jiang et al. (2023) identified the shift in the amounts of organic acids in peaches within the ripening process and the most notable citric, malic, and quinic acids. Such acids are known not only to make the fruits sour, but they also have antimicrobial properties and hence are relevant in food safety. When it comes to cucumbers, the perception of freshness and the general taste of the fruit is also correlated with organic acids, especially citric and malic acid, which further highlights how these acids play a vital role in customer satisfaction (Du et al., 2022).

2.10 Chlorinated Water

Food processing facilities have used chlorinated water since ancient times to achieve sanitation through its application in both water treatment processes and the surface disinfection of fruits and vegetables. The application of chlorine-based disinfectants demonstrates their ability to decrease microbial contamination, but people have expressed worries about the formation of disinfection by-products (DBPs) that occur during their usage (Huang et al., 2022). Chlorine reacts with organic matter to produce these by-products, which contain some DBPs that scientists link to health dangers that include carcinogenic effects (Richardson et al., 2007). Researchers actively explore new sanitizing solutions and existing treatment methods because they want to decrease compound formation while sustaining their ability to kill germs.

Researchers studied different methods to keep chlorine's disinfecting power intact while reducing health dangers that come with its use. Guo et al. (2022) proposed the UV/chlorine process as an advanced oxidation technology with promising applications in water treatment. Through this method, ultraviolet (UV) light activates chlorine compounds which produce highly reactive hydroxyl and chlorine radicals that kill pathogens while decreasing the creation of dangerous waste materials. The combined method enhances control over microorganisms while providing a safer method for disinfecting purposes. Since the fruit industry has maintained a vested the interest of thein food safety as well as environmental sustainability, these advanced treatment methods might be considered as an alternative to the traditional chlorinated water treatment, as they, which provides safer and more effective solution to the issue of pathogen control in fruit production.

2.11 Summary

The literature review explained how the fruit industry is hard-pressed with the problem of control of the pathogens, especially in the presence of microbial biofilms over the surface of contact between fresh produce and surfaces. Organic acids are naturally occurring in fruits and play a crucial role in flavour and preservative purposes. In fact, they have antimicrobial effects that can potentially help lower the level of microbial contamination. Their effectiveness, however, can differ depending on the kind of fruit and how mature the fruit is. Electrolyszed water, including slightly acidic electrolysed water (SAEW), has become a potential substitute for the traditional chemical sanitizers since it has the potential of effectively eliminate pathogens without leaving any negative residues behind. Although quaternary ammonium compounds (QACs) may be effective, there is a risk of microbial resistance developing, and they should be used with care to avoid overuse. This shows the necessity of using other sanitizing or cleaning agents like chlorinated water and 77X, both of which seem to control the pathogens without having the disadvantage of leaving any residue, to offer fruit businesses a more effective yet sustainable alternative and resistance, which will help the fruit business to be sustainable.

The literature reviewed demonstrates that effective sanitation strategies are essential for controlling microbial contamination and biofilm formation at food-contact surfaces in the fruit industry. Researchers have studied conventional sanitizers which use chlorine-based compounds together with new cleaning methods that include electrolyzed water and organic acid-based sanitizers. The methods show different levels of antimicrobial power yet they fail to function properly when organic materials are present and biofilms continue to exist on processing equipment. Researchers need to test different sanitizing products to find their most effective uses in fruit processing facilities. The study tests how well ANK Neutral Anolyte and 77X can kill foodborne pathogens on fruit and stainless-steel surfaces.

2.12 Aim and Objectives

Aim: To evaluate the effectiveness of ANK Neutral Anolyte and 77X in controlling pathogens on pre-harvest citrus fruit and their suitability as sanitizers for fruit processing materials.

Objectives:

- To determine the optimal conditions (time and concentration) for ANK Neutral Anolyte and 77X for killing *E. coli* ATCC 25922, along with *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027.
- To compare the efficacy of ANK Neutral Anolyte and 77X in removing biofilms of *E. coli* ATCC 25922, along with *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027 on plastic *in vitro*.
- To compare the effectiveness of ANK Neutral Anolyte and 77X in sanitizing artificially contaminated harvested citrus fruit and stainless-steel surfaces with *E. coli* ATCC 25922.

3. Sanitizer Optimization

3.1 Introduction

The worldwide fruit market functions as a fundamental component of agricultural production. Microbial contamination of fresh fruits remains a major food safety concern, particularly during post-harvest handling and packaging operations. The study assessed bacterial samples through two standardized antimicrobial susceptibility testing methods, which combine disc diffusion assay with minimum inhibitory concentration testing. The research methods enable testing of antimicrobial effectiveness methods on planktonic bacterial cells, which can provide optimal conditions for sanitation, including concentration and exposure time.

The disc diffusion method, namely the Kirby–Bauer technique, has served as a basic method for testing antimicrobial substances in microbiology labs since Alfred W Bauer and William Kirby established it in 1966. The test requires the application of a standardized inoculum which meets the 0.5 McFarland turbidity standard across the surface of Mueller-Hinton agar before researchers position sterile discs containing test substances onto the agar surface. In this research, they examine the effectiveness of antimicrobials by checking the clear areas on agar plates. These zones of clearing are created by antimicrobials permeating through the agar and preventing bacteria from multiplying. These zones are measured, and resistance is determined by pre-determined breakpoints outlined by Taken from EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing Version 13.0 (January 2025).

For the testing, disc assays investigate *E. coli* ATCC 25922, along with *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027. This should give us a clue about the antimicrobial properties. Different sanitizers could show different inhibition zones, probably because their active ingredients spread out differently and attack the bacteria in their own unique ways (Golin et al., 2020).

The Minimum Inhibitory Concentration (MIC) test determines the lowest dose of an antibiotic required to completely halt bacterial growth. The disc diffusion method facilitates the comparison of sanitizers' performances across various products, and disc diffusion is quicker than MIC (Schuetz et al., 2025).

The disc test is a screening system in order to determine how different antimicrobial agents perform (Feßler et al., 2023), while MIC testing gives specific details on the most effective concentration (Kowalska-Krochmal & Dudek-Wicher, 2021). By following the testing procedures established by disc diffusion and MIC, these methods used for this study comply with international standards. This chapter aims to test two commercially available sanitizers, Anolyte and 77X, against *E. coli* ATCC 25922, along with *S. aureus* ATCC 6538*,* and *P. aeruginosa* ATCC 9027 using these two methods.

3.2 Materials and methods

3.2.1 Culturing and Maintenance of Bacterial Isolates

The bacterial strains *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027 were obtained from the New Zealand Reference Culture Collection (Public Health and Forensic Science, New Zealand). Cultures were grown on a Mueller Hinton Broth for 16-20 hours at 37 °C under aerobic conditions. Stock cultures were stored at -80 ºC in 20% w/v glycerol until required.

3.2.2 Disc Diffusion Methods for Antimicrobial Susceptibility Testing

The disc diffusion assay was executed based on the EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing, which presents its procedures in Version 13.0, which was released in January 2025. The microorganisms tested in this study were *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027. The study used Mueller–Hinton agar (MHA) plates, sterile loops, 0.85% saline solution, 0.5 McFarland standard, sterile cotton swabs, sterile centrifuge tubes, sterile petri dishes, sterile water, Mueller–Hinton broth (MHB), MMG medium 77x, Anolyte, and commercially prepared antibiotic discs of Ciprofloxacin, Augmentin and Meropenem served as controls for *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027*,* respectively.

In this study, several well-isolated colonies from each plate were selected and transferred to sterile saline solution within centrifuge tubes. This study adjusted each suspension's turbidity by adding more colonies or additional saline until the optical density at 600 nm matched a 0.5 McFarland standard, which corresponds to an approximate OD_{600} of 0.1. The agar plates were inoculated within 15 minutes after the preparation of the inoculum. Then, each bacterial suspension was thoroughly mixed, and a sterile cotton swab was immersed until fully saturated. The swab was then pressed against the inner wall of the tube to remove excess fluid and then spread the inoculum across a fresh MHA plate to create uniform lawn growth with no gaps. A new sterile swab for each bacterial strain and plate.

The antibiotic discs were plated directly after they were inoculated with the test samples. The discs were placed on the agar surface using sterile forceps to establish complete contact with the surface. The treatments included sterile water as negative control, and either Meropenem, Augmentin, and Ciprofloxacin as a positive control for *E. coli* ATCC 25922, *S. aureus* ATCC

6538, and *P. aeruginosa* ATCC 9027 respectively. Tests discs were impregnated with 77x and Anolyte. Afterwards, maintained the appropriate disc separation to prevent the inhibition zones from creating overlapping regions. The plates were inverted and incubated at 35 ± 1°C for a total duration of 18 ± 2 hours. After incubation, the diameter of the zones of inhibition around each disc was measured in mm. Measurements were recorded and averaged among biological triplicates for each bacterial strain.

3.3 Determination of the minimum inhibitory concentration (MIC) of Anolyte and 77X against E. coli, S. aureus, and P. aeruginosa

The MIC of Anolyte and 77X sanitizers was determined as per the EUCAST guidelines (2025). Bacterial cultures *of E. coli, S.* aureus and *P. aeruginosa* were cultured in Mueller-Hinton Broth (MHB). The test included three organisms, namely *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027. The assay required Anolyte and 77X Mueller-Hinton Broth (MHB), and sterile flat-bottom 96-well microtiter plates (Fig 2).

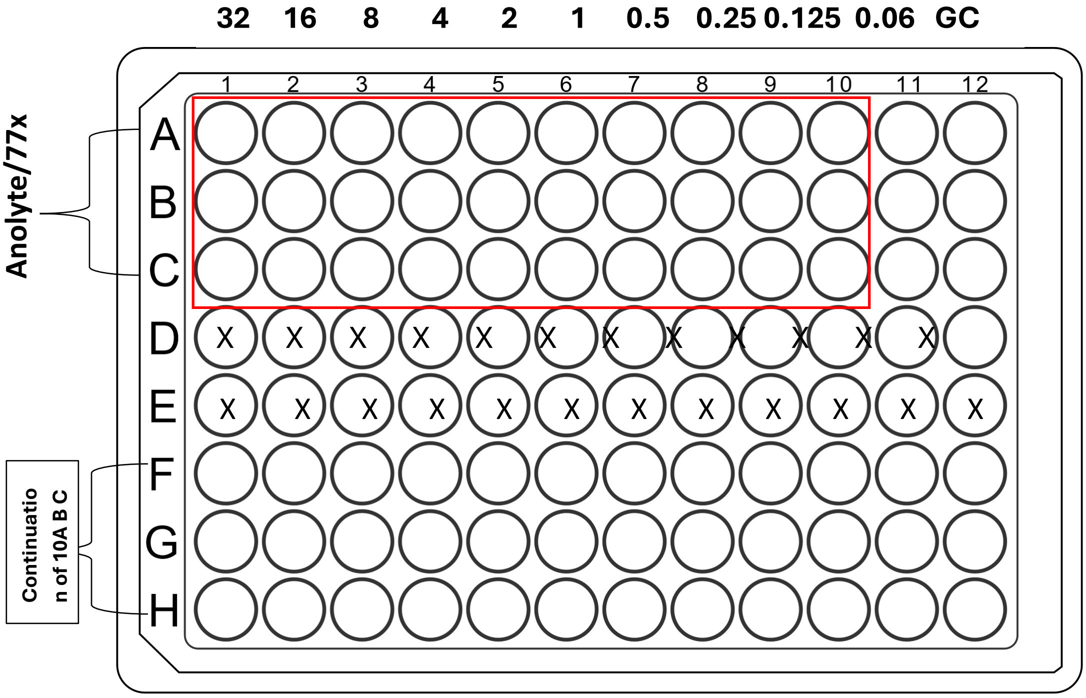

Figure 2. Representation of a layout for a 96-well microtiter plate used for MIC testing.

The Anolyte and 77X testing solutions were prepared by mixing both compounds at a 1:10 ratio with sterile distilled water. Bacterial cultures were prepared by transferring a loopful of glycerol stock culture into MHB and were incubated at 37°C overnight to create fresh growth. Then, the optical density (OD) of each culture was measured at 600 nm, and the inoculum was adjusted until the OD600 value was below 0.1. A sterile 96-well plate was prepared to conduct the broth microdilution assay. The growth control (GC) wells contained inoculated broth that lacked both Anolyte and 77x, while the sterility control (SC) wells contained only sterile MHB. The required empty wells were designated as required. 50 µL of MHB was added to columns 2 through 11 to create serial dilutions that matched concentration levels from 32 to 0.06 µL/mL standard. 100 µL of MHB was added to column 12, which functioned as the sterility control. 100 µL of the prepared Anolyte or 77x working solution was added to column 1 in rows A to C and F to H. A two-fold serial dilution was conducted by transferring 50 µL from column 1 to column 2, followed by thorough mixing. The 50 µL transfer and mixing procedure was executed throughout the plate until column 10 was reached. 50 µL after the mixing process was discarded to keep all wells at equal volume. 50 µL of standardized bacterial inoculum from MHB was introduced into columns 1 through 11 (32-0.06µL/mL). Each test well contained a total volume of 100 µL. OD600 was measured immediately after inoculation or incubated for 24 hours at 37°C before making the measurement. The MIC was identified as the lowest Anolyte or 77x concentration that inhibited visible bacterial growth in comparison with the growth control.

3.4 Results

3.4.1 Disc diffusion assay.

Results of the disc diffusion assay for the activity of 77X and Anolyte against three bacterial isolates are presented in Table 1. For all three isolates, no antimicrobial activity was observed for Anolyte, whereas 77X demonstrated activity as indicated by the zone of inhibition measurements. The sanitizer 77X performed nearly as well as the Augmentin positive control for *S. aureus*.

Table 1: Measurements of the diameters (mm) for the zones of inhibition from the disc diffusion assay for the activity of 77X and Anolyte against the three-bacteria *E. coli* ATCC 25922, along with *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027.

Pathogen *E. coli*	**Antibiotic- Meropenem Positive Control**	**77X**	**Anolyte**	**Sterile- water Negative Control**
1ST plate	35 mm	13 mm	0 mm	0 mm
2ND plate	35 mm	13 mm	0 mm	0 mm
3RD plate	35 mm	14 mm	0 mm	0 mm
Mean ± standard deviation	35 ± 0.0 mm	13.33 ± 0.58 mm	0 ± 0.0mm	0 ± 0.0 mm

Pathogen *S. aureus*	**Antibiotic- Augmentin Positive Control**	**77X**	**Anolyte**	**Sterile- water Negative Control**
1ST plate	20 mm	18 mm	0 mm	0 mm
2ND plate	20 mm	19 mm	0 mm	0 mm
3RD plate	20 mm	19 mm	0 mm	0 mm
Mean ± standard deviation	20 ± 0.0 mm	19 ± 0.58 mm	0 ± 0.0 mm	0 ± 0.0 mm

Pathogen *P. aeruginosa*	**Antibiotic- ciprofloxacin Positive Control**	**77X**	**Anolyte**	**Sterile- water Negative Control**
1ST plate	35 mm	14 mm	0 mm	0 mm
2ND plate	35 mm	15 mm	0 mm	0 mm
3RD plate	35 mm	14 mm	0 mm	0 mm
Mean ± standard deviation	35 ± 0.0 mm	14.33 ± 0.58 mm	0 ± 0.0 mm	0 ± 0.0 mm

In this study, standard antibiotics Meropenem, Augmentin, and Ciprofloxacin were used as positive controls, while sterile water was used as the negative control. The three independent test plates produced measurements, and the mean inhibition diameters are reported in millimeters.

The positive control of Meropenem produced an inhibition zone that measured 35.0 ± 0.0 mm in three tests with *E. coli*. The product 77X produced three test results, which showed inhibition zones measuring 13- and 14-mm. Anolyte and sterile water showed no detectable inhibition (0 mm). The results show that 77X achieved antibacterial effects which worked against *E. coli*, but its effects stayed below the level of standard antibiotics.

The positive control (Augmentin) for *S. aureus* produced a stable inhibition zone that measured 20.0 ± 0.0 mm. The test results showed that 77X produced three different inhibition zones, which reached 18, 19, and 19 mm with an average of 19.0 ± 0.58 mm. Anolyte and sterile water again showed no inhibition (0 mm). The product 77X showed strong antibacterial properties against *S. aureus* because its inhibition zones reached high values that approached those of standard antibiotics.

The positive control (Ciprofloxacin) for *P. aeruginosa* produced the same inhibition zone results, which reached 35 mm for all tests conducted. The 77X test results showed that the product produced three different inhibition zones, which measured 14, 15, and 14 mm while showing an average measurement of 14.33 ± 0.58 mm. Anolyte and sterile water showed no inhibition (0 mm). The product 77X showed antibacterial effects which worked against *P. aeruginosa* but fell short of ciprofloxacin.

The antibacterial efficiency of 77X showed its highest effect against *S. aureus*, which measured 19.0 ± 0.58 mm. The testing results showed that 77X produced its next highest antibacterial efficiency against *P. aeruginosa*, which reached 14.33 ± 0.58 mm and then showed its lowest effect against *E. coli,* which reached 13.33 ± 0.58 mm. The experimental conditions showed that the Anolyte failed to create measurable inhibition zones against any of the organisms which we tested. The assay results showed no inhibition from sterile water, which confirmed the test's accuracy.

The antibacterial activity of 77X was stronger against Gram-positive *S. aureus* when compared to Gram-negative bacteria such as *E. coli* and *P. aeruginosa* because bacterial cell wall structure determines their resistance to antimicrobial agents. However, Anolyte appeared to have no antimicrobial effect against any microorganisms in this susceptibility disc assay.

3.4.2 Minimum Inhibitory Concentration

1.1.1.1 MIC Assay for E. coli

The MIC results for 77X and Anolyte against *E. coli* ATCC 25922 can be seen in Figures 2 and 3. The current study used Optical density (OD600) measurements throughout 24 hours to establish the minimum inhibitory concentration (MIC), which represents the smallest amount required to stop all visible bacterial growth.

The Anolyte-treated *E. coli* showed complete suppression of bacterial growth only when applied the highest concentration of 1/10 as can be seen when the OD_{600} did not change during the testing period. At 1/20 and higher dilutions (1/40–1/5120), bacterial growth resumed after the initial lag phase, with OD_{600} values reaching approximately 1.0–1.4 at 24 h (Fig 3). The intermediate concentrations between 1/40 and 1/160 showed a slight delay in exponential growth, but all other growth periods stayed stable after 1/10 dilution. This study used 1/10 Anolyte as its optimal MIC value against *E. coli* (Fig 3).

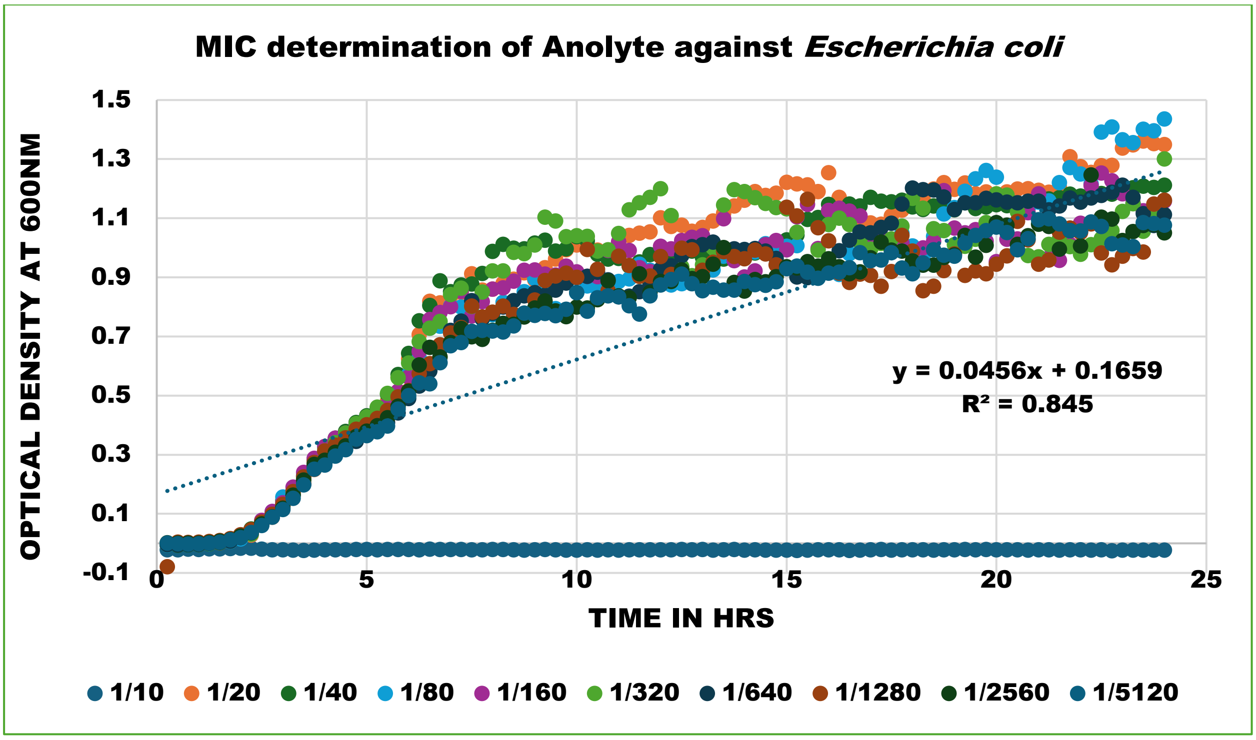

Figure 3: The effect of different concentrations of Anolyte on the growth of *E. coli* 25922 over 24 hours. The study used Anolyte to measure the minimum inhibitory concentration for *E. coli*. The study measured the growth of *E. coli* for 24 hours under different concentrations of Anolyte which ranged from 1/10 to 1/5120, through OD_{600} measurements. The dotted regression line ($y = 0.0456x + 0.1659$; $R^2 = 0.845$) shows the overall growth pattern.

The current study found that Anolyte's minimum inhibitory concentration (MIC) against *E. coli* required a measurement of 1/10. The regression coefficient for Anolyte-treated samples ($R^2 = 0.845$) demonstrated a stronger linear relationship between incubation time and OD increase compared with 77X, indicating more consistent growth progression across sub-inhibitory concentrations. 77X proved to be more effective against *E. coli* than Anolyte because its MIC required a 1/20 dilution to achieve total bacterial growth suppression, whereas Anolyte needed 1/10. The data further demonstrate a clear concentration-dependent inhibitory pattern for both treatments, with bacteriostatic effects diminishing as dilution increased (Fig 3).

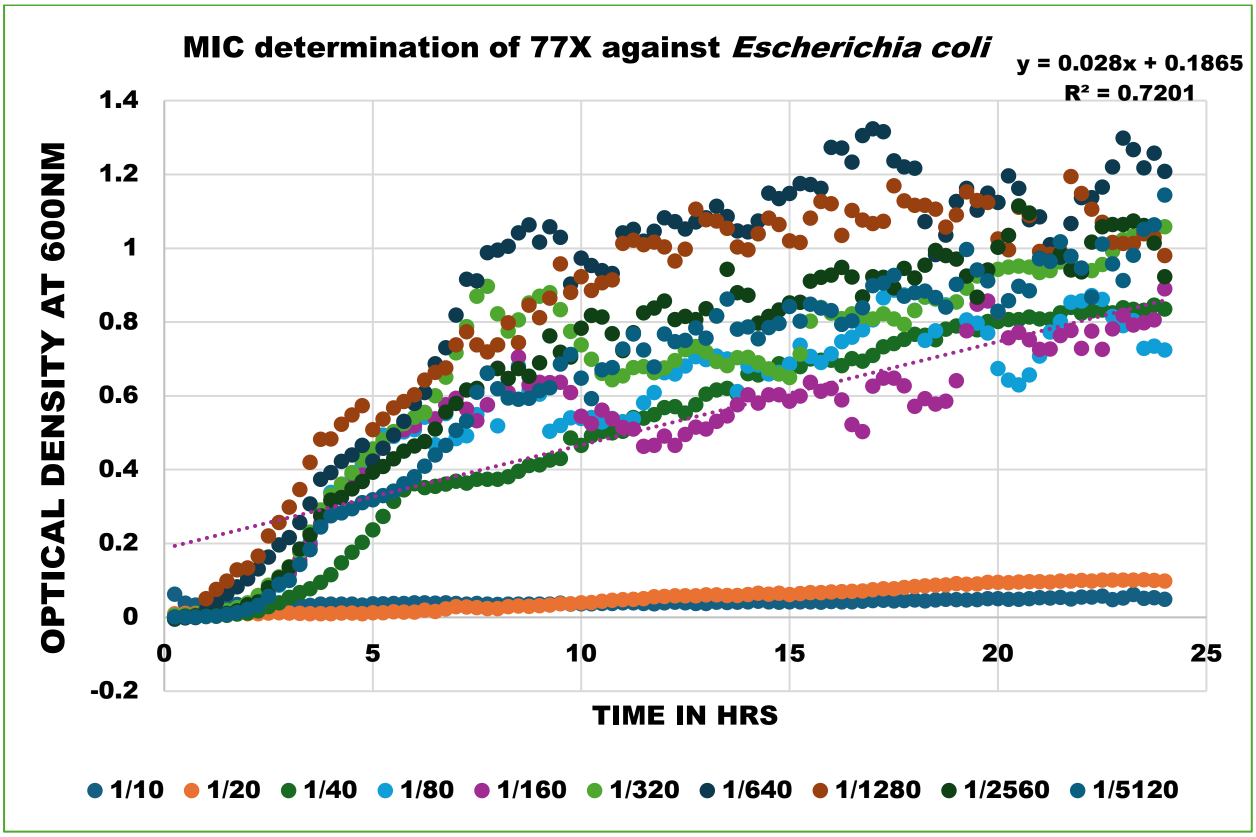

Figure 4. MIC determination of 77X against *E. coli*. The figure displays the growth kinetics of *E. coli* after 24 hours of treatment with serial dilutions (1/10–1/5120) of 77X, which was measured as optical density at 600 nanometers (OD_{600}). The optical density curves display each specific dilution factor through its different curves. The dotted line indicates the linear regression trend ($y = 0.028x + 0.1865$; $R^2 = 0.7201$).

Bacterial growth in 77X showed strong suppression at the two highest test concentrations, which consisted of both 1/10 and 1/20, because the OD600 values stayed below 0.10 during the entire 24-hour testing period. At 1/40 and 1/80 dilutions, delayed growth was observed, with gradual increases in OD after approximately 6–8 h, suggesting partial inhibition. From 1/160 onwards (1/160–1/5120), progressive increases in OD_{600} were recorded, with final values approaching 0.8–1.3, comparable to untreated growth controls. The findings show that 77X achieves MIC against *E. coli* at a dilution range between 1/20 and 1/40 because 1/20 results in complete bacterial growth inhibition. The regression analysis for 77X-treated samples showed a moderate correlation between time and OD increase ($R^2 = 0.7201$), reflecting concentration-dependent growth recovery at higher dilutions (Fig 4).

1.1.1.2 MIC Assay for S. aureus

This current study assessed *S. aureus* growth through 24 hours of OD600 measurements after exposing the bacteria to 77X and Anolyte at serial dilutions from 1/10 to 1/5120.

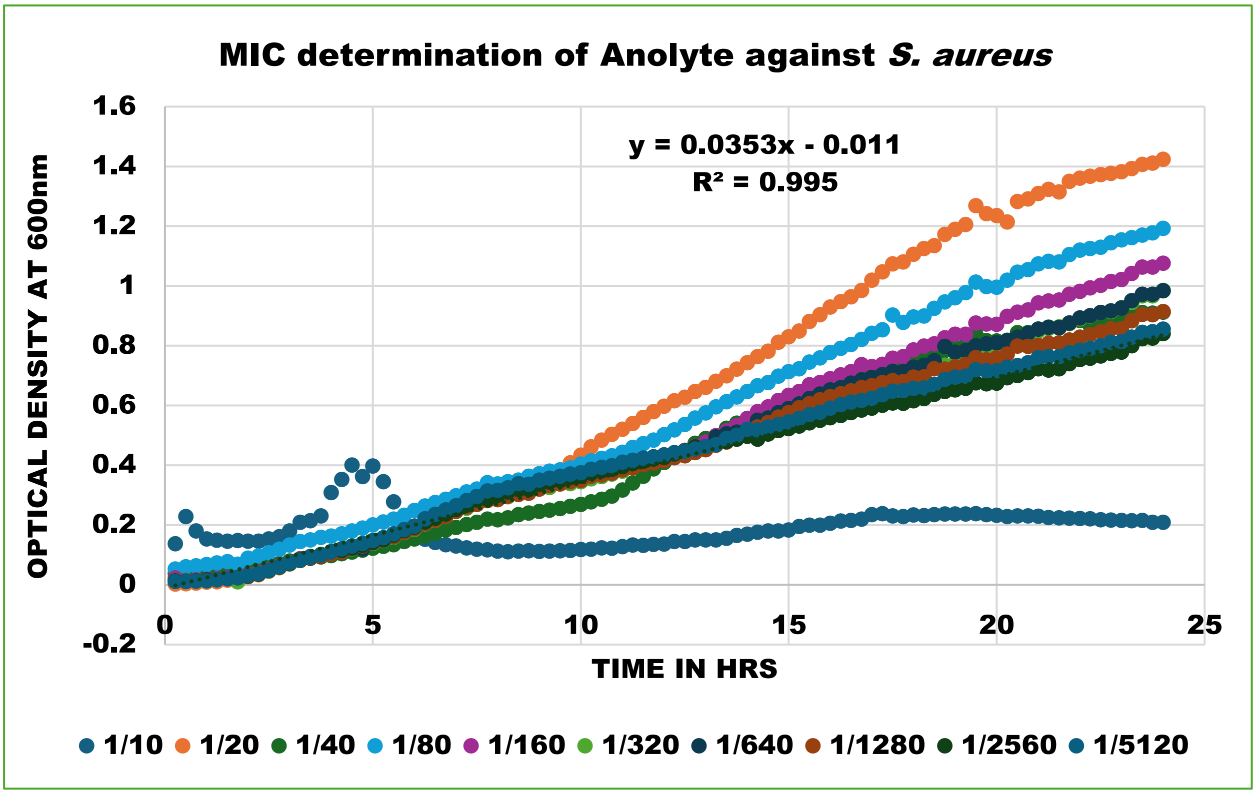

Figure 5. The research shows Anolyte's minimum inhibitory concentration, which was tested against *S. aureus*. The research shows *S. aureus* growth patterns, which were tested with different Anolyte concentrations at 24-hour intervals. The regression line shows bacterial growth patterns which develop with time at various concentrations according to the formula $y = 0.0353x - 0.011$; $R^2 = 0.995$.

The MIC of Anolyte against *S. aureus* remained unmeasured because the testing used dilution amounts below 1/10 (Fig 5). The high regression coefficient ($R^2 = 0.995$) proves that the growth rate developed steadily through each concentration level, which confirms that the bacteria showed only minor resistance. The 77X compound showed significantly greater capacity to inhibit *S. aureus* compared to Anolyte. The 77X product achieved complete inhibition at a 1/20 dilution, but Anolyte remained unable to stop all bacterial growth at any of its tested strengths. Anolyte-treated *S. aureus* showed sustained growth at nearly all dilutions tested. The highest concentration at 1/10 showed OD_{600} values which increased throughout the testing period to reach approximately 0.2 to 0.25 at 24 hours. The 1/20 through 1/5120 dilutions showed typical exponential growth, which produced final OD_{600} values between 0.8 and 1.4. The experimental conditions did not permit any concentration to achieve total suppression of visible growth.

The MIC established the threshold dilution which maintained bacterial growth suppression during the entire testing period. The 1/20 dilution achieved complete growth inhibition for *S. aureus* treated with 77X because OD600 values maintained constant base levels between 0.01 and 0.03 throughout the 24 hours (Fig 6). The 1/10 dilution showed optical density changes because the bacteria did not grow at an exponential rate, which demonstrated that the treatment had strong inhibitory effects. The 1/40 dilution showed partial growth inhibition because OD_{600} values stayed under 0.35 at 24 hours, which resulted in slower growth than what occurs at higher dilutions. The bacteria started to grow after 1/80 because the final OD_{600} readings showed a range of 0.6 to more than 1.1, which demonstrated that the lower concentrations had lost their ability to stop bacterial growth. The MIC of 77X against *S. aureus* reached a value of 1/20 based on collected data. The regression analysis ($R^2 = 0.9752$) established a strong linear growth relationship between sub-inhibitory concentrations, which showed that bacteria recovered their growth capacity at lower treatment levels (Fig 6).

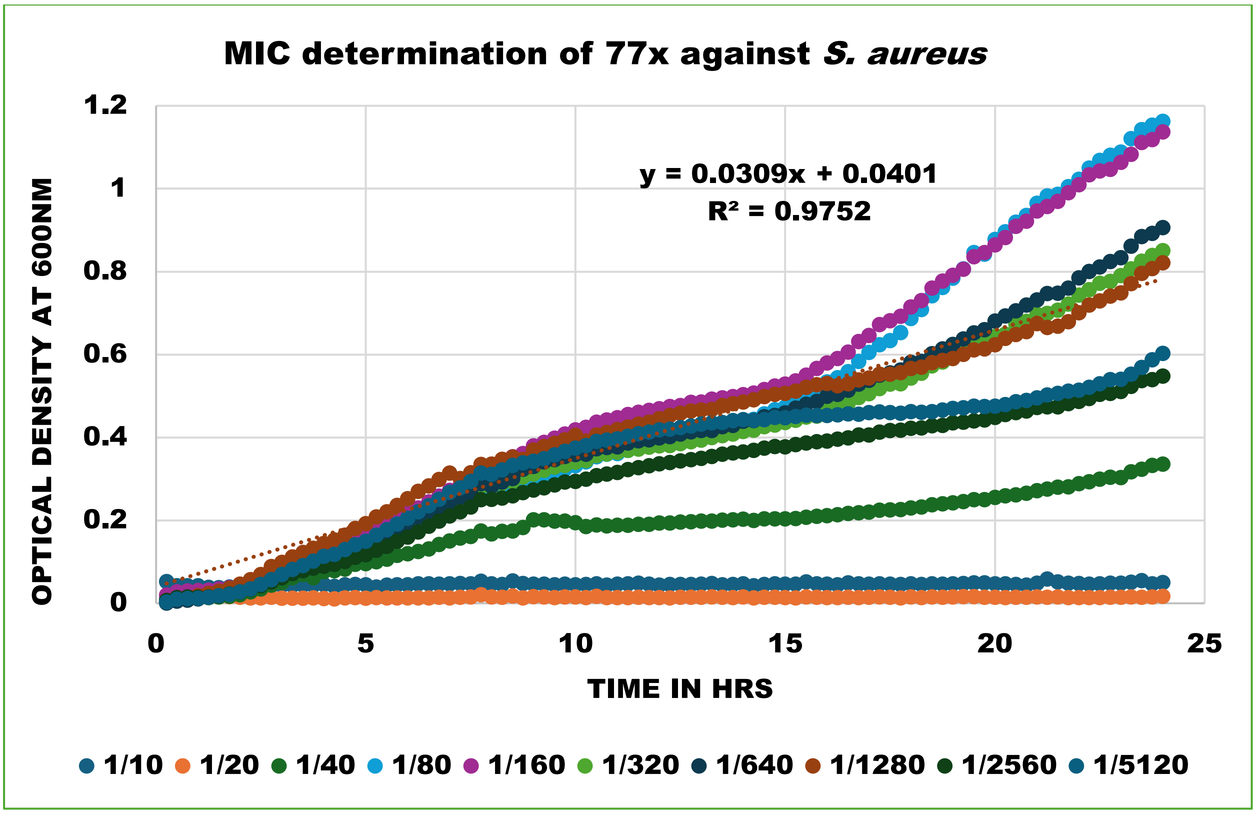

Figure 6. The study used 77X to measure minimum inhibitory concentration against *S. aureus* and measured as OD600 for 24 hours through serial dilutions between 1/10 and 1/5120. The linear regression trend line ($y = 0.0309x + 0.0401$; $R^2 = 0.9752$) shows that time influences.

1.1.1.3 MIC Assay for P. aeruginosa

The growth patterns of *P. aeruginosa* were examined through two experiments, which used 77X and analyte as treatments at dilutions of 1/10 to 1/5120 over 24 hours. The optical density measurements showed how bacteria multiplied, which scientists used to establish patterns of inhibition and determine minimum inhibitory concentration (MIC) values (Jorgensen & Ferraro, 2009).

The MIC Assay for *P. aeruginosa* (Fig. 7) with analyte demonstrated that treatment results established a consistent pattern of blocking. The OD_{600} results showed stable performance across different dilutions because they showed time-based decreases which extended through the mid-to-high dilution range of 1/320 to 1/5120. The regression analysis showed a stronger negative correlation ($R^2 = 0.6356$), which showed a clear time-dependent decrease in bacterial density.

Anolyte demonstrates stronger and more consistent inhibitory activity against *P. aeruginosa* than 77X because its MIC values show lower levels, and its bacterial growth suppression abilities across time show greater strength. The OD decline at higher concentrations for both treatments suggests that bactericidal effects may occur at concentrations above the determined MIC.

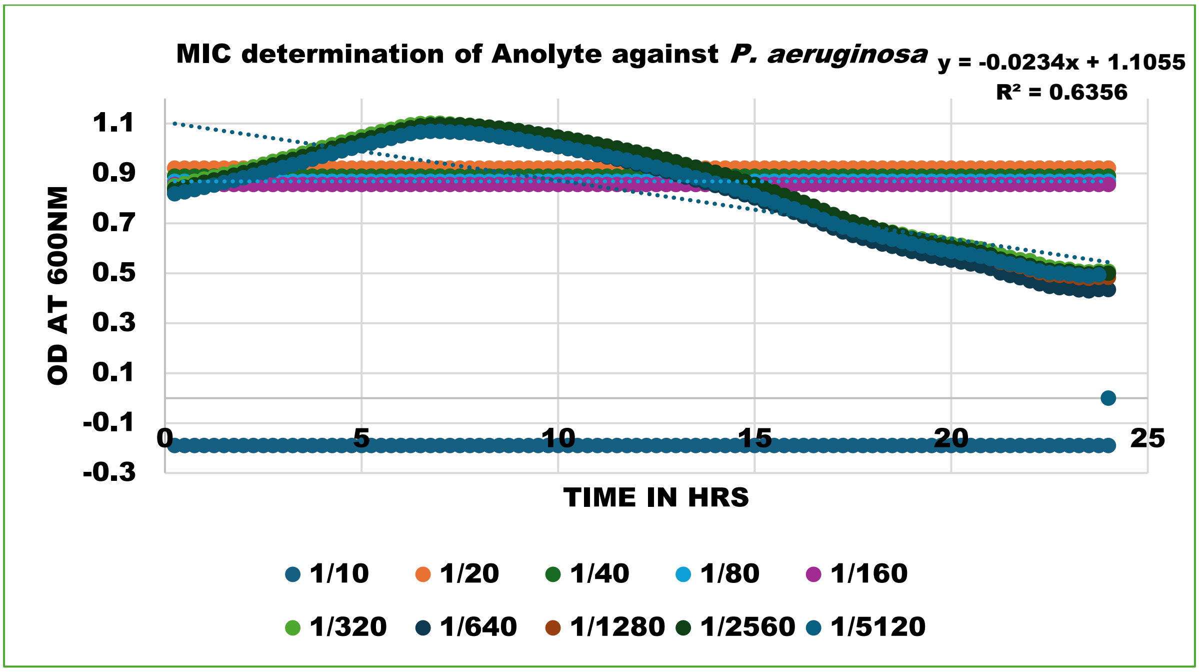

Figure 7. The experiment used optical density measurements at 600 nm to assess the antimicrobial effectiveness of *P. aeruginosa* growth after treatment with anolyte through various serial dilutions, which ranged from 1/10 to 1/5120, over 24 hours, to study the effectiveness of the antimicrobial agent. The anolyte product showed better growth control than 77X because it produced continuous growth inhibition across mid-to-high dilution ranges between 1/320 and 1/5120, which reached its peak at 10 to 12 hours after treatment. The dotted line represents the linear regression trend ($y = -0.0234x + 1.1055$; $R^2 = 0.6356$), which shows stronger time-dependent inhibition.

The treatment of 77X through the application of Fig 8 showed that bacterial growth experienced only partial control because lower dilutions from 1/10 to 1/80 produced rising OD_{600} results that reached maximum bacterial growth between 1.2 and 1.6 after 8 to 14 hours. Bacterial growth experienced a delay at intermediate dilutions between 1/160 and 1/640, but the bacteria continued to grow because their OD_{600} values showed reduced yet ongoing growth, which deviated from normal untreated development patterns. The higher dilutions between 1/1280 and 1/5120 produced more effective treatment because OD_{600} values dropped after the mid-log phase, while some cases reached lower than normal values during later measurements, which indicates that these levels produced bacteriostatic and potentially bactericidal effects. The study used linear regression analysis to identify a relationship between time and growth suppression, which showed weak results ($R^2 = 0.1734$) because different concentrations produced different blocking effects. The growth curves show that 77X reaches its minimum inhibitory concentration at higher dilutions, which maintain visible growth inhibition against lower dilutions.

The study showed that bacterial growth started to decrease after 10-12 hours at higher dilutions because several treatment groups reached OD_{600} values below 0.6 at 24 hours, which indicated they maintained their ability to suppress growth. The cultures treated with anolyte showed different growth patterns from 77X because their exponential growth peaks were less intense and their growth suppression occurred consistently across all tested concentrations. The MIC for anolyte appears to occur at a lower dilution because visible growth reduction moves between 1/320 and 1/640 and continues through all incubation time. The decline in OD_{600} at later time points demonstrates that higher concentrations of the substance can kill bacteria.

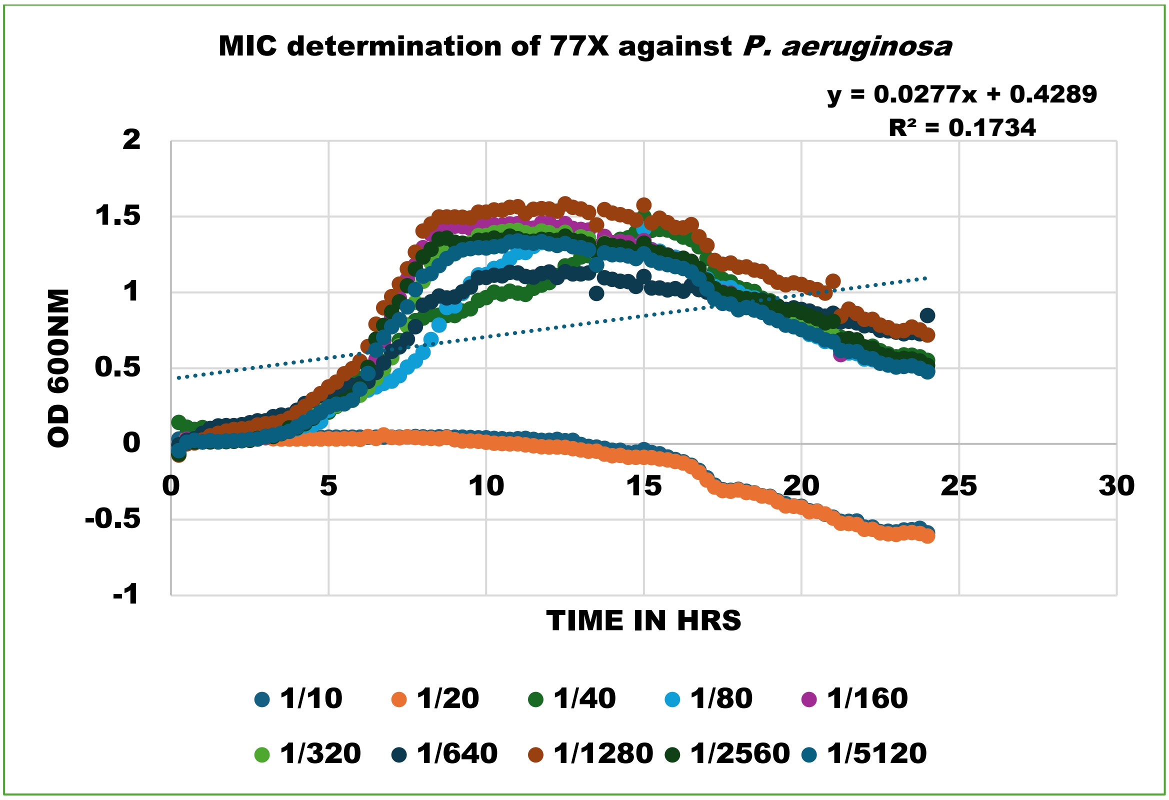

Figure 8. The experiment used optical density measurements at 600 nm to determine the antimicrobial effectiveness of *P. aeruginosa* growth after treatment with 77X through various serial dilutions, which ranged from 1/10 to 1/5120. The dotted line represents the linear regression trend ($y = 0.0277x + 0.4289$; $R^2 = 0.1734$), which shows weak time-dependent inhibition. The 77X concentration data demonstrates its growth-inhibiting effects on *P. aeruginosa*.

3.5 Discussion

The disc diffusion assay showed that 77X exhibits antibacterial properties against all three tested pathogens, with the strongest effectiveness against *S. aureus*. Microbiological research supports this finding because it shows that Gram-positive bacteria lack an outer membrane for protection, which serves as a second defence barrier for Gram-negative bacteria, when they face antimicrobial substances (Bauer et al., 1966; CLSI, 2023). The *E. coli* and *P. aeruginosa* inhibition zones decrease because the outer lipopolysaccharide membrane of Gram-negative bacteria prevents antimicrobial compounds from entering the bacteria, which makes the bacteria harder to kill (Nikaido, 2003). *P. aeruginosa* develops multidrug resistance through its efflux pumps and low membrane permeability, which may be why it exhibited smaller inhibition zones against the sanitizers (Breidenstein et al., 2011).

The current research shows that Anolyte lacks measurable inhibition power against *E. coli*, *S. aureus*, and *P. aeruginosa* in the disc diffusion assay, which conflicts with previous research that states electrolyzed oxidizing solutions only show antimicrobial effects when chlorine levels, pH values and contact duration reach specific levels (Rahman et al., 2016). The assay performance shows valid test results through the standard antibiotic controls Meropenem, Augmentin, and Ciprofloxacin, which produced larger inhibition zones that confirmed bacterial strains' susceptibility under test conditions. The 77X inhibition zones produced smaller results, which could be duplicated because the product showed some antimicrobial capacity, yet its effectiveness remained much lower than standard antibiotics.

This research results showed that 77X exhibited stronger antibacterial effects toward Gram-positive bacteria than Gram-negative bacteria, while Anolyte produced no antibacterial activity under the study conditions. The complete study of 77X's antimicrobial properties needs further research, including time-kill experiments (Jain et al., 2016).

The MIC results show that 77X provides better *E. coli* growth control than Anolyte under the experimental conditions. The concentration-dependent bacterial growth suppression that occurred for each treatment matched established antimicrobial kinetics, which demonstrate that higher sanitizers concentrations lead to stronger inhibition and bactericidal effects according to CLSI 2023 standards. The 1/20 dilution of 77X completely inhibits *E. coli* growth, which indicates that 77X possesses strong antimicrobial properties. Anolyte needed its highest concentration (1/10) to reach the same level of inhibition. Electrolyzed oxidizing solutions, which include Anolyte, generate antimicrobial effects through their reactive chlorine species together with their oxidative stress mechanisms (Rahman et al., 2016). Nonetheless, their antimicrobial performance depends on three factors, which include the organic load and pH and the active species that maintain their effectiveness throughout time (Rahman et al., 2016). The observed fast regrowth at lower Anolyte concentrations shows that active oxidants become less available as time passes.

This study demonstrates that growth recovery occurs at sub-MIC dilutions for both agents because their bacteriostatic effect ceases once the antimicrobial treatment ends (Pankey & Sabath, 2004). The outer membrane of *E. coli* offers protection by preventing antimicrobial compounds from entering the cell (Nikaido, 2003). The lower MIC value of 77X indicates it has greater antibacterial potency against *E. coli* than Anolyte. The antibacterial activity of 77X is due to its fatty acid components, including caprylic acid, which researchers have discovered kills bacteria by damaging their cell walls. Caprylic acid acts as an amphiphilic molecule, allowing it to penetrate bacterial membrane lipid bilayers, thereby increasing membrane permeability, leading to loss of intracellular material, which eventually causes cell death or partial metabolic impairment. This interaction destabilises the lipid bilayer, resulting in membrane damage that causes either growth inhibition or bactericidal effects depending on the concentration (Wang et al., 2020; Yoon et al., 2015). The presence of fatty acids enables them to bind with lipopolysaccharides in Gram-negative bacteria, disrupting the outer membrane and facilitating entry into the cytoplasmic membrane (Wang et al., 2020).

The study found that 77X caused more harm to *E. coli* because fatty acids destroyed bacterial membranes and made Gram-negative bacteria more accessible to their outer protective layers. Amphiphilic fatty acids can enter the lipid layers of Gram-negative bacteria, which normally prevent drugs from reaching their outer membrane, leading to growth restriction through direct membrane damage. This finding highlighted the need to conduct further tests, which is discussed in the following section of minimum bactericidal concentration (MBC) assays and time–kill kinetics and mechanistic studies to assess whether 77X has bactericidal or bacteriostatic properties. The two agents showed antibacterial effects that depended on their concentration, which matched the recognized rules of antimicrobial resistance testing.

77X demonstrates strong antibacterial effects against *S. aureus*, while Anolyte shows only minor inhibitory effects in the same experimental conditions. The MIC shows the lowest concentration that stops visible organism growth according to standardized antimicrobial susceptibility principles (CLSI, 2023). The absence of an outer membrane barrier in *S. aureus* enables antimicrobial substances to enter the bacteria more easily than they do with Gram-negative bacteria (Nikaido, 2003). The structural weaknesses of these bacteria make them more vulnerable to disinfectants and antimicrobial substances. The low effectiveness of Anolyte shows that its active oxidizing substances, including hypochlorous acid failed to maintain their ability to kill or stop bacterial growth for 24 hours.

The antimicrobial effects of electrolyzed oxidizing solutions operate through their ability to produce oxidative damage, which destroys essential cellular elements, and their effectiveness decreases because of their unstable nature and their reaction with organic materials and their neutralisation (Rahman et al., 2016). The results obtained from Anolyte-treated groups demonstrate continuous growth, which provides evidence to support this explanation.

The concentration-dependent regrowth of the bacteria at sub-MIC dilutions of 77X is characteristic of a typical bacteriostatic effect in which the inhibitory activity of the antimicrobial agent decreases as the antimicrobial pressure decreases (Pankey & Sabath, 2004). Additional studies are necessary to determine if 77X has bactericidal activity against S. aureus or only bacteriostatic activity. This can be achieved through the determination of the minimum bactericidal concentration of the drug.

The bacterium *S. aureus* showed a clear minimum inhibitory concentration (MIC) of 1/20 for 77X, whereas Anolyte failed to achieve complete bacterial inhibition at any of the tested concentrations. The current experimental conditions demonstrate that 77X exhibits greater antibacterial effectiveness against Gram-positive bacteria than the other tested substances.

The available evidence indicates that anolyte inhibits *P. aeruginosa* more effectively than 77X under the experimental conditions examined in this study. The stronger time-dependent decline in OD_{600} and higher regression coefficient observed for anolyte suggest more consistent antimicrobial activity across dilutions. 77X showed inconsistent inhibition, permitting bacterial multiplication at lower concentrations while achieving only partial control at higher levels. Anolyte is an electrochemically activated solution that generates reactive oxygen species (ROS), hypochlorous acid (HOCl), and other oxidizing substances (Block, 2001; Thorn et al., 2012). The research demonstrates that hypochlorous acid enters bacterial cells through their walls, disrupting crucial metabolic functions (Winter et al., 2008). The continuous decline in OD_{600} throughout the experiment supports the idea that oxidative damage leads to permanent bacterial inactivation.

P. aeruginosa exhibits inherent resistance to various antimicrobial drugs because its outer membrane acts as a permeability barrier, and it possesses bacterial expulsion systems and biofilm production capabilities (Breidenstein et al., 2011). The ability of anolyte to maintain its inhibitory effect despite multiple dilutions indicates that oxidative processes can partially counteract these resistance mechanisms. The 77X product offered less predictable performance because its active ingredients differ in stability and effects on bacteria.

The decrease in optical density at longer incubation times for both elevated-concentration treatments indicates that the results likely reflect bactericidal effects, but these findings require verification through minimum bactericidal concentration testing by subculturing onto non-selective media. The optical density method provides minimum inhibitory concentration results that need to be verified by viable plate count testing to improve understanding, as the method has limitations when testing samples containing cell debris. The anolyte showed lower apparent minimum inhibitory concentration results, yet it inhibited *P. aeruginosa* growth by more than 77X during the experimental testing. The results of this study demonstrate that electrochemically activated solutions should undergo additional testing because they show potential to function as antimicrobial agents against Gram-negative bacteria.

3.6 Conclusion

The disc diffusion and MIC tests show that 77X provides consistent antibacterial effects that are evident across all studied bacteria. The two methods produced the greatest inhibition of bacterial growth against *S. aureus*, indicating that this Gram-positive bacterium is particularly vulnerable to treatment. The antibacterial effect of 77X against Gram-negative bacteria was moderate, resulting in lower sensitivity in both *E. coli* and *P. aeruginosa*. The disc diffusion assay showed distinct zones of inhibition for 77X, with the greatest effect on *S. aureus*. The MIC growth-curve analysis showed that bacterial growth was inhibited at specific concentration levels. The results demonstrate that 77X inhibits bacterial growth through two mechanisms, namely diffusion in solid media and continuous suppression of broth culture growth. The inhibitory activity diminished progressively with increasing dilution, establishing a strong concentration–response relationship.

The disc diffusion assay did not show any measurable inhibition zone for Anolyte, implying that the active components of Anolyte were unable to diffuse into the agar medium, likely due to limited diffusion capacity and instability of the active components in the solid state. The broth microdilution susceptibility tests showed that specific growth patterns emerged at high Anolyte concentrations, particularly during the initial stages of development. The antimicrobial evaluation shows varying results between the two systems, as Anolyte must come into contact with surfaces to work effectively and does not spread into the environment. The differing susceptibility of Gram-positive and Gram-negative organisms further supports the role of bacterial cell wall structure in the antimicrobial effect. Gram-positive bacteria, with a thicker peptidoglycan layer and no outer membrane, are 77X more susceptible than Gram-negative bacteria, which have an outer membrane that limits the compound's penetration and reduces its effectiveness.

4. Biofilms and Citrus

4.1 Introduction

Biofilms are organised communities of microorganisms that permanently adhere to natural or artificial surfaces and reside within their self-produced extracellular polymeric substance (EPS) protective matrix. The ability of bacteria to form biofilms is a crucial element of their virulence, enabling chronic infections, antimicrobial resistance, and persistence on medical equipment and in outdoor environments. The bacteria *E. coli*, *S. aureus*, and *P aeruginosa* create biofilms, which commonly lead to contamination problems within food processing facilities. These microorganisms can adhere to food-contact surfaces, which include stainless steel, plastic, rubber and glass equipment found in food production and handling areas to create biofilms. Biofilms create permanent contamination sources, which allow bacteria to break away and spread to food during all stages of processing, storage and handling. Cross-contamination events ultimately lead to foodborne illnesses, which affect consumers (Bai et al., 2021; Galié et al., 2018).

E. coli can stick to various surfaces in food processing facilities, which leads to the contamination of foods during production and post-harvest handling of raw vegetables, meat and dairy products (Brás et al., 2024; Giaouris et al., 2014). *S. aureus* has been found on equipment surfaces and throughout food processing chains where it spreads contamination to food products while producing heat-stable enterotoxins, which cause food poisoning (Bai et al., 2021). *P. aeruginosa* and related *Pseudomonas* species serve as essential biofilm-producing bacteria in food environments because they maintain their presence on food-contact surfaces and they lead to spoilage and contamination of processed foods (Galié et al., 2018).

The 96-well microtiter plate biofilm assay is an established laboratory technique used to quickly screen bacterial biofilm growth *in vitro* and then test antimicrobial compound effectiveness against the biofilm. This method enables testing multiple antimicrobials and antibiofilm substances by assessing bacterial biomass, measured either through crystal violet staining or metabolic activity using XTT, followed by spectrophotometric analysis (Stepanović et al., 2007). It allows comparative testing across bacterial species, producing reliable results while remaining affordable and flexible (Stepanović et al., 2007). In recent years, scientists have begun studying new antimicrobial agents, including oxidising solutions and fatty acid-based compounds, which show potential to combat biofilms.

Anolyte is an electrolysed oxidising solution that generates reactive oxygen species capable of damaging microbial cell walls and disrupting biofilm matrix structure. These oxidising agents have been shown to kill various microorganisms and reduce the resistance conferred by biofilms (Block and Rowan, 2020). Scientists have studied caprylic acid (known as 77X) to determine its ability to kill microorganisms and inhibit biofilm growth (Yoon et al., 2018). Medium-chain fatty acids can disrupt bacterial membranes, alter permeability, and interfere with metabolic processes, thereby inhibiting planktonic growth and biofilm formation (Desbois & Smith, 2010). Their amphipathic nature enables interaction with lipid bilayers, making them promising candidates for biofilm control strategies (Desbois & Smith, 2010). The present study aims to test Anolyte and caprylic acid against *E. coli*, *S. aureus*, and *P. aeruginosa* using 96-well microtiter plate biofilm testing. This research examines how different bacterial strains respond to reductions in biofilm biomass to identify new methods for controlling infections and biofilm-related contamination.

4.1.1 Antimicrobial Assessment on Mandarin fruit:

Fresh produce and minimally processed foods have gained recognition as potential sources of foodborne pathogens. As mentioned earlier (Beuchat, 2002; Olaimat & Holley, 2012), fresh produce serves as a pathway for foodborne pathogens to spread. The peel surface of citrus fruits serves as the main entry point for contamination, while the acidic internal tissues remain uncontaminated. Although the internal tissues of citrus fruit remain acidic, which prevents enteric pathogens from surviving, external peel contamination can occur through multiple processes, including cultivation, harvest, postharvest handling, transport and retail display (Parish, 1997). Microorganisms present on peel surfaces may subsequently be transferred to the edible portion during peeling or cutting.

The structural elements of citrus peels include their rough and porous surface structure, create a barrier that protects microorganisms while making their removal and elimination more challenging during sanitation activities (Beuchat, 2002; Sapers, 2001). The mandarin peel surface displays a textured design that includes natural pores and oil glands, enabling bacteria to adhere and survive on its surface. Bacterial attachment to produce surfaces has been shown to significantly influence sanitizer efficacy, as microorganisms may become embedded within surface irregularities, thereby reducing exposure to antimicrobial agents (Beuchat, 2002).

Microbial survival and resistance to inactivation treatments are affected by environmental conditions, organic matter, and surface topography (Olaimat & Holley, 2012).

Fresh produce requires effective postharvest sanitation methods. The antimicrobial effectiveness of chemical sanitizers used on fruit surfaces depends on the concentration of the active ingredient, the duration of application, the product formula, and the product being disinfected (Gil et al., 2009; Parish et al., 2003). Fresh produce wash systems need to achieve two operational goals, which require assessment through controlled efficacy tests, and maintaining product quality (Gil et al., 2009). The assessment of antimicrobial activity requires two steps, which involve counting the microorganisms that survived and determining the decrease in colony-forming units through log reductions. Log reduction values serve as a standardised method that enables the comparison of sanitizer effectiveness across different treatment periods and contact durations.

The current research assessed the effectiveness of 77X and ANK Neutral Anolyte against *E. coli*, which was deliberately introduced onto mandarin peel surfaces. It measured both CFU reduction and log reduction across various exposure durations. The study also assessed microbial survival rates at different treatment durations to provide numerical evidence supporting the effectiveness of surface sanitizers in postharvest citrus handling operations.

4.1.2 Introduction to Antimicrobial Assessment on Stainless Steel Coupons

The presence of dangerous bacteria on the surfaces of food-processing equipment poses a critical barrier that challenges both safe food handling and public health protection. According to Hage et al. (2021), stainless steel exhibits both mechanical strength and corrosion resistance, making it suitable for food-contact applications. Stainless steel surfaces remain contaminated with pathogens after cleaning procedures because proper sanitation methods are required to achieve contamination-free results. However, bacteria can adhere to these surfaces and form biofilms. Evaluating antimicrobial effectiveness on stainless steel surfaces is essential for developing and testing cleaning methods and protective coatings that will be applied in actual contact areas.

The established method for testing antimicrobial effectiveness requires researchers to use stainless-steel coupons as standardized testing surfaces to measure bacterial binding and survival rates after various treatments. The stainless-steel coupons enable researchers to conduct controlled testing of disinfectants by applying different antimicrobial agents to bacterial cells that have attached to metal surfaces and then assessing the reduction in live bacteria (Saini et al. 2013). The testing procedure simulates real food production environments where microorganisms contaminate clean surfaces, requiring disinfectants to remove them.

Multiple studies have shown that stainless steel coupon testing for microbial control has direct practical applications. The Saini et al. (2013) study examined the efficacy of lauric arginate against *L. monocytogenes* on stainless steel coupons and found that higher treatment concentrations and longer contact times led to greater reductions in bacterial populations (Saini et al., 2013). The research framework creates a simulated environment that replicates how food production facilities experience their actual hygienic conditions when microorganisms colonise their hygiene barriers, which then undergo disinfection procedures.

The importance of stainless-steel coupon testing for microbial control has been established through multiple research studies. Saini et al. (2013) demonstrated that lauric arginate killed *L. monocytogenes* when applied to stainless steel coupons, and their experiments revealed that increasing treatment levels and extending contact times led to improved bacterial destruction.

Previous research has examined the use of antimicrobial coatings for the surface protection of stainless steel via surface functionalization (Hage et al., 2021). A review of peptide-based and biocide-infused coatings indicates that their built-in antimicrobial properties reduce bacterial adhesion and biofilm formation, thereby protecting the entire surface area. The combination of bactericidal activity and permanent surface attachment makes antimicrobial peptides suitable for grafting onto stainless steel (Hage et al., 2021). The research shows that both chemical sanitizers and surface modifications require direct performance evaluation through coupon testing to assess their effectiveness in real-world field conditions. Thus, the aim of this current study, i.e., comparing the effectiveness of ANK Neutral Anolyte and 77X in sanitizing artificially contaminated harvested citrus fruit and stainless-steel surfaces with *E. coli* ATCC 25922, was fulfilled.

4.2 Materials and methods

4.2.1 Biofilm XTT Assay

The 96-well biofilm XTT-menadione metabolic assay was used to evaluate the effects of Anolyte and 77X on mature biofilms by measuring metabolic activity via XTT reduction. The test organisms were *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027. The materials included sterile 96-well flat-bottom microtiter plates, Mueller–Hinton Broth (MHB), phosphate-buffered saline (PBS), 0.85% saline, XTT powder, menadione, 100% acetone, 0.22 µm syringe filters, aluminum foil, sterile pipette tips, sterile centrifuge tubes, a microplate reader set at 590nm, 77X, and Anolyte.

In order to create biofilms, overnight cultures of the test organisms in MHB were incubated at 37°C for 16 to 20 hours. The bacterial suspension concentration was calculated to be approximately 1×10^6 cells/mL using the $C_1V_1 = C_2V_2$ formula based on the optical density at 600 nm. Subsequently, 100 µL of the prepared bacterial suspension was added to wells in Rows 3 through 11 of a sterile 96-well plate. Incubation at 37°C for 48 hours facilitated the formation of mature biofilms. After 24 hours of incubation, the spent medium was aspirated and carefully replaced with fresh MHB, while maintaining the integrity of the developing biofilm.

Once biofilms were established, the sanitizer treatment began after the 48-hour incubation period. The culture medium was aspirated, and the wells were washed three times with sterile saline to remove planktonic cells. 100 mL of the respective sanitizers was applied according to the experimental layout, which assigned 77X for biofilm treatment to Rows 3 through 6 (100 µL) and Anolyte to Rows 7 through 10 (100 µL). The sanitizers were allowed to remain in contact with the biofilms for defined exposure times of 1 min, 5 min, 15 min and 30 min. Separate plates were used for each contact time, maintaining strict timing control throughout the experiment. The sanitizers were aspirated after the designated contact period, and the wells were washed three times with sterile saline to remove any remaining sanitizer.

The XTT-menadione reagent was prepared in the laboratory immediately before use. All experimental procedures were performed under dim light, wrapping XTT solutions in aluminum foil. The XTT stock solution was prepared at 0.5 g/L in PBS and filter-sterilized through a 0.22 µm syringe filter. The menadione stock solution was prepared at 10 mM in 100% acetone and stored at −70°C in 50 µL aliquots until required. The working solution was prepared by adding 1 µL of menadione stock solution to 10 mL of XTT solution.

The 96-well plate layout included Column 1, which was tested as the 77X control and contained pure 77X material; Column 11 served as the positive control, showing biofilm without sanitizer treatment; and Column 12 tested the Anolyte control, containing pure Anolyte material to assess contamination and intrinsic color development. The study washed the samples before applying the XTT–menadione working solution to each well and incubated the plates under suitable conditions until color development occurred. The study measured biofilm metabolic activity by absorbance at 490 nm and 450 nm using a microplate reader.

4.2.2 Evaluation of 77X and ANK Neutral Anolyte Efficacy on Mandarins

The antimicrobial effectiveness of 77X and ANK Neutral Anolyte against *E. coli* ATCC25922 on mandarin peel surfaces over time was determined. An overnight *E. coli* culture was introduced into nutrient broth and incubated under aerobic conditions at 37°C for 24 hours. A culture was standardized to 10^7 CFU/mL using sterile water after completing the incubation process.

The fresh mandarins of nearly identical size and undamaged peels were selected for the investigation. Subsequently, the fruit was rinsed using autoclaved deionized water to remove any debris, and then air-dried in a biosafety cabinet for at least 1 hour.

Each mandarin was placed individually in sterile Petri dishes for inoculation. The fruit surface received one milliliter of the standardized 10^7 CFU/mL *E. coli* suspension through pipetting. The suspension was applied slowly over the top and sides to maximize surface coverage and ensure even distribution across the peel. A one-hour air-dry period using the biosafety cabinet for the inoculated mandarins to ensure that bacteria were adhered to the surface.

This study prepared sanitizer solutions by diluting 77X and ANK Neutral Anolyte 1:10 with sterile water. Then, each inoculated mandarin was placed in a new sterile petri dish before 1 mL of the appropriate diluted sanitizer was applied directly to the surface area that needed treatment. The fruit remained inside the petri dish for the entire contact period. The evaluation examined exposure times of 1 min, 5 min, 15 min, and 30 min.

After completing the mandated exposure period, each mandarin sample was transferred into a Whirl-Pak bag that maintained sterile conditions. Then, 10 mL of Letheen Broth was added as a neutralizing agent. The bags were sealed and shaken vigorously for five minutes to force bacteria from the porous peel surface.

The drop plate method was used to count living bacteria that remained after the experiment. Then, ten-fold serial dilutions of the Letheen were prepared in recovery liquid by transferring 1 mL into 9 mL of 0.1% peptone water and performing sequential serial dilutions. A micropipette was used to transfer 5 times 10 µL from each dilution onto Nutrient Agar plates (Fort Richard, NZ) to create drop plates. The plates in triplicate were incubated at 37°C for 24 hours before counting the colonies.

4.2.3 Antimicrobial Assessment on Stainless Steel Coupons

This study assessed the efficacy of 77X and ANK Neutral Anolyte against *E. coli* by testing their activity on bacteria spread across stainless-steel coupons, measuring colony-forming unit (CFU) reduction and log reduction across various exposure durations. An overnight culture of *E. coli* was prepared by inoculating the bacterium into nutrient broth and incubating at 37°C for 24 hours to reach optimal growth. The bacterial suspension was prepared by incubating the culture until it reached 10^7 CFU/mL, using sterile nutrient broth or saline solution to achieve the desired concentration. Stainless-steel coupons were cleaned with 70% ethanol until all surface dirt and contaminants were removed, then autoclaved to achieve complete sterility. Coupons were transferred from the sterilization process into sterile Petri dishes using aseptic methods. Each sterile coupon was inoculated with 250 µL of the standardised *E. coli* suspension (10^7 CFU/mL). The inoculum was spread across the surface at the required locations to create an even distribution. The inoculated coupons were placed inside a biosafety cabinet to dry for one hour, which helped the bacteria bind to the stainless-steel surface.

The sanitizing agents were prepared by making a 1:10 dilution of 77X and ANK Neutral Anolyte using sterile distilled water. Then, 1 mL of the diluted sanitizer was applied directly onto each inoculated coupon located in the petri dish. The coupons in triplicate were tested with sanitizers which were applied during four specific contact times at 1 min, 5 min, 15 min, and 30 minutes. 10 mL of sterile distilled water was added to rinse each coupon after its exposure period ended, which stopped any remaining antimicrobial effects.

4.2.4 Statistics

Statistical analysis was performed using IBM SPSS Statistical Software v 31 (SPSS [Statistical Package for the Social Sciences], Inc., Chicago, IL). The effect of sanitizers against biofilms and contaminated fruit and stainless steel was investigated by analysis of variance, followed by a multiple means comparison procedure using Tukey grouping. All tests were performed with a confidence level of 95%.

4.3 Results

4.3.1 Biofilm metabolic activity of *E. coli*

The growth control wells of *E. coli* isolates demonstrated substantial growth through multiple *E. coli* isolates, which reached an optical density of 1.58. The treatment with 77x showed variations in remaining growth throughout different time periods. The time intervals of 1 and 5 minutes yielded moderate optical density readings, ranging from 0.25 to 0.33. The antimicrobial effect reached its peak at half an hour, when the treatment showed lower effectiveness because growth levels decreased after 15 minutes to approximately 0.12, but then surged to approximately 0.79 at 30 minutes (Fig 9).

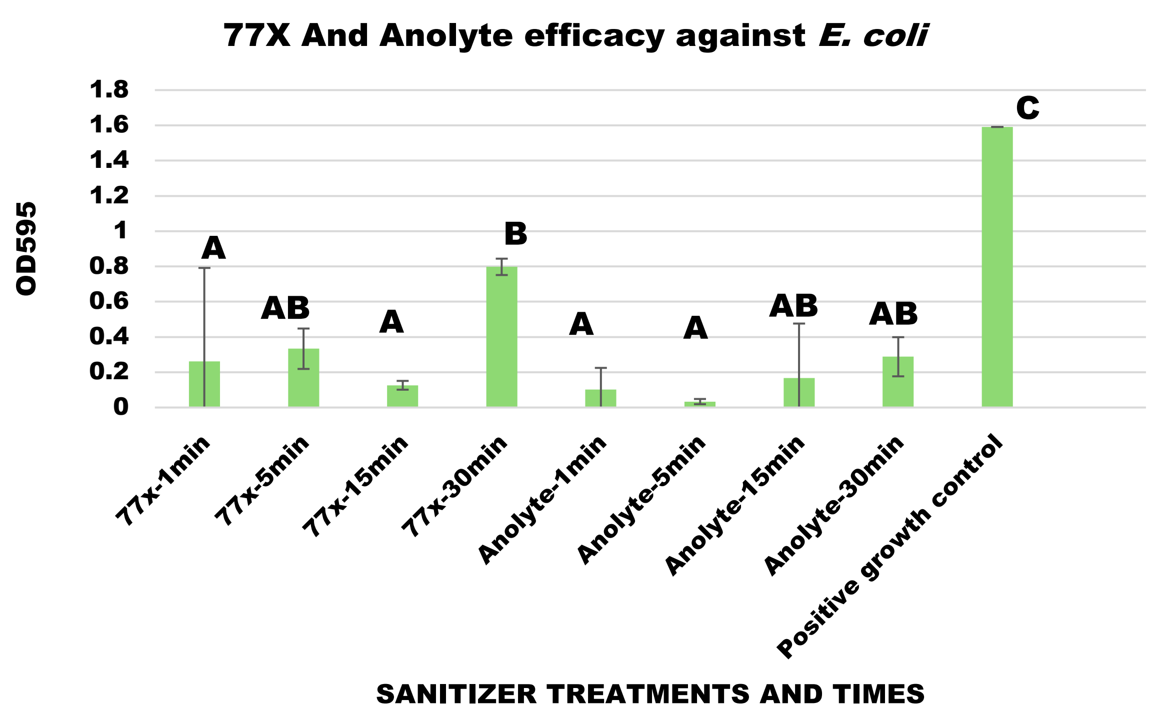

Figure 9. Biofilm metabolic activity (mean ±standard deviation) of *E. coli* with treatments of sanitizer at different time intervals. One-way ANOVA statistics with Tukey HSD at a $p<0.05$ level of significance for the Biofilm metabolic activity of *E. coli* analyzed. Values represent the means of three replicates, while error bars represent the standard deviation of the means. Points exhibiting no common letter are significantly different according to Tukey's grouping ($p < 0.05$).

The anolyte treatment produced lower optical density results, which persisted throughout all measurement intervals. The optical density measurement at one minute was 0.10, but it dropped to 0.03 at five minutes. The optical density measurements showed slight increases at 15 and 30 minutes, ranging from 0.16 to 0.28, but remained lower than those of the positive control. The anolyte treatment demonstrated stronger suppression of *E. coli* growth than the 77X treatment during most time intervals, especially at shorter treatment durations.

4.3.2 Biofilm metabolic activity of *S aureus*

The growth control for *S. aureus* showed high biofilm formation ($OD_{595} \approx 1.58$). The OD values increased after the 77X treatment was applied throughout the experimental period. The minimal growth at one minute was approximately 0.04, yet the biofilm formation rate increased to approximately 0.67 at five minutes, then to approximately 0.39 at 15 minutes and approximately 0.47 at 30 minutes (Fig 10).

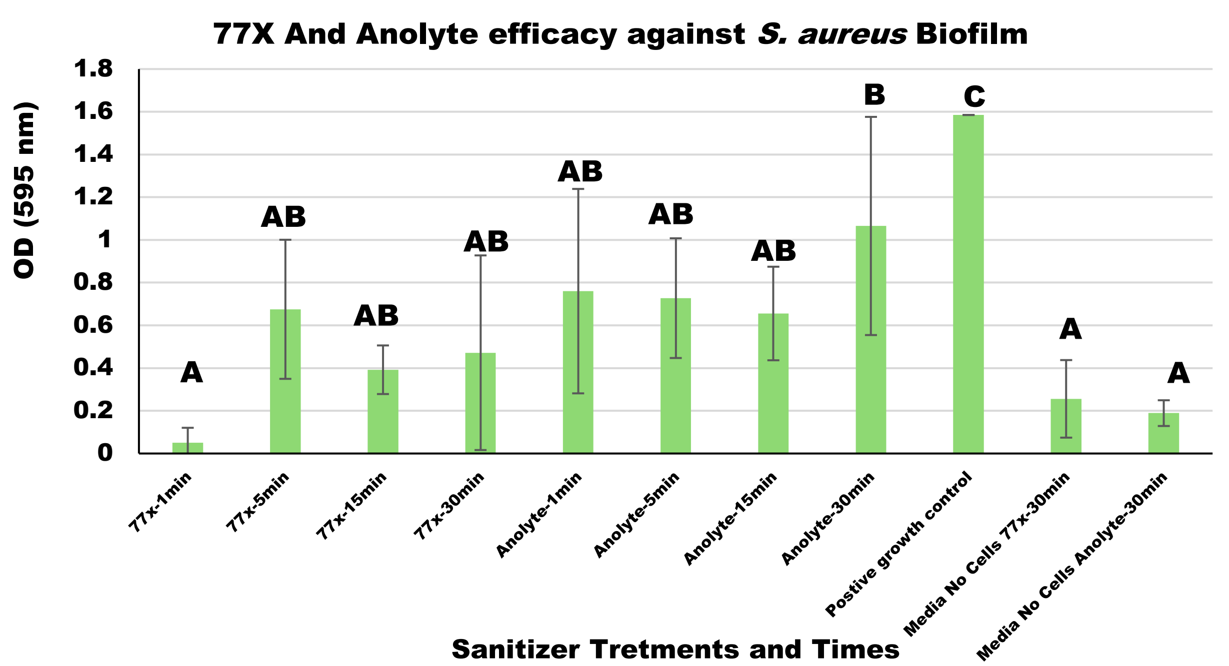

Figure 10. Biofilm metabolic activity of *S. aureus* (mean ±standard deviation) with treatments of sanitizer at different time intervals. One-way ANOVA statistics with Tukey HSD at a p<0.05 level of significance for the Biofilm metabolic activity of *E. coli* analysed. The different letters above the bars indicate significant differences between treatments over time at a p<0.05 level of significance. Values represent the means of three replicates, while error bars represent the standard deviation of the means. Points exhibiting no common letter are significantly different according to Tukey's grouping ($p < 0.05$).

The anolyte-treated wells showed directly higher optical density measurements compared to the 77X treatment at multiple measurement intervals. The optical density measurement reached approximately 0.75 at one minute, then decreased to approximately 0.72 and 0.65 at five and fifteen minutes, respectively, before rising to approximately 1.05 at thirty minutes. The media-only controls showed low optical density at 30 minutes, ranging from 0.18 to 0.25, thus confirming that bacterial growth caused the absorbance measurements.

The 77X treatment suppressed *S. aureus* biofilm development more effectively than anolyte treatment during the first stage of their interaction. The 1-minute exposure period showed the highest effectiveness of 77x treatment against biofilms, but neither treatment could completely block biofilm development when compared to the positive control.

4.3.3 Biofilm metabolic activity of *P. aeruginosa*

The growth control exhibited measurable growth ($OD_{595} \approx 0.24$). The 77x treatment produced minimal optical density results, which ranged from approximately −0.02 to 0.04 throughout all measurement intervals (Fig 11). The anolyte treatment produced optical density results which approached zero or slightly negative values at 5 and 30 minutes, which demonstrated its ability to fight against bacteria. The two sanitizers demonstrated better performance than the positive control by decreasing *P. aeruginosa* growth throughout all exposure durations. The treatment results produced no evidence of increased growth, which occurred at specific time intervals, because both agents maintained their inhibitory power.

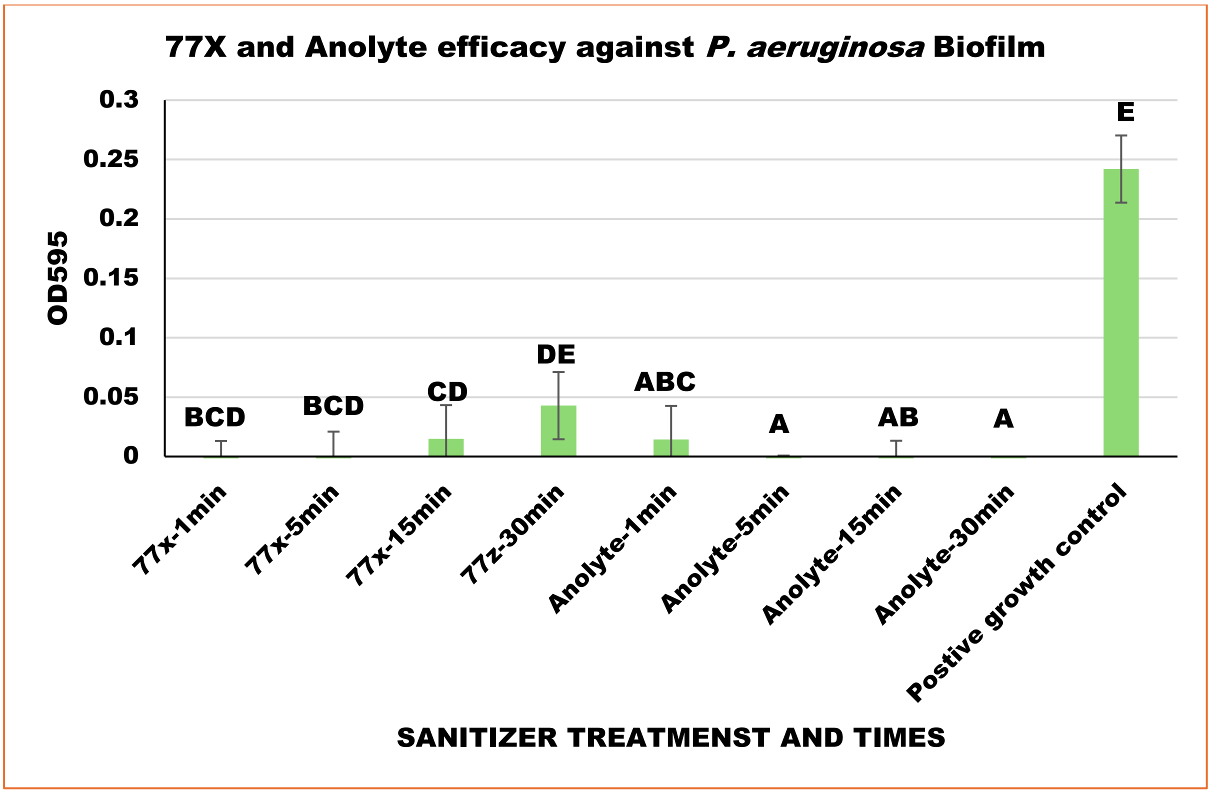

Figure 11. Biofilm metabolic activity of *P. aeruginosa* (mean ±standard deviation) with treatments of sanitizer at different time intervals. The different letters above the bars indicate significant differences between treatments over time at a p<0.05 level of significance. Values represent the means of three replicates, while error bars represent the standard deviation of the means. Points exhibiting no common letter are significantly different according to Tukey's grouping ($p < 0.05$).

4.3.4 Evaluation of 77X and ANK Neutral Anolyte Efficacy on Mandarins

The study evaluated the antimicrobial effectiveness of 77X and ANK Neutral Anolyte against *E. coli* ATCC 25922 on inoculated mandarin peel surfaces by measuring the Log_{10}CFU counts and log reductions at exposure times of 1, 5, 15, and 30 minutes. The positive control group, comprising inoculated but untreated samples, showed substantial bacterial recovery, confirming successful introduction of the organism to the test surfaces and its viability. Mandarin samples treated with 77X, and ANK Neutral Anolyte showed no colony growth on either nutrient agar or Fort Richard agar plates throughout all tested exposure times (Fig 12). The complete absence of detectable growth across the serial dilutions indicates that all standardized 10^7 CFU/mL *E. coli* inocula became inactive under the experimental conditions. The two sanitizers produced a bactericidal effect, reducing bacterial counts by at least 7 $\log_{10}$

within 1 minute of first contact with the bacteria, with killing continuing for 30 minutes. The results demonstrate that 77X and ANK Neutral Anolyte exhibit potent and rapid antimicrobial activity against contaminated mandarin peel surfaces.

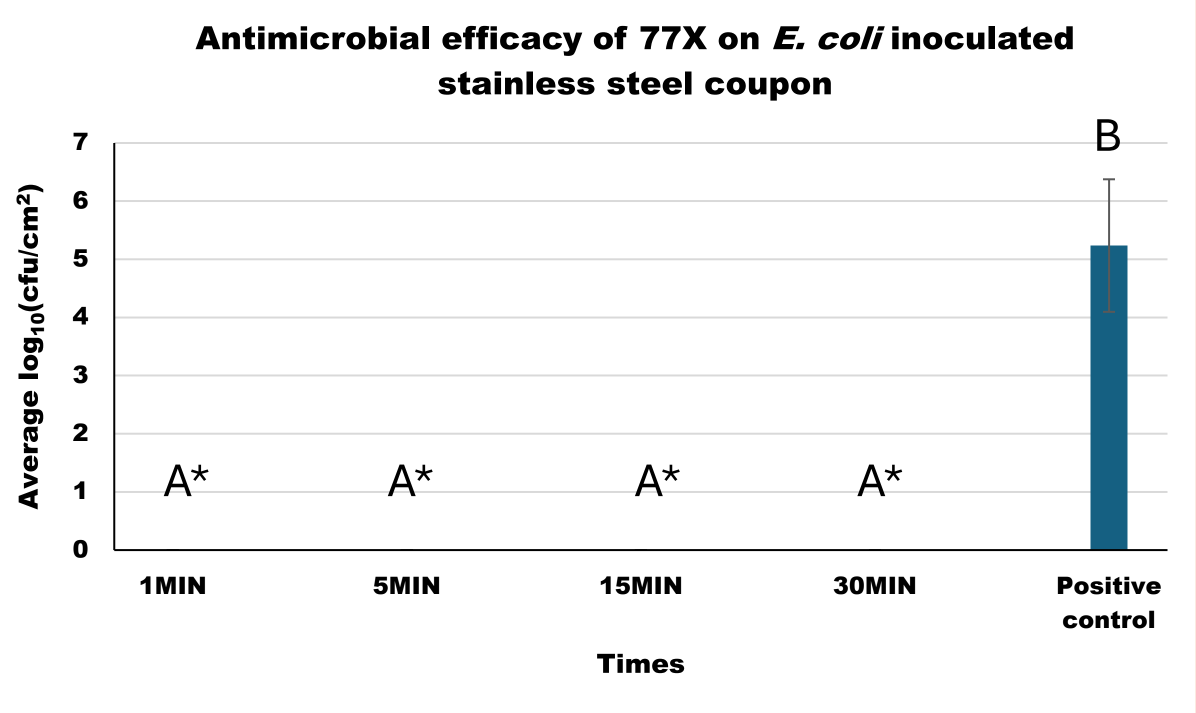

Figure 12. Antimicrobial efficacy (mean ± standard deviation) of 77X on *E. coli* inoculated stainless steel at different time intervals. The different letters above the bars indicate significant differences between treatments over time at a p<0.05 level of significance. Values represent the means of three replicates, while error bars represent the standard deviation of the means. Points exhibiting no common letter are significantly different according to Tukey's grouping ($p < 0.05$). * Denotes those counts were below detection limit of 1 $\log_{10}$ CFU/cm^{-2}.

4.3.5 Antimicrobial efficacy of anolyte on *E. coli* inoculated stainless steel coupon.

The antimicrobial activity of ANK Neutral Anolyte against *E. coli* on stainless steel coupons appears in Figure 13. The positive control that did not receive treatment showed a bacterial load of about 5.2 $\log_{10}$ CFU/cm^2, which demonstrated that *E. coli* successfully attached to the coupon surface. The application of sanitizer resulted in a significant decrease in bacterial populations. The mean count after 1 minute of exposure maintained high levels at approximately 7.8 $\log_{10}$ CFU/cm^2, which showed that the treatment had only slight effects. The results showed considerable reductions, which became visible after prolonged contact periods. After 5 minutes, the bacterial load decreased to about 2.6 $\log_{10}$ CFU/cm^2, which represented a 2.6 log reduction from the positive control. The 15-minute mark showed a similar decrease to 2.7 $\log_{10}$ CFU/cm^2. The count after 30 minutes reached about 3.1 $\log_{10}$ CFU/cm^2, which displays more variability according to the larger error bars. ANK Neutral Anolyte showed its antimicrobial effects on stainless steel surfaces through time-dependent activity, which reached substantial reductions after 5 minutes of exposure and continued during extended contact times.

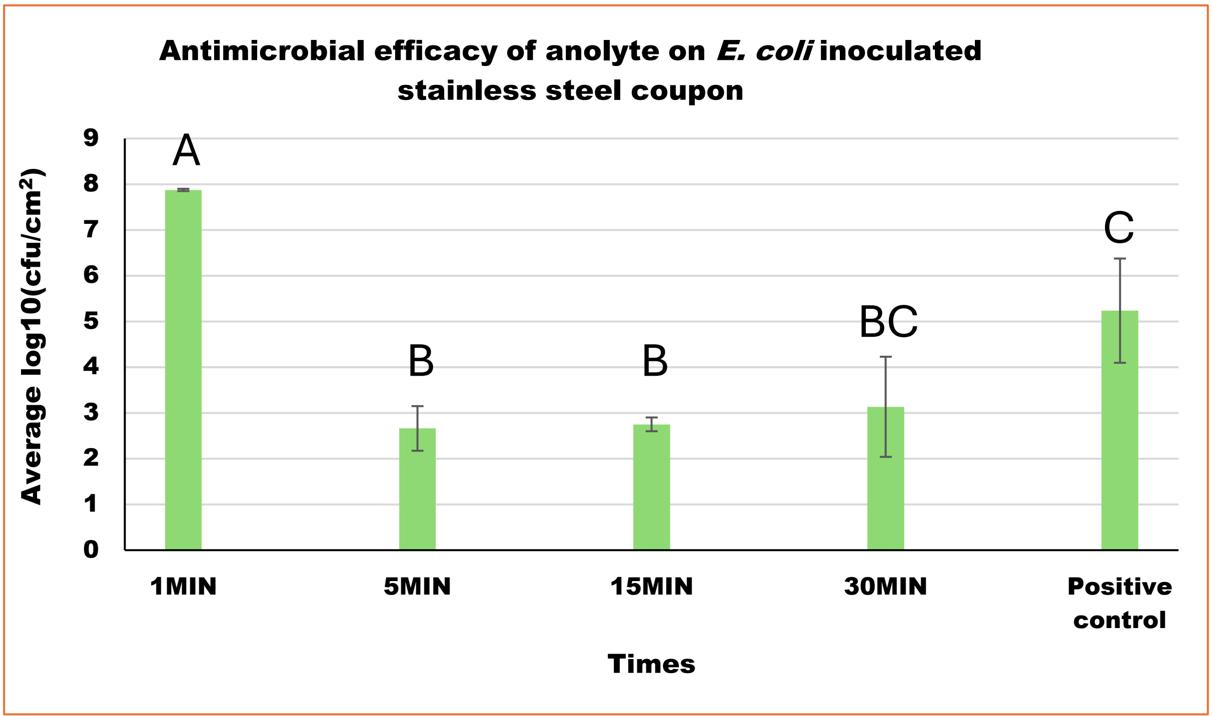

Figure 13. Antimicrobial efficacy (mean ± standard deviation) of anolyte on *E. coli* inoculated stainless steel at different time intervals. The different letters above the bars indicate significant

differences between treatments over time at a $p<0.05$ level of significance. Values represent the means of three replicates, while error bars represent the standard deviation of the means. Points exhibiting no common letter are significantly different according to Tukey's grouping ($p < 0.05$).

4.4Discussion

4.4.1 XTT Assay

The current chapter investigated the antibacterial efficacy of 77x and Anolyte against *E. coli*, *S. aureus*, and *P. aeruginosa,* as measured by OD_{595} after culturing biofilm samples.

Anolyte demonstrated better ability to inhibit *E. coli* growth than 77x did, especially during brief contact periods. Electrolyzed oxidizing water (Anolyte) operates as a bactericide by producing reactive oxygen species and hypochlorous acid, which damage bacterial membranes and disrupt their metabolic systems (Huang et al., 2008). The 5-minute time point showed reductions of growth, confirming that short exposure periods enable effective inactivation of Gram-negative organisms. The 30-minute OD increase in the 77x treatment group suggests that the treatment may have lost its effectiveness, or that the bacteria developed new survival strategies. While there was a reduction compared to the control, no statistical difference could be seen between the different time points. However, 77x demonstrated greater initial growth-prevention capabilities against *S. aureus* than anolyte did. The thicker peptidoglycan layer of Gram-positive bacteria creates a barrier that reduces their vulnerability to oxidizing agents (McDonnell and Russell, 1999). The higher OD results from anolyte treatment indicate that the treatment could not completely penetrate the existing biofilm structures. Biofilm-associated cells exhibit greater disinfectant resistance because their extracellular polymeric substances restrict the movement of antimicrobial agents (Costerton et al., 1999).

The two treatments further achieved complete elimination of *P. aeruginosa*, resulting in OD measurements reaching zero across all testing intervals. *P. aeruginosa* exhibits a well-known tendency to form biofilms while also showing natural resistance to various treatments (Hall-Stoodley et al., 2004). The experimental conditions revealed substantial strength from both sanitizers which led to the observed major reduction.

4.4.2 Efficacy of sanitizers on artificially contaminated Mandarin with *E. coli*

The study found no *E. coli* growth after using both 77X and ANK Neutral Anolyte, supporting existing research showing that electrochemically activated and electrolyzed water-based sanitizers effectively kill pathogens on produce and contaminated surfaces. Electrolyzed or anolyte solutions act as bactericidal agents by utilizing three distinct pathways that induce oxidative stress, damage cellular structures, and halt essential microbial processes through chemical means. Cloete et al. (2009) demonstrated that anolyte treatments caused *E. coli* protein breakdown and oxidative damage, resulting in total microbial death at both 10^0 dilution and undiluted treatment levels. The sanitizers used in the current study have high oxidative potential, leading to immediate and permanent destruction of bacterial cells and preventing recovery, even after brief contact.

The study of electrolyzed waters shows how *E. coli* reacts to them: slightly acidic electrolyzed water (SAEW) causes bacterial membrane destruction and increases membrane permeability, resulting in necrosis and apoptosis through reactive oxygen species generation and membrane disruption within minutes of contact (Ye et al., 2017). Their study found that all contact times led to complete cell death because the bacteria could not survive any contact duration. Research has demonstrated that electrolyzed oxidizing waters with optimized chlorine levels and ORP and pH settings can achieve ≥7-log reductions in *E. coli* and other pathogens that contaminate produce surfaces and water suspensions within practical times (Venkitanarayanan et al., 1999).

Research demonstrates that the physicochemical characteristics of electrolyzed anolyte or electrolyzed solutions create conditions with high ORP and strong oxidants, such as hypochlorous acid, which enable microorganisms to breach bacterial protective mechanisms and kill bacteria on the surfaces of produce (Rebezov et al., 2022). Studies show that electrolyzed water systems for food safety depend on these specific characteristics, which provide antimicrobial protection and enable the safe cleaning of high-risk foods while maintaining produce quality (Rebezov et al., 2022). The literature shows that anolyte solutions cause rapid oxidative damage to E. coli cell structures and metabolic functions, resulting in the elimination of colony growth on treated mandarins. The established antimicrobial mechanisms that electrolyzed sanitizers use to exert their disinfecting power show this.

4.4.3 Efficacy of stainless steel

The time-dependent decrease in *E. coli* on stainless steel coupons after ANK Neutral Anolyte treatment can be explained by the established antimicrobial properties of electrolyzed oxidizing water and neutral anolyte solutions. Neutral anolyte usually contains hypochlorous acid as its main active component, which, together with the solution's high oxidation-reduction potential (ORP), enables rapid microbial elimination. The electrical neutrality of HOCl allows it to cross bacterial cell membranes, where it oxidizes sulfhydryl groups in vital enzymes, disrupts membranes, and damages nucleic acids, resulting in cell death (Albrich et al., 1981). Neutral anolyte formulations exhibit strong bactericidal properties because HOCl is more effective than hypochlorite ions at near-neutral pH (Len et al., 2002).

The *E. coli* counts, which decreased at 5-, 15-, and 30-minute intervals, matched previous studies showing that electrolyzed water produced antimicrobial effects on food-contact surfaces that depended on contact duration. Park et al. (2002) demonstrated that electrolyzed oxidizing water achieved substantial reductions in *E. coli* O157:H7 and *L. monocytogenes* on stainless steel surfaces within minutes of exposure, with greater reductions as contact time increased. Kiura et al. (2002) showed that *E. coli* inactivation occurred rapidly via membrane disruption by acidic and neutral electrolyzed waters, confirming that oxidative stress, together with structural damage, functions as the main mechanism of action.

The first-minute reduction shows a small decrease because bacteria remain protected by surface attachments, which contain tiny surface defects that prevent sanitizer from reaching them. Surface-attached cells typically display higher tolerance levels than planktonic cells because they develop distinct physiological traits while maintaining some protection in surface-based areas (Frank & Koffi, 1990). The protective mechanisms of the human body become overwhelmed when people experience prolonged oxidative stress, leading to significant declines in their red blood cell count. The procedure of rinsing with sterile distilled water after each contact period has interrupted the subsequent antimicrobial effects of the sanitizer, revealing its actual time-based effectiveness rather than its total time-based effects.

Thus, the study results confirm existing research showing that neutral electrolyzed water demonstrates its antibacterial properties through two main mechanisms. The study results demonstrate that five-minute tests and additional testing periods effectively validate anolyte solutions as disinfectants, which maintain their effectiveness on stainless steel surfaces that contact food.

4.4.4 Discussion for mandarin and stainless-steel experiment

Microbial recovery from mandarin peels shows different results than from stainless steel coupons because their different surface structures affect how bacteria stick to them. The stainless-steel surface is a smooth, inert material with no porosity, but its microscopic irregularities create safe areas that protect against sanitizer contact and enable gradual sanitizer inactivation (Frank & Koffi, 1990). The data shows that 1 minute of testing produced only minor results, while the following intervals produced increasing log reductions at 5, 15, and 30 minutes. The research shows that *E. coli* and other pathogens exhibit time-dependent decreases on stainless steel surfaces because electrolyzed oxidizing water requires longer contact times to achieve better microbial elimination (Park et al., 2002).

The sanitizer’s oxidative effects, together with the physicochemical characteristics of citrus peel, create a situation in which mandarin peels show a total absence of observable colonies. The natural antimicrobial compounds present in mandarin peel, including essential oils containing limonene and other terpenoids, exert an effect that fights *E. coli* through their combined action. Moreover, the presence of acidic microenvironments on fruit surfaces increases HOCl formation, which acts as a more potent antimicrobial agent than hypochlorite ions, which exhibit lower antimicrobial activity (Len et al., 2002). Their study shows that electrolyzed water achieves high log reductions in *E. coli* on fresh produce when proper contact and concentration requirements are met, supporting the findings of this study (Park et al., 2002).

5. Conclusion

The current research tested the antimicrobial and antibiofilm effectiveness of ANK Neutral Anolyte and 77X against *S. aureus*, *P. aeruginosa* and *E. coli* on stainless steel surfaces and pre-harvest citrus fruit surfaces. The findings support the study hypothesis that both test sanitizers exhibit measurable antimicrobial activity, as their effectiveness varies with microbial structure, biofilm maturity, and application conditions. The three tests, disc diffusion, MIC, and biofilm metabolic assays, showed that 77X maintained more effective antibacterial properties against all bacteria compared to ANK Neutral Anolyte, with greater strength against *S. aureus*. The results showed that the bacteria were killed at a certain treatment concentration, as inhibition zones and lower MIC values indicated a concentration-dependent effect. The bacterium showed moderate activity against Gram-negative organisms because their outer membrane barriers prevented effective penetration by the antimicrobial. The product 77X demonstrated the ability to reduce mature biofilms, proving its effectiveness as a hard-surface sanitizer for plastic crates that handle fruit.

The ANK Neutral Anolyte solution showed specific antibacterial activity against certain microorganisms. The substance showed no results in disc diffusion tests due to decreased stability and rapid loss of active chlorine through neutralization in agar. Still, it showed antimicrobial activity when tested in broth and through direct contact with bacteria. The system demonstrates effectiveness only when parameters such as concentration, contact time, and organic load are applied. The results demonstrate that electrolyzed oxidizing water systems operate as established, because their antimicrobial properties depend on exposure to reactive oxygen and chlorine species, which require specific conditions to reach their maximum effect. The two sanitizers achieved their best pathogen-elimination results when higher doses were used with extended exposure times at room temperature and at slightly higher-than-normal temperature. The biofilm removal process on stainless steel surfaces and pre-harvest citrus fruit requires both mechanical surface coverage and sufficient wet contact time to achieve practical results, according to the direct exposure testing results.

The 77X product showed comparable antimicrobial effectiveness against *S. aureus* and *E. coli* ATCC 25922 to that of existing sanitizers used by citrus producers for pre-harvest cleaning, including chlorine-based sanitizers and quaternary ammonium compounds. ANK Neutral Anolyte's hygienic performance requires careful parameter management to achieve results comparable to those of traditional sanitizers. The system offers environmental advantages through lower chemical residue emissions, improving safety during fresh produce handling.

Overall, the study demonstrates that both ANK Neutral Anolyte and 77X possess antimicrobial properties that protect against essential fruit pathogens. The 77X product delivers extended, dependable performance across different testing environments. The effectiveness of antimicrobial agents depends on their concentration, which in turn affects their ability to penetrate bacterial cell walls. The citrus fruit industry can use both products as effective sanitizers for hard surfaces, although 77X proves more effective when used alone, while ANK Neutral Anolyte requires specific conditions to work. The research questions have been successfully answered, and the hypothesis that ANK Neutral Anolyte and 77X serve as effective sanitizers for plastic crates and pre-harvest citrus fruit has been proven correct, with 77X showing better and steadier antimicrobial capabilities. This research study carried out testing in a laboratory environment which assessed three different bacterial strains and three distinct surface materials. Future research should assess how well sanitizers work in commercial packinghouse settings while testing their effectiveness against various microorganisms and examining contact time, organic load, and methods used in large-scale applications.

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