Antimicrobial efficacy of TiO_2 against foodborne pathogens in microgreen systems

by

Chiara Lynn Abou Elias

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Major Professor
Dr. Valentina Trinetta

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Abstract

Microgreens are superfoods that have grown in popularity due to their high nutritional content. Recent recalls have highlighted several food quality and safety concerns since microgreens are harvested shortly after germination in a warm and moist environment. Titanium dioxide (TiO_2) has been used to inactivate microorganisms through the photocatalytic generation of reactive oxygen species. This technology is stable, non-toxic, inexpensive, and operates under ambient conditions. Hence, this study aimed to (1) Evaluate the efficacy of TiO_2 as a potential antimicrobial agent for water disinfection against *E. coli* and *Listeria* in a controlled laboratory setting; and (2) Verify the effect of TiO_2 on the growth of experimentally inoculated microgreens on a soilless substrate by evaluating microbial reduction and chemical-physical parameters. TiO_2 antimicrobial activity was tested against *E. coli* and *Listeria* at 0.1% w/v for up to 4 hours *in vitro* conditions. At appropriate time intervals, the bacterial population was enumerated. The experiments were performed in triplicate, with statistical significance set at a P value < 0.05. Both *E. coli* and *Listeria* populations exhibited significant reductions ($P < 0.05$) with log CFU/mL reductions of 3.59 and 4.66, respectively. The best conditions for microbial reduction were used in the microgreen growing systems where arugula seeds or mats were experimentally inoculated with *E. coli* and *Listeria*. Germination and growth were monitored for 14 days, and bacterial count, color, pH, height, weight, and titratable acidity were evaluated. Two tower systems were run in parallel: a control where no TiO_2 was applied, and a treatment where 0.05% w/v TiO_2 was integrated into the water reservoir system. Experiments were conducted in triplicate with statistical significance set at a P value < 0.05. By the end of the experiment, *Listeria* populations were reduced by 2.17 log CFU/cm^2 in samples grown from contaminated mats and by 1.91 log CFU/cm^2 in seed-contaminated samples, both showing statistically

significant reductions as compared to the control ($P < 0.05$). *E. coli* populations were significantly reduced only in the seed-inoculated samples treated with TiO_2 with a 1.57 log CFU/cm^2 reduction as compared to the control ($P = 0.05$). These results suggest that both contamination routes pose a potential risk in microgreens as pathogens can survive. No significant differences ($P > 0.05$) were observed in the pH, height, weight, or the a* and L* color values. There was a significant difference in titratable acidity between control and mat-inoculated samples with *L. innocua* and the b* color value between control and mat-inoculated samples with *L. innocua* and *E. coli* ($P < 0.05$). This study provides insight into the potential efficacy of TiO_2 to reduce the risk of foodborne pathogens in microgreen systems.

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Chapter 1 - Review of the Literature

1.1. Introduction to microgreens

Microgreens are “superfoods” that have become increasingly popular in the food industry due to their high nutritional value (Sharma et al., 2022). In recent years, they have garnered the attention of scientists, industries, and farmers all around the globe (Partap et al., 2023) and have been considered as a dietary alternative that can contribute to the nutritional security of large populations, especially in urban areas (Mishra et al., 2022). Microgreens offer outstanding nutritional elements, contributing significant health benefits to those who consume them (Sharma et al., 2022). Studies have shown that these plantlets contain higher nutrient and phytochemical concentrations than their fully-grown counterparts (Komeroski et al., 2023). These nutrient-dense greens are concentrated in vitamins, such as vitamin C (or ascorbic acid), Vitamin K (or Phylloquinone), Vitamin E (or tocopherol), Carotenoids (β-carotene is the precursor of Vitamin A), minerals (Ca, Mg, Na, K, Fe, Zn, among others), polyphenols, and glucosinolates (Bhaswant et al., 2023). Therefore, they are valued for their capacity to provide essential nutrients to diets lacking in nutritional variety, thereby enhancing consumer diets (Bhaswant et al., 2023).

Microgreens are renowned for their vivid colors, distinctive textures, and unique flavors, making them a popular choice for garnishing a variety of dishes such as soups, sandwiches, and salads (Sharma et al., 2022). Moreover, they can come in a variety of colors, ranging from green, yellow, and purple, to red, crimson, and multicolor, which further enhances their appeal to consumers (Bhaswant et al., 2023).

1.2. Microgreen varieties, composition, and growing practices

There is a wide diversity of microgreen plants that can be produced from a variety of crops such as vegetables, herbs, grains, and even some wild species. Variations in bioactivity and antioxidant capacity have been observed across different varieties of microgreens (Choe et al., 2018). These varieties include arugula, celery, red beet, green basil, and Tartary buckwheat, belonging to the families Brassicaceae, Apiaceae, Amaranthaceae, Chenopodiaceae, Lamiaceae, and Poaceae, respectively (Du et al., 2022). However, it is important to note that some plants, including but not limited to potatoes, tomatoes, eggplants, and peppers, contain antinutrients and could be toxic when consumed in an immature state, and thus, they are not suitable for microgreen growth (Jagatheeswari, 2014; Du et al., 2022).

The compositional variation of microgreens from different families has been analyzed by Sharma et al. (2022). Brassica microgreens showed the highest lipophilic and hydrophilic antioxidant activities, while Lamiaceae and Apiaceae microgreens exhibited high phenolic content. Regardless of the family the plantlets belong to, microgreens are made of three main components: a central stem, two cotyledon leaves, and true leaves (Choe et al., 2018). Although microgreens share similar characteristics with sprouts, they are distinct specialty crops and should not be confused even though both are consumed in an immature state (Kyriacou et al., 2016).

Microgreens have a rather short production cycle, (Sharma et al., 2022), harvested 7 to 21 days post-germination. The seedlings show species-specific production cycles, with most species collected at the appearance of the first true leaves and cotyledons fully expanded, usually when they attain a height of 2 to 8 cm (Bhaswant et al., 2023). Growers harvest the seedlings by manually or mechanically cutting them at the base of the hypocotyls (Kyriacou et al., 2016).

Microgreens typically have a short shelf life once harvested, due to the high respiration rate of these young seedlings (Chandra et al., 2012). To maximize the shelf life of the microgreens and extend their freshness, growers also harvest microgreens along with the growth medium after the plants have reached the desired height and appearance (Bhaswant et al., 2023). This harvesting method ensures that the highest shelf life of microgreens is achieved (Turner et al., 2020). The post-harvesting techniques of microgreens should follow good handling practices and should be done with the utmost care under the food safety standards (Bhaswant et al., 2023). Growers can voluntarily request audits of their farm food safety practices under Good Agricultural Practices (GAPs) and Good Handling Practices (GHPs) to demonstrate compliance (Riggio et al., 2019). Additionally, Riggio et al. (2019) highlighted that non-binding guidelines, such as the 1998 "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" (FDA, 1998) and the 2011 "Produce GAPs Harmonized Food Safety Standard" (United States Department of Agriculture, 2018), help growers comply with the Produce Safety Rule.

1.3. Microgreen systems

In a world where the population is ever-increasing, the economic, industrial, and agricultural sectors need to adapt to accommodate and feed the anticipated 9 billion residents of planet Earth (Godfray et al., 2010). Studies have estimated a 40% rise in the world population by 2050, which gives the agricultural segment the responsibility to increase its production by 70% (Ebert, 2014). However, the competition over limited land resources has greatly increased (Godfray et al., 2010), which led professionals to look for alternative solutions. Controlled Environment Agriculture (CEA) has emerged as one of the promising solutions that can meet the food safety and security demands of the growing population. Such systems ensure a more

reasonable and balanced use of resources with minimal damage to the environment (Lada et al., 2018).

There are several types of urban horticulture systems including greenhouses, roof-top farming, backyard gardening, as well as vertical farming that meet sustainable agriculture principles (Lada et al., 2018). Although traditional agriculture, or soil-based cultivation, is one of the most globally used farming techniques, soil-substitute growing systems have been gaining popularity (Du et al., 2022).

The microgreen business has been growing exponentially, and since these plants are relatively new food entities, novel production methods continue to emerge (Xiao et al., 2015). Microgreens are favored for their versatility in production. They can be grown on a commercial scale, or planted at home, an aspect that emphasizes their appeal (Ebert et al., 2015). These seedlings can be grown indoors in a controlled environment, such as a greenhouse or high tunnel via vertical agriculture (Singh, 2018). However, the majority of industrial farmers prefer to grow microgreens via soil-based methods (Figure 1.1) or hydroponic techniques (Figure 1.2) which are the leading production systems in the United States (Abaajeh et al., 2023; Xiao et al., 2015). Soil-substitute growing systems (Figure 1.3) essentially consist of trays of seeds sown on mats that are periodically irrigated with water which drains back into a reservoir. This provides a continuous flow of water throughout the system.

The growing substrate is fundamental for the proper germination and growth of microgreens, and different seedlings require different conditions and media for growth. The growing media can be organic, such as peat and coco coir, or inorganic, like perlite and vermiculite (Mishra et al., 2022). Other soil alternatives include gravel, burlap, jute fabric, fibrous mats, sand, biodegradable felt, and hemp (Misra & Gibson, 2020) (Figure 1.4). Several

factors are used to assess the suitability and quality of the soil substitutes as growing substrates which include the air and water-holding capacity, particle and dry bulk density, and the total pore space (Du et al., 2022). Each of these growing substrates is suitable for particular species of microgreens and has different effects on the yield and plantlet characteristics as demonstrated by Du et al. (2022). Their studies showed that planting red basil on coconut fiber yields a higher sucrose level than when planted on other substitutes. Then again, when grown on vermiculite or jute fiber, the anthocyanin concentrations are greater instead. Similarly, Kyriacou et al. (2020) studied the effects of natural and synthetic fiber substrates on the characteristics of microgreens. Their findings suggested that cabbage, kohlrabi, and cilantro grown on peat had greater width during growth, whereas the cilantro microgreens grown on coconut fiber were short as a result of the high salt sensitivity, which differs from that of cabbage or kohlrabi.

For this reason, choosing an adequate growing substrate when planting microgreens is very important, as it can stimulate the nutritional quality and functional capacity of these micro-vegetables (Kyriacou et al., 2020) and affect the final yield. Other significant factors to consider are the sowing density of the seeds, the type and method of irrigation (immersion or spraying), the pH of the water, the light quality, as well as the environmental conditions of growth, such as temperature and relative humidity (Moraru et al., 2022). In order to ensure consistency between different cycles, the temperature and relative humidity should periodically be measured to detect deviations.

1.4. Obstacles in microgreen production

In recent years, there have been several notable recalls of microgreens in North America due to contamination with pathogens such as *Salmonella* and *Listeria monocytogenes* (Du et al., 2022; Topalcengiz et al., 2024; Riggio et al., 2019). Since 2016, both the Canadian Food

Inspection Agency (CFIA) and the U.S. FDA's Food Recalls, Withdrawals, and Safety Alerts Database have reported multiple microgreens recalls (Table 1.1) due to contamination with either *Salmonella* or *L. monocytogenes* in the final product (Topalcengiz et al., 2024; Riggio et al., 2019; Yeargin et al., 2023). A Kansas-based American company voluntarily recalled their organic microgreens in 2016 due to a potential health risk from *Salmonella* contamination (U.S Food and Drug Administration, 2016). The following year, another Kansan company experienced a recall for their Asian mix microgreens, also due to *Salmonella*. In 2018, a Canadian company cultivating several types of microgreens and mixes had to recall products on three separate occasions due to contamination with *L. monocytogenes* (CFIA, 2018a, 2018b, 2018c, 2018d, 2018e; U.S. FDA, 2018). These products were distributed in Alberta, British Columbia, Ontario, and Washington State. In the years that followed, various Canadian producers also recalled a range of microgreens, including daikon radish, arugula (both organic and non-organic), salad mixes, broccoli (both organic and non-organic), and coriander, following the detection of *L. monocytogenes* and *Salmonella* (CFIA, 2018f, 2018g, 2019, 2020, 2021a, 2021b, 2021c). These reported recalls highlight the importance and the urgent need for enhanced, proactive, and preventive pathogen control strategies in microgreen production (Topalcengiz et al., 2024). Furthermore, contaminated seeds pose a systemic risk to the safety of these seedlings, which urges the implementation of guidelines for seed microbiological quality (Kyriacou et al., 2016). This is particularly important with regard to microgreens since they are mainly eaten raw (no microbial killing step is present in the process) to preserve their nutritional value and maintain their fresh, crisp texture (Turner et al., 2020).

The current U.S. Food and Drug Administration regulation does not mandate seed sanitization treatment being used in microgreen production, unlike in that of sprouts (Xiao et al.,

2015), which makes them more susceptible to microbial contamination. The Produce Safety Rule (81 FR 57784) of the Food Safety Modernization Act (FSMA) provides specific guidelines for sprouts but does not include any for microgreens (U.S. Department of Health and Human Services, 2015). Instead, microgreens, along with fresh herbs and edible flowers, are covered in another section in the Produce Safety Rule Part 112 titled: "Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption", which is specific to produce eaten raw (Riggio et al., 2019). Although microgreens are similar to sprouts, the FDA considers them distinct due to differences in harvest age and practices, so they are not included under the sprout requirements of Part 112 Sub-part M. Alternatively, because microgreens are susceptible to similar microbial risks as sprouts during cultivation, harvest, and marketing (Reed et al., 2018), the FDA recommends that microgreen growers follow sprout safety guidelines. For microgreens grown in hydroponic or aquaponic systems, the FDA recommends that growers follow the agricultural water and soil amendment guidelines outlined in Part 112, Sub-parts E and F (Riggio et al., 2019). Since microgreens are usually collected by hand, workers' hygiene and training as per Good Hygiene Practices (GHPs) are fundamental (Riggio et al., 2019).

Although contamination can happen in several ways, contaminated seeds are often the principal cause of foodborne illnesses linked to sprouts and are the most common source of such contamination. It has been theorized that pathogens, such as bacteria and viruses, could hide in the crevices of the seed surface, and also become internalized in the seed during germination (Riggio et al., 2019). Another possible source of contamination is irrigation water, which can contribute to contamination during the preharvest phase. Microgreens are susceptible to contamination from pathogens via many routes including seeds, workers, and irrigation water, as previously mentioned, as well as the facilities used to grow the plants (Işık et al., 2024). R. et al.,

(2008) found that the presence of *Salmonella Montevideo* on hydroponic tomatoes was correlated with the invasion of wild animals into the greenhouse. Lopez-Galvez et al. (2014) identified species of *Salmonella*, *Listeria*, and *Escherichia coli* on a hydroponic farm that used reclaimed and surface water for irrigation. Recycling the water in CEA systems is sustainable and reduces water usage but can also provide a pathway for pathogen survival, enabling microorganisms to spread throughout the system. Wang & Kiel (2016) reported a study where water in a microgreen hydroponic system was experimentally inoculated with human norovirus and after 12 days the pathogen still persisted in the recirculating water.

Climate-controlled greenhouses and indoor commercial spaces easily allow for the transfer of microorganisms into the microgreens' edible part due to the conducive environment that supports microorganisms' growth and survival (Işık et al., 2024). Several studies have proven that microorganisms such as *L. monocytogenes*, *Salmonella,* and *E. coli* O157:H7 can transfer from soilless substrates and from contaminated seeds to microgreens (Işık et al., 2020; Wright and Holden, 2018). *L. monocytogenes* are Gram-positive, rod-shaped, non-spore-forming, facultative anaerobic bacteria that are widespread in the environment (Wiktorczyk-Kapischke et al., 2021). These persistent microorganisms are responsible for a severe invasive illness known as listeriosis (Ferreira et al., 2014). In the United States, *L. monocytogenes* is the cause of around 1,460 hospitalizations and 260 deaths annually (Ferreira et al., 2014). On the other hand, *Salmonella* species are Gram-negative, non-spore-forming, facultative anaerobic rod-shaped bacteria that are ubiquitous in the environment (Oludairo et al., 2022). They are widely recognized foodborne pathogens that cause significant illness and death globally and were estimated to be the leading cause of foodborne bacterial illnesses and deaths in the U.S. (Moreno Switt et al., 2009). *E. coli* is a Gram-negative, facultative anaerobic, non-sporulating, and

coliform-forming bacterium (Basavaraju & Gunashree, 2023). Although most *E. coli* strains are harmless, certain strains can cause severe illness (Yang et al., 2017). Pathogenic *E. coli* alternates between the intestines of warm-blooded animals and environmental habitats like water, soil, and sediment (Basavaraju & Gunashree, 2023).

Işık et al. (2024) have demonstrated in their study that the transfer of any of the pathogens mentioned above is quite likely when any material used during cultivation such as the water, seeds, nutrient solutions, or soilless substrates, becomes contaminated. In fact, Reed et al. (2018) and Xiao et al. (2015) found that microgreens grown on hydroponic mats had higher bacterial populations than those grown in a soil-like medium. Wang et al. (2015) studied the growth of *L. monocytogenes* on 10-day-old microgreens and found that the bacteria increased by 0.7 logs on seed coats in soil-based systems and by 1.3 logs in hydroponic systems. Reed et al. (2018) demonstrated that microgreens grown in hydroponic systems had high levels of *Salmonella*-positive samples, in the mats and the microgreens. In a similar study, Di Gioia et al. (2017) compared the microbial populations across various growth media including textile-fiber mats, Green-Felt P500 containing a mix of recycled jute (85%), and kenaf (15%), peat, and Sure to Grow (STG) mats consisting of polyethylene terephthalate. They observed that microgreens grown on recycled fiber mats had lower microbial counts as compared to peat-based mixes and microgreens grown on peat. Di Gioia et al. (2017) also observed that microgreens grown on textile fibers and STG mats had no detectable levels of *Enterobacteriaceae* or *E. coli* in their edible parts, suggesting that these microorganisms are less likely transferred from the growing media to the plants. Conversely, jute-kenaf fiber media did not show detectable levels of *Enterobacteriaceae;* this group of bacteria was found in significant amounts in microgreens (Di Gioia et al., 2017).

Therefore, it is important to develop innovative technologies to address the microbial safety concerns that microgreens face and ensure the safety of these immature plants. These innovative solutions are necessary to restore consumer confidence in these delicate but highly nutritious greens and to support their broader market expansion (Du et al., 2022). Implementing effective hurdle technologies will allow producers to mitigate the microbial risks associated with the production of microgreens and improve the quality and safety of these plantlets.

1.5. Titanium dioxide

1.5.1. What is titanium dioxide?

In recent years, particular attention has been given to nanomaterials and nanoparticles (Subhapriya & Gomathipriya, 2018) due to their remarkable surface area to volume ratio, which allows an easier combination with other particles (Anbumani et al., 2022). They have been used in biomedical, pharmacological, and therapeutic applications, owing to their bioactivity, biodegradability, and bioavailability (Diana & Mathew, 2022). Metal oxide nanoparticles (MONPs) are a type of nanoparticle characterized by their distinct physio-chemical attributes (Diana & Mathew, 2022). These metallic nanoparticles have also been used in the packaging of food products and have achieved great results in terms of antimicrobial activity (Mesgari et al., 2021). These inorganic materials, including CuO, ZnO, MgO, CaO, and TiO_2 (Anbumani et al., 2022), are recognized for their significant impact in inhibiting foodborne pathogens (Mesgari et al., 2021) thanks to their exceptional effectiveness and reactivity as antimicrobial agents (Anbumani et al., 2022). Among the metal oxide nanoparticles, titanium dioxide nanoparticles (TiO_2 NPs) have been applied in several industries, such as food preservation, active food packaging, wound healing, environmental pollution repair, solar cells, biological imaging, and biosensing (Diana & Mathew, 2022; Mesgari et al., 2021). TiO_2 is known for its high

photocatalytic activity, corrosion resistance, high refractive index, mechanical and chemical stability, excellent photochemical properties, and antibacterial activity (Diana & Mathew, 2022; Srisitthiratkul et al., 2011). The Food and Drug Administration categorizes TiO_2 as Generally Recognized as Safe, meaning that it can be utilized safely in food products when kept within recommended limits (FDA, 2023). Nevertheless, it is advised to use it within safe toxicity limits as an antimicrobial agent (Mesgari et al., 2021).

1.5.2. Mechanism of action of titanium dioxide

The photocatalytic properties of TiO_2 were first identified more than 90 years ago and gained prominence with the discovery of the photoelectrolysis of water (Shirai et al., 2016). Studies have shown that TiO_2 exhibits high photoactivity under near-UV light, and can render microorganisms nonviable, including bacteria and spores (Yeung et al., 2009). Its antimicrobial properties are associated with its photo-induced superhydrophilicity and the generation of reactive oxygen species (ROS) upon the photoactivation of TiO_2 (Yeung et al., 2009).

The primary mechanism leading to the formation of radicals involves TiO_2 nanoparticles reacting with hydroxyl groups (-OH) and oxygen (O_2) which are produced from the breakdown of water and oxygen. This reaction leads to the formation of hydroxyl free radicals and superoxide anions (Mesgari et al., 2021). This process occurs after the nanoparticles are activated by UV light in the 254-385 nm range, which excites their electrons (Huang et al., 2016; Subhapriya & Gomathipriya, 2018, Shirai et al., 2016).

The resulting free radicals, known as ROS, are powerful oxidizing agents that can cause significant oxidative damage to the cell membranes and cell walls of bacteria, eventually leading to increased cell permeability and cell death (Shirai et al., 2016; Anbumani et al., 2022) (Figure 1.5). Furthermore, the increased surface area of the nanoparticles, which constitutes the area of

interaction with pathogenic microorganisms, allows these nanoparticles to work as antimicrobial agents (Subhapriya & Gomathipriya, 2018).

1.5.3. Titanium dioxide as an antimicrobial

There has been a significant amount of research done on the antimicrobial efficacy of TiO_2 NPs, which shows their potential use in various applications. Several studies have highlighted the effectiveness of TiO_2 NPs synthesized using different extracts and their impact on a wide range of pathogens. For example, Alasmary et al. (2022) examined the antibacterial efficiency of TiO_2 NPs prepared from lichen *Parmotrema austrosinense*, which exhibited high antimicrobial activity against bacterial (*Xanthomonas phaseoli*) and fungal (*Fusarium oxysporum*) plant pathogens. Similarly, Alavi et al. (2019) found that TiO_2 NPs fabricated from *Protoparmeliopsis muralis* lichen effectively inhibited quorum sensing in multidrug-resistant *Staphylococcus aureus* (MRSA), emphasizing their potential against resistant bacterial strains.

The biosynthesis of nanoparticles has been garnering attention because of the evolving necessity of using nontoxic and renewable solvents and materials that use low-cost methods and are eco-friendly. Subhapriya and Gomathipriya (2018) synthesized TiO_2 NPs using *Trigonella foenum-graecum* leaf extract, revealing promising antibacterial properties compared to the control, thereby demonstrating the nanoparticles' effective antimicrobial activity. In a similar study, Abu-Dalo et al. (2019) prepared green TiO_2 NPs and evaluated their ability to disinfect water by removing bacteria. According to their findings, TiO_2 NPs reduced the growth of both Gram-positive and Gram-negative bacteria, with the level of inhibition dependent on the concentration of TiO_2 NPs. Anbumani et al. (2022) reported that TiO_2 NPs biosynthesized using *L. acutangula* leaf extract exhibited substantial inhibition against a wide range of bacteria (*E. coli, S. aureus, P. aeruginosa, E. faecalis, K. pneumoniae, B. subtilis*) and fungi (*A. niger, A.*

flavus, R. oryzae, S. rolfsii). These findings demonstrated the effectiveness of TiO_2 NPs as antibacterial and antifungal agents, suggesting their potential use in clinical and drug applications. A study performed by Shirai et al. (2016) showed that TiO_2 continues to exhibit a photocatalytic effect even after UV light exposure is halted and has antimicrobial activity against periodontal pathogens, such as *Porphyromonas gingivalis*. These nanoparticles could be an effective strategy for bacterial decontamination in the treatment of peri-implantitis, which is an inflammatory reaction of the soft tissues around an implant, that causes nearby bone loss (Shirai et al., 2016).

The prevention of biofilm formation is another important area where TiO_2-based materials have shown promise. Santhosh and Natarajan (2015) demonstrated that epoxy/TiO_2 and epoxy/Ag-TiO_2 nanocomposites effectively inhibited biofilms formed by *Staphylococcus aureus* (Gram-positive) and *E. coli* (Gram-negative) when exposed to UV light. Ansari et al. (2020) further explored the antibacterial and antibiofilm properties of TiO_2 nanofibers (NFs) and found them effective against drug-resistant pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. Their findings suggest that TiO_2 NFs could potentially be used in the coatings of medical devices and equipment to prevent biofilm development. In a related study, Ansari et al. (2022) recommended the use of biosynthesized TiO_2 NPs as therapeutic agents in the medical field, given their demonstrated high inhibition rates against human pathogenic microorganisms: *B. subtilis* and *S. aureus* (Gram-positive) as well as *P. aeruginosa* and *E. coli* (Gram-negative).

Several studies found optimistic results by combining TiO_2 with other components which allowed enhanced activity. For instance, coupling TiO_2 with FeS_2 nanocomposites showed a strong antibacterial effect compared to those treated with TiO_2 or FeS_2 nanoparticles alone

(Mutalik et al. 2020). A probable explanation was attributed to an enhanced production of ROS from the TiO_2-FeS_2 combination. Another pairing was described by Mesgari et al. (2021) with chitosan and TiO_2 composites. This review showed a broad range of antimicrobial activity against foodborne pathogens, notably *Staphylococcus aureus, E. coli, S.* Typhimurium, and *P. aeruginosa*, due to the ability of TiO_2 to improve chitosan's natural antimicrobial properties. This coupling of chitosan-TiO_2 bio-composites could be applied in food packaging and preservation systems, creating affordable eco-friendly films. Huang et al. (2016) found that the combination of TiO_2 with polylactide under UV-A light catalyzed the formation of ROS and improved the microbial inactivation of *Listeria.*

These studies show the influential and versatile antimicrobial properties of TiO_2 NPs which are effective against a wide range of microorganisms, in combinations with various components, and numerous fields, whether in medicine, agriculture, or food safety.

Figure 1.1. Soil-based Microgreen System.

A multi-tiered shelving system with trays of microgreens under LED grow lights, arranged for efficient indoor cultivation.

Source: Reddit. (2022).

Figure 1.2. Hydroponic Microgreens Growing System.

Hydroponic setup designed for water-based cultivation, allowing microgreens to thrive without soil.

Source: NFT Hydro. (n.d.).

Figure 1.3. Soil-substitute Microgreen Growing System.

Indoor cultivation of microgreens in a greenhouse environment.

Source: Home Grown Passion. (2021).

Figure 1.4. Different Growing Mediums for Microgreens.

Various growing mediums for microgreens including soil, coco noir, and hydroponic mats.

Source: React Greens. (n.d.).

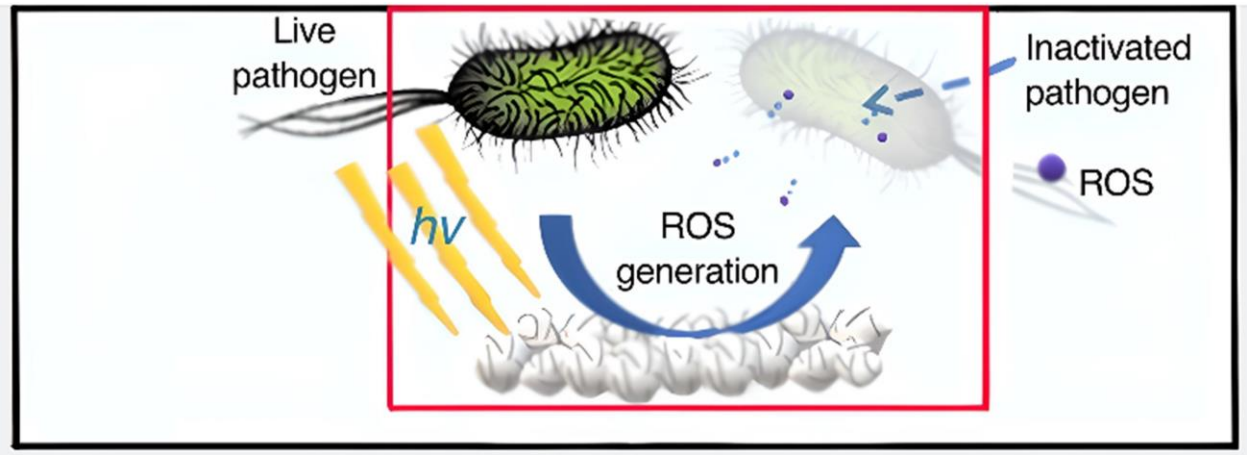

Figure 1.5. Schematic representation of photocatalytic inactivation of a live pathogen via interactions with photogenerated ROS.

Table 1.1. Recalls of foodborne disease associated with microgreens grown in North America.

Microgreen Type	**Pathogen**	**Location**	**Date**	**Reference**
Organic Microgreens	*Salmonella*	Colorado, Kansas, USA	October 7, 2016	U.S Food and Drug Administration (USFDA), 2016
Asian Mix Microgreens	*Salmonella*	Colorado, Kansas, Missouri, USA	March 23, 2017	U.S Food and Drug Administration (USFDA), 2017
Arugula microgreens, Broccoli microgreens, Fresh Microgreen Mix, Sweet and Crunchy Microgreen Mix, Spicy Microgreen Mix, Pea Shoots Microgreens, Sunflower Microgreens, Wheatgrass, Spring Pea microgreen Mix	*Listeria monocytogenes*	Alberta, British Columbia, Northwest Territories, Canada; Washington, USA	April 24, 2018, and April 30, 2018, May 1, 2018	Canadian Food Inspection Agency (CFIA), 2018a-c U.S Food and Drug Administration (USFDA), 2018
Broccoli microgreens, Radish microgreens, Spicy micro & lettuce mix	*Listeria monocytogenes*	Ontario, Canada	August 25, 2018, and September 22, 2018	Canadian Food Inspection Agency (CFIA), 2018d, e
Daikon Radish Microgreens	*Listeria monocytogenes*	New Brunswick, Nova Scotia, Prince Edward Island, Canada	June 28, 2018	Canadian Food Inspection Agency (CFIA), 2018f
Arugula Microgreens	*Salmonella*	Quebec, Canada	June 29, 2018	Canadian Food Inspection Agency (CFIA), 2018g
Mix Spicy Microgreens	*Listeria monocytogenes*	Quebec, Canada	May 22, 2019	Canadian Food Inspection Agency (CFIA), 2019
Organic Broccoli Microgreens, Organic Arugula Microgreens, Organic Coriander Microgreens	*Salmonella*	New Brunswick, Quebec, Canada	August 28, 2020,	Canadian Food Inspection Agency (CFIA), 2020
Broccoli Microgreens	*Salmonella*	Alberta, Canada	November 8, 2021	Canadian Food Inspection Agency (CFIA), 2021a
Broccoli Microgreens, Salad Mix Microgreens	*Salmonella*	Ontario, Canada	November 9, 2021	Canadian Food Inspection Agency (CFIA), 2021b
Broccoli Microgreens	*Salmonella*	Ontario, Canada	November 9, 2021	Canadian Food Inspection Agency (CFIA), 2021c

Microgreens	*Salmonella*	NY, PA, MA, NJ, VA, MD & NC, USA	December 23, 2022	U.S Food and Drug Administration (USFDA), 2022

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Chapter 2 - Research Gaps

While many studies have explored the chemical compositions, nutritional values, and health benefits of microgreens (Bhaswant et al., 2023, Işık et al., 2022), microbiological-focused studies concerning microgreens remain scarce (Işık et al., 2022).

To bridge this gap, there is an urgent need for research focused on developing intervention strategies that preserve the quality and safety of microgreens (Deng et al., 2021). Such research is crucial for helping the microgreen industry overcome challenges similar to those previously faced by the mature produce and sprout industries (Turner et al., 2020). Sanitation is a key intervention strategy that plays a critical role in the development of industrial microgreen production (Kyriacou et al., 2016). Researchers have investigated various sanitizing treatments for microgreens, such as chlorine washes (Lee et al., 2009; Xiao et al., 2014), calcium treatments (Kou et al., 2015), peracetic acid (Fuzawa et al., 2021), citric acid, ascorbic acid, and combinations such as citric acid with ethanol or ascorbic acid (Chandra et al.,2012; Dalal et al. 2020).

Although chemical disinfectants are effective, they might produce undesirable and harmful byproducts and pose environmental risks (Elgohary et al., 2021; Laxma Reddy et al., 2017; Arun et al., 2023). For this reason, there is a growing need for sustainable and eco-friendly alternatives that can successfully control microorganisms (Arun et al., 2023; Elgohary et al., 2021). Researchers have recently investigated the potential of microbial photocatalytic disruption through the use of semiconductor nanoparticles, such as TiO_2 (Laxma Reddy et al., 2017). This technique is highly efficient, reusable, cost-effective, safe, and non-toxic (Elgohary et al., 2021; Nadeem et al. 2018). TiO_2 generates ROS upon interacting with microbial cells, causing

membrane leakage by disrupting cell wall integrity through phospholipid oxidation and ionic balance alteration (Laxma Reddy et al., 2017; Nadeem et al., 2018).

Therefore, to address the identified research gaps and the urgent need for effective intervention strategies, this research aims to achieve the following objectives:

- Evaluate the efficacy of TiO_2 as a potential antimicrobial agent for water disinfection against *E. coli* and *Listeria* in a controlled laboratory setting;
- Verify the effect of TiO_2 on the growth of experimentally inoculated microgreens on a soilless substrate by evaluating microbial reduction and chemical-physical parameters.

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Chapter 3 - Assessing the efficacy of TiO_2 as a potential antimicrobial agent for disinfecting water contaminated with *E. coli* and *L. innocua*

Abstract

Microgreens are superfoods that have gained attention among farmers, consumers, and scientists due to their rich nutritional profile. However, several recalls have been reported over the years, raising concerns about the safety of microgreen production. Titanium dioxide (TiO_2) has been explored as a means of inactivating microorganisms through photocatalytic generation of reactive oxygen species. This technology is stable, non-toxic, inexpensive, and operates under ambient conditions. Therefore, this study aimed to evaluate the antimicrobial efficacy of TiO_2 for water disinfection against *Escherichia coli* and *Listeria* in a controlled laboratory setting. TiO_2 antimicrobial activity was tested against *E. coli* and *Listeria* at 0.1% w/v for up to 4 hours *in vitro* conditions. At appropriate time intervals, the bacterial population was enumerated. Experiments were conducted in triplicate and a significant *P* value was set < 0.05. By the end of the experiment, both *E. coli* and *Listeria* populations were significantly reduced ($P < 0.05$), with *Listeria* showing a 4.66 log CFU/mL reduction compared to the control, while *E. coli* exhibited a 3.59 log CFU/mL reduction. This study provides insight into the potential efficacy of TiO_2 to reduce the risk of foodborne pathogens in microgreen systems.

3.1. Introduction

Microgreens have gained significant popularity throughout the food industry, notably due to their dense nutritional profile, earning them the title of "superfoods" (Sharma et al., 2022). They are known for their vivid colors, varied textures, and unique flavors, and are often used to elevate the taste and presentation of dishes such as soups, sandwiches, and salads (Sharma et al., 2022). Additionally, microgreens are recognized for improving overall dietary quality by providing essential nutrients in diets lacking diversity (Bhaswant et al., 2023).

Microgreens are versatile in their production methods but are typically grown indoors in soil-based or hydroponic systems often within controlled environment agriculture (CEA) settings for optimal growth conditions (Singh, 2018; Abaajeh et al., 2023). Nevertheless, microgreens present several food safety concerns, which led to numerous recalls over the past decade (Du et al., 2022; Topalcengiz et al., 2024). Since microgreens are immature plants, they are more prone to pathogen internalization than mature plants, as their internal protective structures are not fully developed, allowing easier access for bacteria colonization (Warriner et al, 2003). Furthermore, contaminated seeds and soil-substitute growing media can also lead to microgreen contamination (Turner et al., 2020; Wright & Holden, 2018; Xiao et al., 2015; Reed et al., 2018; Misra et al., 2021). Pathogens can be introduced through various routes, including cutting tools used during harvesting or from the workers (Riggio et al., 2019; Turner et al., 2020). Irrigation water poses one of the largest risks of contamination of microgreens, especially in hydroponic systems (Işık et al., 2024). While the water in these systems is recycled for sustainability, it can also become a pathway for pathogen survival, potentially spreading contamination throughout the system (Riggio et al., 2019).

For these reasons, it is crucial to develop efficient intervention strategies to ensure safe microgreens for consumption (Deng et al., 2021). Various preharvest and postharvest interventions have been tested throughout the literature, such as calcium treatments (Kou et al., 2015), chlorine washes (Lee et al., 2009; Xiao et al., 2014), peracetic acid (Fuzawa et al., 2021), ascorbic acid, citric acid, ethanol, and their combinations (Chandra et al., 2012; Dalal et al. 2020). Additionally, modified atmosphere packaging and temperature control have been used to maintain the quality and extend the shelf life of microgreens (Turner et al., 2020).

Nonetheless, there have been growing concerns in recent years about water contamination from organic pollutants resulting from the use of chemical disinfectants in agricultural production (Wang et al., 2016). Consequently, the attention of consumers and scientists has shifted toward chemical alternatives, such as TiO_2 (Wang et al., 2016), which is a metal oxide semiconductor that has been used for its ability to inhibit pathogens thanks to its photocatalytic activity when activated by UV light (Mesgari et al., 2021). The antimicrobial activity of TiO_2 stems from its ability to generate reactive oxygen species (ROS) which can inactivate microorganisms (Yeung et al., 2009; Laxma Reddy et al., 2017). ROS are powerful oxidizing agents that can cause oxidative damage to bacterial cell membranes and cell walls, leading to membrane leakage, ionic imbalances, and eventually cell death (Shirai et al., 2016; Anbumani et al., 2022; Nadeem et al., 2018). The key process behind ROS formation involves TiO_2 interacting with hydroxyl groups (-OH) and oxygen (O_2) generated from water and oxygen decomposition, which leads to the formation of hydroxyl free radicals and superoxide anions (Mesgari et al., 2021). Due to its promising attributes, TiO_2 has been widely used in recent years as an eco-friendly photocatalyst valued for its chemical stability and low toxicity (Anbumani et al., 2022) in food preservation, active food packaging, environmental pollution repair, wound

healing, biosensing, biological imaging, and water disinfection (Diana & Mathew, 2022; Mesgari et al., 2021). Nevertheless, the application of TiO_2 to disinfect water reservoirs of microgreen systems remains unexplored. Therefore, this study aimed to evaluate the efficacy of TiO_2 as a potential antimicrobial agent for water disinfection against *E. coli* and *Listeria* in a controlled laboratory setting.

3.2. Materials and Methods

3.2.1. Bacterial strains and inoculum preparation

Two BSL-1 strains were used in this study: *Escherichia coli* TVS 353 and *Listeria innocua* ATCC 33090. Two days before the experiment, strains were retrieved and activated from the frozen stock collection stored at -80℃, streaked onto Tryptic Soy Agar (TSA; BD, Franklin Lakes, NJ, USA), and incubated at 37℃ for 24h. For the experiment day, a bacterial inoculation solution in phosphate buffer saline (PBS; EMD Millipore, Burlington, MA, USA) was obtained by centrifuging at 4000 x g for 10 minutes an overnight culture in Tryptic Soy Broth (TSB; BD, Franklin Lakes, NJ, USA) at 37℃ on a rotary shaker set to 70 rpm (prepared by using an isolated colony) to achieve an inoculation level of 9 $\log_{10}$ CFU/ml: 0.1% w/v of *E. coli* and 0.2% w/v of *Listeria* in 110 L of water. Counts were checked by serial dilution on TSA, Modified Oxford Agar (MOX; BD, Franklin Lakes, NJ, USA) supplemented with the *Listeria* selective supplement (Oxford formulation) (Hardy Diagnostics, Santa Maria, CA, USA). and MacConkey Agar (BD, Franklin Lakes, NJ, USA).

3.2.2. Titanium dioxide system

In order to evaluate the ability of TiO_2 to reduce *E. coli* and *Listeria* populations in a microgreen system, experiments in a lab setting were conducted. As mentioned before, TiO_2 is activated by UV light, therefore a UV-TiO_2 treatment system was built by the Technology

Development Institute at Kansas State University. The system consisted of a submersible 1/4-horsepower thermoplastic utility pump (Barracuda pumps, Menards®, Eau Claire, WI, USA) fixed on a stand and placed in a 189 L plastic container (Sterilite, Townsend, MA, USA) (Figures 3.1 and 3.2). The pump was connected to a 36-watt UVC lamp (The Pond Guy Inc., Bellevue, NE, USA) covered in an outer housing that allows water circulation. The UV clarifier operated by directing the water with the TiO_2 into a vortex chamber, optimizing its exposure to high-intensity UV light. The ultraviolet rays triggered the photoactivity of the TiO_2 nanoparticles which inactivated microorganisms through the photocatalytic generation of reactive oxygen species, thus purifying the water before it left the unit and returned to the container (The Pond Guy©, 2024). The system contained a Programmable Logic Controller (PLC) that pumped water until the "low water level" float had been triggered. A single push button connected to the PLC was pressed at a pre-determined time, initiating the programmed system to operate for 4 hours (based on our preliminary experiments). The powder was left to settle until the next experiment day. The system was clean between runs.

3.2.3. Inoculation, treatment, and sampling

Prior to microbial inoculation, a 1 mL sample was taken from the water in the system to confirm no pathogen presence (negative control). The water system inoculated with bacteria but without the addition of TiO_2 powder was considered a positive control and monitored at the same sampling times as the treatment samples. For the treatment samples, TiO_2 powder (1 g/L) and bacteria suspension were added to the system which was allowed to run for a few minutes to facilitate the mixing, before sampling. Sampling was performed for all the conditions every hour for a total of 4 hours of experiment.

3.2.4. Microbial enumeration

At the appropriate sampling times, 1 ml aliquot was vortexed for 1 minute and transferred into 9 mL peptone water (BD, Franklin Lakes, NJ, USA) for serial dilution. From the selected dilution, 100 µL were plated on MacConkey Agar for *E. coli*, or on MOX for *Listeria.* Plates were incubated at 37°C for 24h and colonies were counted and expressed as CFU/mL.

3.2.5. Experimental Design and Statistical Models

This experiment consisted of a one-way treatment structure including two sanitizer treatments (positive control, and TiO_2) in a repeated measures design. The experiment was conducted separately for each microorganism (*E. coli* and *L. innocua*). The bacterial survival of each microorganism, measured as log CFU/mL, was measured at five time points (0, 1, 2, 3, and 4 hours after inoculation) for all the conditions tested. The data were analyzed using a linear mixed-effects model to assess the effects of treatment and time on bacterial survival. The model included fixed effects for treatment and time, and their interaction, to evaluate how different treatments affected bacterial survival over time. Note that time was considered a continuous covariate. Random effects were incorporated to account for variability among replicates. Expected marginal means and standard errors were obtained for post-hoc mean comparisons with a significance level of 0.05. The analyses were performed using the lme4 (Bates et al., 2015) and emmeans (Lenth, 2024) packages in R, version 4.4.1 (R Core Team, 2024).

3.3. Results

E. coli and *L. innocua* populations were monitored over a 4-hour period in a water system using two treatments: a control group without an antimicrobial agent and a treatment group where TiO_2 was added as an antimicrobial agent.

The ANOVA analysis for both *E. coli* and *L. innocua* (Tables 3.1 and 3.2) shows that time had a significant effect on bacterial reduction ($P < 0.05$). Also, the treatment was found to be a significant factor ($P < 0.05$), with the lowest counts obtained when TiO_2 was used compared to the control. A significant interaction was observed between time and treatment ($P < 0.05$).

Figure 3.3 illustrates the trend in *E. coli* populations (log CFU/mL) across three trials for both control and TiO_2-treated groups. At the start of the experiment (0 hours), the TiO_2-treated group had an initial bacterial population of 5.95 ± 0.38 log CFU/mL, while the control group started at 9.17 ± 0.06 log CFU/mL. During the 4-hour period, the TiO_2-treated group experienced a significant reduction in *E. coli* populations, achieving an overall reduction of 3.59 log CFU/mL ($P < 0.05$). In contrast, the control group showed only a slight decline which was not statistically significant ($P > 0.05$). Furthermore, the 95% confidence interval for the control group ranged from -0.244 to 0.208, which indicates that no significant reduction occurred over time. On the other hand, the 95% confidence interval for the TiO_2-treated group ranged from -1.121 to -0.669, which confirms a significant reduction in the bacterial population over time.

Figure 3.4 displays the log CFU/mL reduction in *L. innocua* populations under the same experimental conditions described for *E. coli*. The TiO_2-treated group showed an initial *L. innocua* population of 6.06 ± 0.32 log CFU/mL, while the control group began at 9.19 ± 0.28 log CFU/mL. Similar to *E. coli*, the TiO_2 treatment caused a significant reduction in the *L. innocua* populations, resulting in an overall reduction of 4.66 log CFU/mL ($P < 0.05$). The control group exhibited a non-significant decrease ($P > 0.05$). The 95% confidence interval for the control group (0.282 to 0.127), indicating no significant change, while the TiO_2-treated group had a 95% confidence interval from -1.419 to -1.011, which means that this group had a significant reduction in bacterial populations over time. These results highlight the effectiveness

of TiO_2 as an antimicrobial agent, emphasizing its potential application in water treatment systems to control bacterial contamination.

3.4. Discussion

This study investigated the application of TiO_2 as an antimicrobial agent as a means of reducing *E. coli* and *L. innocua* populations experimentally inoculated into a water system. Past FDA investigations into romaine lettuce outbreaks indicate that agricultural water, including irrigation canals and surface reservoirs, may be a vector of crop contamination (Bell et al., 2021). For this reason, it is crucial to find sustainable sanitation and disinfection methods to reduce or eliminate pathogens in such systems. TiO_2 has emerged as a chemical alternative in recent years due to its antimicrobial properties, which stem from its photocatalytic activity (Wang et al., 2016; Mesgari et al., 2021). When activated by UV light, TiO_2 generates reactive oxygen species (ROS) that are oxidizing agents capable of causing oxidative damage to bacterial cells, leading to cell wall disruption and ultimately resulting in cell death. (Shirai et al., 2016; Anbumani et al., 2022; Nadeem et al., 2018; Yeung et al., 2009; Laxma Reddy et al., 2017).

Bell et al. (2021) reported that irrigation water disinfection has become one of the crucial parameters to monitor for produce safety, as it is important to tighten the microbial quality standards of irrigation water after the deadly 2006 multistate outbreak of *E. coli* linked to bagged spinach. For this reason, the Leafy Greens Marketing Agreement (LGMA) for water metrics in California and Arizona now mandates that overhead irrigation water, such as canals, used within 21 days of harvest, must be treated for pathogen elimination (Bell et al., 2021). Additionally, research by Machado-Moreira et al. (2021) demonstrated that a single inoculation event using overhead spray irrigation was enough to contaminate plants, which allowed *E. coli* and *L. innocua* to attach and persist in the water samples for up to 28 days until the plants reached a

marketable stage. Machado-Moreira et al. (2021) also emphasized the significance of using water of good microbiological quality in fresh produce production. It is crucial to have in place a good sanitizing system to reduce pathogens.

In our experiment, we were able to achieve 3.59 log CFU/mL reduction in the *E. coli* populations after 4 hours of experiment, and 4.66 log CFU/mL of *L. innocua.* Miranda et al. (2016) compared advanced oxidation processes (AOPs), including H_2O_2/UV, TiO_2/UV, and N-TiO_2 (TiO_2 doped with nitrogen)/UV, with chlorination for the disinfection of surface water and inactivation of antibiotic-resistant *E. coli*. Their findings showed that the H_2O_2 control achieved a 1-log reduction after 90 minutes, while UV alone resulted in a 4-log inactivation after 75 minutes. On the other hand, the combined H_2O_2/UV treatment led to a total inactivation of the antibiotic-resistant *E. coli*, with a 6-log reduction, which they attributed to ROS generation. These findings are supported by Cho et al. (2004) who identified a strong linear correlation between hydroxyl radicals and the inactivation rate of *E. coli*. Miranda et al. (2016) also tested N-TiO_2 as a photocatalyst, achieving 4.5-log and 6.5-log reductions at synthesis temperatures of 0°C and -20°C, respectively. They attributed the greater reduction to a larger specific surface area, which can enhance light exposure and increase the photocatalytic inactivation rate (Miranda et al., 2016). The differences between our results and those of Miranda et al. (2016) may be due to experimental conditions, as they used 500 mL of water and a TiO_2 concentration of 0.05 g/L, while we used 110 L of water with a TiO_2 concentration of 1 g/L.

Similarly, Ogunniyi et al. (2019) compared the effectiveness of different sanitizers, including pH-neutral electrolyzed oxidizing water (EOW), chlorine dioxide (ClO_2), and sodium hypochlorite (NaClO), in artificially contaminated water with *E. coli*, *Listeria*, and *Salmonella*, across various pH levels and organic matter content. Their findings revealed that EOW was

effective in reducing microbial population with 4 to 6 log reductions in water free from organic matter, and its ability was equivalent or even better in some instances than the chlorine-based disinfectants (NaClO, ClO_2). In our study, we found comparable results, with log reductions of 3.59 log CFU/mL for *E. coli* and 4.66 log CFU/mL for *L. innocua,* using 1 g/L of TiO_2.

Mensah et al., (2024) also studied the effectiveness of chemical sanitizers to eliminate *Salmonella* Typhimurium, a gram-negative bacterium, from surfaces in commercial hydroponic systems. Their results revealed that sanitizers such as quaternary ammonium compounds, SaniDate 12.0 (200 ppm), Zerotol (5%), and Virkon (1%) successfully removed *Salmonella* from surfaces, whereas chlorine-based sanitizers failed to eliminate the pathogen, yielding results statistically comparable to the water treatment. Nevertheless, it is important to note that although effective, chemical disinfectants might produce harmful and undesirable byproducts leading to environmental concerns (Elgohary et al., 2021; Laxma Reddy et al., 2017; Arun et al., 2023). In our study, we obtained log reductions (CFU/mL) of 3.59 and 4.66 for *E. coli* (gram-negative) and *L. innocua* (gram-positive) respectively, using a concentration of 1 g/L of TiO_2. Chlorine-based sanitizers have traditionally been the preferred means of surface disinfection among growers, but the regular use of such sanitizers in high concentrations can damage components such as pumps aerators, and tubing (Mensah et al., 2024). For this reason, further research should investigate the use of TiO_2 in commercial applications. TiO_2-based photocatalysis is highly efficient, reusable, cost-effective, and both safe and non-toxic (Elgohary et al., 2021; Nadeem et al., 2018). Its photocatalytic activation makes it a sustainable alternative for long-term use in hydroponic systems, especially since it avoids equipment degradation and safety risks associated with chlorine-based disinfectants, thereby reducing both environmental impact and operational costs.

A study by Bonetta et al. (2013) investigated the photoactivated antibacterial properties of surfaces coated with TiO_2 against *Staphylococcus aureus, Pseudomonas putida, E. coli*, and *L. innocua*. Their findings revealed that *L. innocua* was the most sensitive to inactivation by TiO_2, although the TiO_2-coated surfaces successfully inhibited all tested microorganisms under UV light (Han et al., 2016; Bonetta et al., 2013). Our findings align with those of Bonetta et al. (2013), as we observed substantial reductions in *E. coli* (3.59 log CFU/mL) and *L. innocua* (4.66 log CFU/mL) ($P < 0.05$), with *L. innocua* showing the greatest reduction.

Similarly, Helali et al. (2014) studied the disinfection of *E. coli* in water through photocatalytic and photolytic processes under natural light, using various photocatalysts, including three samples of TiO_2: TiO_2 P-25, TiO_2 PC500, TiO_2 Ruana, and one sample of Bismuth tungstate (Bi_2WO_6) at different concentration levels. They were able to achieve a total inactivation of 6 logs between 3 to 5 hours using solar disinfection (SODIS). However, when the photocatalysts were introduced to the water, the bactericidal effect of solar disinfection was enhanced, reducing the disinfection time, which varied based on the catalyst used. Their findings align with the results obtained in our study, although they achieved a higher reduction, which could be attributed to the experimental conditions used in their research.

In a similar context, Sichel et al. (2007) investigated the photocatalytic water disinfection of *E. coli* K12 in a compound parabolic collector using a TiO_2-coated paper matrix made of synthetic fiber. They evaluated how factors such as flow rate, water quality, and microbial load affected bacterial survival under both solar photocatalysis and in the absence of light. Their results revealed that TiO_2 significantly enhanced solar disinfection, achieving up to a 6-log reduction in 90 minutes. On the other hand, when conducting the experiment in the dark, 99% of the *E. coli* bacteria were inactivated after 90 minutes. Their significant inactivation could be

attributed to the use of a paper matrix, which was absent from our experiment, as we used a TiO_2-water suspension at a concentration of 1 g/L. Additionally, they observed the highest reduction in dark conditions, which were not included in our experiments.

In line with this, Fernández-Ibáñez et al. (2015) used a specific brand of TiO_2 called Evonik Aeroxide P25, or TiO_2 P25 for short, as well as TiO_2 modified with reduced graphene oxide (TiO_2-RGO) to disinfect water contaminated with *E. coli* and *Fusarium solani* spores. Their findings demonstrated rapid disinfection for both microorganisms, with a notably improved inactivation rate for *E. coli* when using the TiO_2-RGO composite compared to P25 alone. In our study, we utilized TiO_2 alone without any combinations with other materials. Future research could explore the potential benefits of integrating TiO_2 with other substances to enhance its photocatalytic activity.

3.5. Conclusion

In conclusion, this study highlights the efficiency of TiO_2 as an antimicrobial agent in reducing *E. coli* and *L. innocua* populations in water systems. The effect of time had a significant impact on the bacterial counts throughout the experiment, with the TiO_2 treatment group exhibiting greater reductions compared to the control group. Additionally, there was a significant interaction between time and the TiO_2 treatment. This research indicates that TiO_2 could serve as a valuable antimicrobial solution to mitigate bacterial populations in water treatment systems, such as those used in hydroponic microgreen cultivation. Nevertheless, further research is essential to understand the long-term efficacy of TiO_2 under various environmental conditions, such as laboratory conditions and field trials in commercial settings.

3.6. Acknowledgments

The authors extend their gratitude to the Kansas Department of Agriculture Specialty Crop Block and the USDA Specialty Crop Block Grant Program for providing the funding for this project.

3.7. Figures and Tables

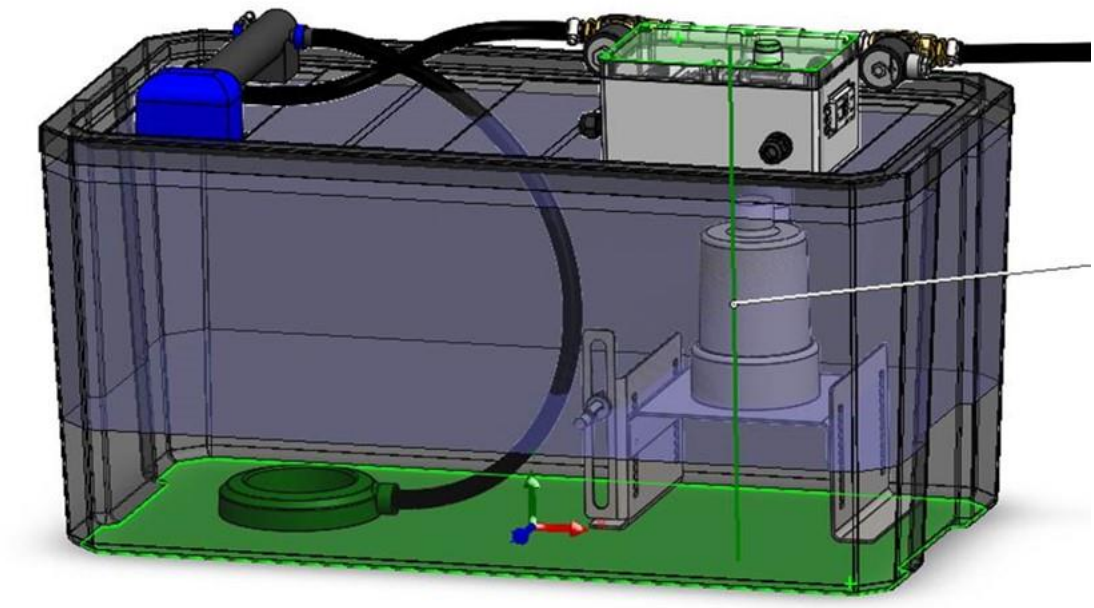

Figure 3.1. Schematic design of the titanium dioxide treatment system, showing the components of the system.

Source: TDI at KSU (2023).

Figure 3.2. Vortex Flow UV Sterilizer Diagram.

This image illustrates the internal flow dynamics of a vortex-style UV sterilizer, where water enters through the inlet, passes through a spiral flow chamber, and is exposed to UV light for sterilization before exiting through the outlet.

Source: The Pond Guy©. (2024).

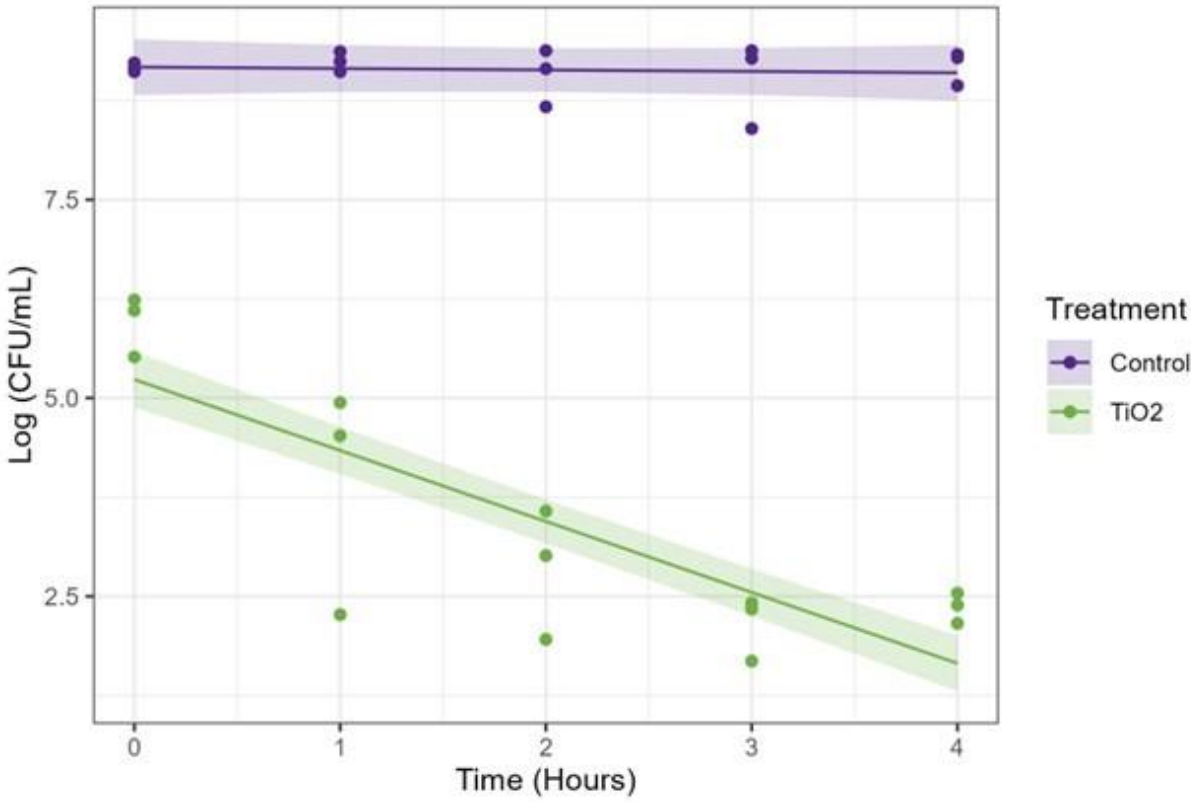

Figure 3.3. Log CFU/mL of *E. coli* TVS 353 over 4 hours across three trials comparing control (purple line) and TiO_2 treatment (green line) in the water system.

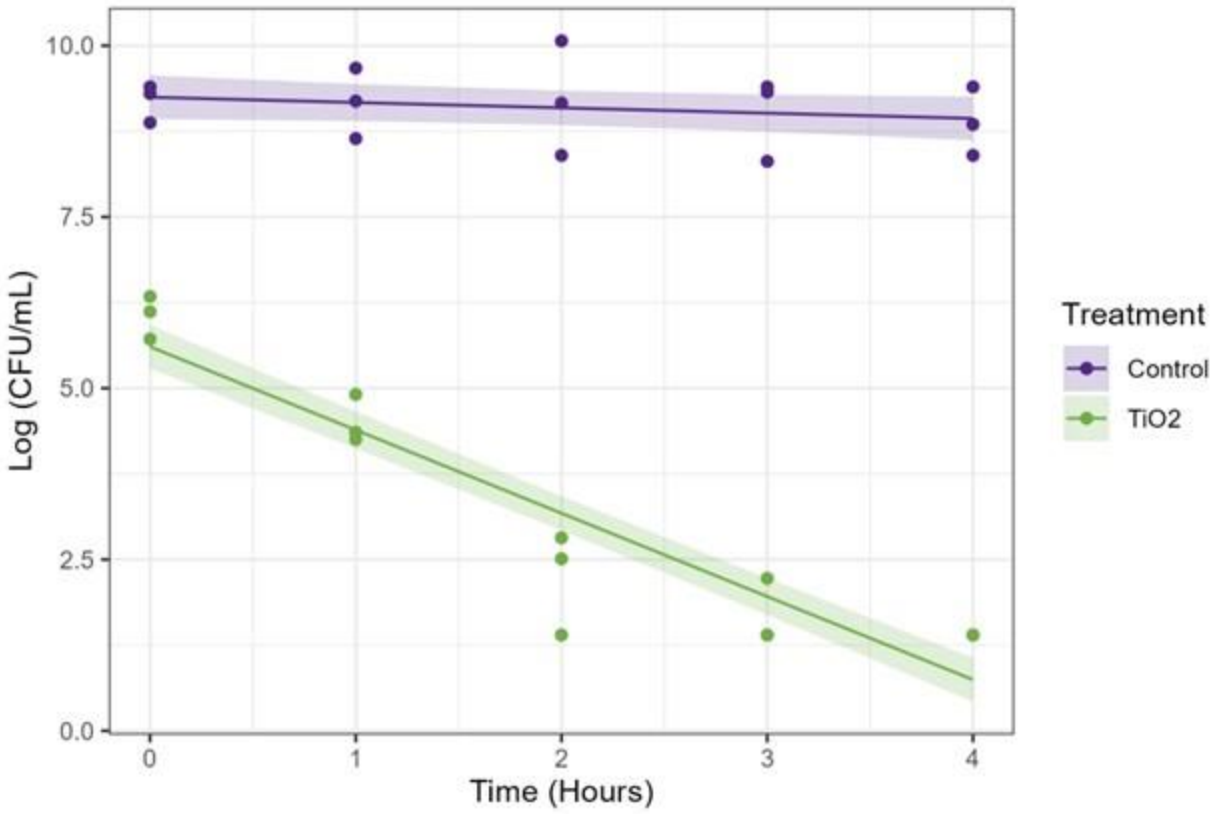

Figure 3.4. Log CFU/mL of *L. innocua* ATCC 33090 over 4 hours across three trials comparing control (purple line) and TiO_2 treatment (green line) in the water system.

Table 3.1. Statistical analysis (ANOVA) of the parameters affecting the log CFU/mL of *E. coli* TVS 353 over a 4-hour period across three trials in the water system.

Parameters	**P-value**
Time (Hours)	< 0.05
Treatment	< 0.05
Time (Hours) × Treatment	< 0.05

Table 3.2. Statistical analysis (ANOVA) of the parameters affecting the log CFU/mL of *L. innocua* ATCC 33090 over a 4-hour period across three trials in the water system.

Parameters	**P-value**
Time (Hours)	< 0.05
Treatment	< 0.05
Time (Hours) × Treatment	< 0.05

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Chapter 4 - Antimicrobial efficacy of TiO_2 on the growth of experimentally inoculated microgreens

Abstract

Microgreens have been attracting the interest of farmers, consumers, and scientists as superfoods rich in nutrients and phytochemicals. Nevertheless, several notable recalls in recent years have highlighted several food quality and safety concerns related to microgreen production. Titanium dioxide (TiO_2) has been used to inactivate microorganisms through photocatalytic generation of reactive oxygen species. This technology is stable, non-toxic, inexpensive, and operates under ambient conditions. This study aims to test the effect of TiO_2 on the growth of experimentally inoculated microgreens on a soilless substrate by evaluating microbial reduction and chemical-physical parameters. The antimicrobial activity of TiO_2 was tested in a microgreen growing system where arugula seeds or burlap growing substrates were experimentally inoculated with Rifampicin-resistant *L. innocua* and *E. coli*. Germination and growth were monitored for 14 days, and bacterial count, color, pH, height, weight, and titratable acidity were evaluated. Two growing systems were run in parallel: a control tower where no TiO_2 was applied, and a treatment tower where 0.05% w/v TiO_2 was integrated into the water reservoir system. Experiments were conducted in triplicate and a significant *P* value was set at 0.05. Pathogens were detected in the edible part of the microgreens. By the end of the experiment, *L. innocua* populations were reduced by 2.17 log CFU/cm^2 in samples grown from contaminated mats and by 1.91 log CFU/cm^2 in seed-contaminated samples, both showing statistically significant reductions as compared to the microgreen that was not treated with TiO_2 ($P < 0.05$). *E. coli* populations were significantly reduced only in the seed-inoculated samples treated with TiO_2 with a 1.57 log CFU/cm^2 reduction compared to the control ($P = 0.05$). These

results suggest that both contamination routes pose a relative risk as pathogens can survive. No significant differences ($P > 0.05$) were observed in color, pH, height, or weight, except for a significant difference in titratable acidity between control and treated samples at day 14 ($P < 0.05$). This study provides insight into the potential efficacy of TiO_2 to reduce the risk of foodborne pathogens in microgreen systems.

4.1. Introduction

In recent years, microgreens have gained widespread popularity throughout the industry due to their high nutritional content (Sharma et al., 2022). Studies have shown that these young plants contain greater concentrations of nutrients and phytochemicals compared to fully-grown vegetables (Komeroski et al., 2023). They have been praised for their ability to supply essential nutrients to diets that lack variety, thus improving overall dietary quality (Bhaswant et al., 2023). Microgreens are distinguished by their vibrant colors, diverse textures, and distinctive flavor profiles, which makes them a popular addition to dishes like soups, sandwiches, and salads to enhance taste and presentation (Sharma et al., 2022).

Studies have projected that by 2050, agriculture will face the significant challenge of boosting production by 70% (Ebert, 2014) to feed the global population that has been on the rise in the last couple of decades (Godfray et al., 2010). The unsustainable nature of conventional agricultural practices, and the growing scarcity of land resources, have driven researchers and professionals to explore alternative solutions (Godfray et al., 2010; Lada et al., 2018). Indoor and vertical farming have become popular since they optimize resource use and do not require additional space (Lada et al., 2018).

Microgreen systems grown via soil-based or hydroponic techniques are the most common methods to grow microgreens (Abaajeh et al., 2023). These young plants are harvested 7 to 21 days post-germination, once the first true leaves appear and the cotyledons are fully expanded (Du et al., 2022; Bhaswant et al., 2023). Although microgreens are often mistaken for sprouts due to their similar characteristics, they are distinct specialty crops (Kyriacou et al., 2016).

Even if in the past decade, there have been numerous recalls linked to microgreens (Du et al., 2022; Topalcengiz et al., 2024), few studies have examined microgreens' food safety concerns (Riggio et al., 2019; Işık et al., 2022). The majority of available research focused on the physiology, nutritional values, and health benefits of microgreens.

Nevertheless, microgreens present several food safety concerns. One of the main risks in microgreens production is irrigation water, especially in hydroponic systems. The recirculated water can facilitate the spreading of microorganisms, which accelerates their colonization (Riggio et al., 2019). Also, microgreens' tissues are more delicate than mature ones, which makes post-harvest sanitation challenging, emphasizing the importance of maintaining strict food safety practices throughout the pre-harvest phase (Turner et al., 2020). Similarly, microgreens are immature plants that are more susceptible to bacterial internalization and colonization than mature vegetables (Warriner et al. 2003). Their internal protective structure is not fully developed, which makes it easier for bacteria to infiltrate the xylem (Warriner et al. 2003). The harvesting method also adds a risk, as the cutting tools or the workers can introduce contamination (Riggio et al., 2019; Turner et al., 2020). Additionally, microgreen systems are more prone to microbial colonization when the seeds are contaminated (Turner et al., 2020; Wright & Holden, 2018; Xiao et al., 2015; Reed et al., 2018), and the choice of growing media can also play an important role in the contamination of microgreens (Misra et al., 2021). Studies have shown that certain soilless substrates like peat and hemp are more prone to microbial growth compared to recycled fiber mats or coco noir (Misra & Gibson, 2020; Turner et al., 2020).

Therefore, there is a pressing need to develop and implement effective intervention strategies to ensure the safety and quality of microgreens (Deng et al., 2021). Various

intervention strategies, including sanitizing treatments, have been used, such as peracetic acid (Fuzawa et al., 2021), chlorine washes (Lee et al., 2009; Xiao et al., 2014), ascorbic acid, citric acid, ethanol, and their combinations (Chandra et al., 2012; Dalal et al. 2020). However, the use of chemical disinfectants can raise concerns for consumers and the environment (Elgohary et al., 2021; Laxma Reddy et al., 2017; Arun et al., 2023).

Recently, Titanium dioxide (TiO_2) has been used to inactivate microorganisms through the photocatalytic generation of reactive oxygen species (Laxma Reddy et al., 2017). This technology is stable, non-toxic, inexpensive, and operates under ambient conditions (Elgohary et al., 2021; Nadeem et al. 2018). The generation of ROS causes cell membrane leakage, phospholipid oxidation, and ionic imbalance, ultimately disrupting the integrity of the cell wall (Laxma Reddy et al., 2017; Nadeem et al., 2018).

Despite these promising attributes, the application of TiO_2 in microgreen farming has not been studied. Therefore, the objective of this research was to evaluate the efficacy of TiO_2 as a potential antimicrobial in microgreen systems by evaluating microbial reduction as well as chemical and physical parameters.

4.2. Materials and Methods

4.2.1. Seeds, burlap, and titanium dioxide

Arugula seeds (*Eruca vesicaria* ssp. *sativa* (Mill.) Thell.) were obtained from a commercial supplier (Country Creek Acres, Chesterfield, MO, USA) and stored in the dark until use. A microgreen system was set up (see detailed below) in the greenhouses in Throckmorton Hall at Kansas State University (KSU). Tight-weave jute burlap fabric (Clever Delights, Waco, TX) was used as a soilless substrate for both control and treatment. Titanium dioxide (TiO_2) (Thermo

Fisher Scientific, Fair Lawn, NJ, USA) was dissolved in water (0.5g/L) and used as an antimicrobial agent to evaluate the effective control of microbial pathogens in CEA systems.

4.2.2. Bacterial strains

Since experiments were conducted in a BSL-1 greenhouse facility, two BSL-1 organisms were used for experimentally inoculating samples: *Escherichia coli* TVS 353 and *Listeria innocua* ATCC 33091. Both strains were made rifampicin-resistant, following the protocol described by Murphy et al. (2023). Two days before the experiment in the greenhouse, the strains were activated from frozen cultures. Bacteria were retrieved from the frozen stock collection, streaked onto Tryptic Soy Agar (TSA; BD, Franklin Lakes, NJ, USA), supplemented with 80 µg/ml rifampicin, and incubated at 37℃ for 24h. An isolated colony was then transferred into 10 mL 80 µg/ml rifampicin Tryptic Soy Broth (TSB; BD, Franklin Lakes, NJ, USA) and incubated overnight at 37℃ to achieve an inoculation level of 9 $\log_{10}$ CFU/ml. For the experiment day, 200 mL TSB supplemented with rifampicin was inoculated with 200 µl from the culture and incubated at 37℃ for 24h.

4.2.3. Microgreen systems

Microgreens were cultivated in 3-meter trays using Farmtek HydroCycle vertical hydroponic microgreen growing systems (Farmtek, South Windsor, CT, USA) composed of 4 layers. The towers were located in the climate-controlled Throckmorton Greenhouses at KSU at a temperature of 23.9 +/- 2.8 °C between 6:00 AM and 8:00 PM and 15.6 +/- 2.8 °C between 8:00 PM and 6:00 AM. The lights operated on a 16-hour cycle, from 6 AM to 10 PM. Two identical towers were used for these experiments: a control tower (sourced by water only) and a treatment tower (sourced by water containing TiO_2). Only one-layer growing system per tower was used in our experiments (Figure 4.1). Each layer was composed of four trays, each lined

with burlap fabric measuring 22.86 cm in width, 305 cm in length, and 0.1 cm in thickness. The burlap was sectioned into five plots, each approximately 57 cm long, separated by 5 cm-wide strips of BioStrate™ felt (GrowTech LLC., Portland, ME, USA). Arugula seeds (50g) were added to each plot on the day of the experiment. The water was sourced from the Manhattan municipal water supply. Irrigation was managed through an automated system, operating for 4 minutes every two hours from 6:30 AM to 6:30 PM throughout the experiment (until harvest). Water was delivered via 0.635 cm polyethylene tubing, with a flow rate of 150 mL per minute. At the end of each day and only for the treatment tower, a UV-TiO_2 system (Figure 4.2) was activated for 4 hours. This system consisted of a 379L tank where TiO_2 (0.5 g/L) circulated through a 36-watt UV lamp (The Pond Guy Inc., Bellevue, NE, USA) for disinfection purposes. At the end of the 4-hour treatment (based on our preliminary results), the powder was allowed to settle and at the beginning of the next sampling day, the water was pumped back into the system based on the irrigation schedule. The control tower received non-sanitized water daily.

4.2.4. Inoculation of seeds and burlap growth substrate

Since pathogens can contaminate both the seeds and the matting substrates (Wright & Holden, 2018), in this study we decided to evaluate both contamination routes. Therefore, in our experimental setup, two trays were designated for seed inoculation, and two trays were allocated for the inoculation of the burlap in each tower system (control and treatment). Each tray was divided into 5 plots (as mentioned before) and 50 g of seeds were used for each plot.

For the seeds' inoculation procedure, arugula seeds were visually inspected, and any seeds with visible defects, such as discoloration, cracks, shrinkage, wrinkling, or insect damage, were removed. Seeds (50 g for each plot) were placed in 200 mL of inoculum suspension in a stomacher bag, which was massaged for 5 minutes to ensure the seeds were thoroughly coated.

The bag was left at room temperature for 1 hour. Afterward, the seeds were drained using a strainer and evenly distributed on the plots.

For the inoculation of the growth substrate, each plot was thoroughly moistened with 200 mL of inoculum suspension until visibly wet. Then, 50 g of seeds were evenly sown onto each plot.

4.2.5. Sampling and harvesting

On designated sampling days (0, 3, 7, and 14), 2 cm^2 of burlap was cut using a sterile blade. Sampling locations for each day and each repetition were randomly assigned. Out of the 5 plots per tray, 3 plots were randomly selected for microbiological analysis; leading to 6 replicates per treatment combination. These samples were collected in 50 mL centrifuge tubes containing 15 mL of phosphate-buffered saline (PBS; EMD Millipore, Burlington, MA, USA). The remaining 2 plots were sampled for the measurement of chemical and physical parameters, leading to 4 replicates per treatment combination. The microgreens were harvested during the week following day 14.

4.2.6. Microbial enumeration

Sample solutions were vortexed for 1 minute, and 1 mL was transferred into 9 mL peptone water (BD, Franklin Lakes, NJ, USA) for serial dilution. From the desired dilution, 100 µL aliquots were plated on TSA plates supplemented with rifampicin and incubated at 37°C for 24h. Colonies were counted and expressed as CFU/cm^2.

4.2.7. Measurement of physical parameters

Weight

Samples from each plot were collected and plant material was carefully removed from the burlap mats and weighed on a scale (Accuris Instruments, Edison, NJ, USA).

Color

After measuring the weight, a colorimeter gun (HunterLab, Reston, VA, USA) was used to assess the color. The colorimeter gun was positioned directly above the microgreens, perpendicular to the surface, to avoid angle-related inaccuracies, and the color was measured once for each sample. The results were expressed using the CIELAB color system: L* (lightness from dark to light), a* (color component from green to red), and b* (color component from blue to yellow).

Height

To measure plant height within the plots, a consistent location was selected within each plot. The plants were gently separated to make space for the ruler, which was inserted vertically and aligned perpendicularly to the burlap to ensure accurate measurements. Plant height was recorded from the surface of the burlap to the top of the plant leaves. Measurements were taken once for each plot on days 7 and 14.

4.2.8. Measurement of chemical parameters

pH

For samples before germination (Days 0 and 3), the seeds were mixed with 15 mL of PBS and vortexed for 1 minute, and pH was measured using a pHmeter (Mettler Toledo, Columbus, OH, USA).

For samples after germination (Days 7 and 14), the plant material was transferred into 15 mL of PBS. Samples were homogenized using a blender for 1 minute, filtered through a sterile strainer to remove any remaining particulate matter, and the pH was measured.

Titratable acidity

The titratable acidity was measured on Days 7 and 14 (Siomos et al., 2000), Briefly, 5 mL of the filtrate used for pH measurement was mixed with 50 mL of deionized water and 5 drops of phenolphthalein indicator. A burette was filled with 0.1 N NaOH solution, and the sample was titrated gradually with the NaOH while swirling the mixture between drops to ensure thorough mixing. The titration continued until the solution reached a persistent pink-magenta color, signaling that the endpoint had been achieved. The volume of 0.1N NaOH used to achieve this color change was then recorded. The following equation was used to calculate the titratable acidity of the samples:

$$\%TA = \frac{(Volume\ of\ NaOH)*0.1*0.064*100}{(Volume\ of\ sample)}$$

with 0.064 being a factor for citric acid equivalency.

4.2.9. Experimental design and statistical models

This experiment consisted of a 2x2 factorial treatment structure including two sanitizer treatments (control and TiO_2) and two inoculation strategies (inoculating the mat and inoculating the seeds) in a split-plot design with 3 repetitions, subsampling, and repeated measures in time. The sanitizer treatment was at the whole plot level, and the inoculation strategy was at the subplot level. The experiment was conducted separately for each microorganism (*E. coli* and *L. innocua*). The load of each microorganism (recorded in log CFU/cm^2), color, pH, and weight were measured at four points in time (i.e., 0, 4, 7, and 14 days after inoculation), and the height and titratable acidity were measured at two points in time (i.e., 7 and 14 days after inoculation). The data was fitted to general linear mixed effects models, where the observed traits (i.e., log CFU/cm^2, height, weight, color, pH, and titratable acidity) were described using the treatment, inoculation method (sample type), time (day), and their double and triple interactions as fixed effects. In addition, we

included the random effects of the repetition and the random effects of the tray subdivision in each repetition. All random terms were assumed to be independent. Note that time was considered a continuous covariate for the microbial load, pH, weight, and color, while it was considered a categorical covariate for the height and titratable acidity. Expected marginal means, expected marginal trends and standard errors were obtained for post-hoc comparisons with a significance level of 0.05. The analyses were performed using the lme4 (Bates et al., 2015) and emmeans (Lenth, 2024) packages in R, version 4.4.1 (R Core Team, 2024).

4.3. Results

4.3.1. Microbial analysis

The *E. coli* and *L. innocua* populations were monitored for 14 days in a microgreen system using two inoculation methods (mat and seed) and two treatments, a traditional microgreen tower without any antimicrobial agent as the control, and a tower in which TiO_2 was integrated into the water reservoir as an antimicrobial agent, with the water treated daily for 14 days.

The ANOVA table analyzing the factors affecting the log reduction (CFU/cm^2) of *E. coli* indicated that there was a significant three-way interaction between the treatment, the sample type, and time (i.e., day) ($P < 0.05$) (Table 4.1). Thus, the slopes of log CFU/cm^2 versus time were compared between the different treatment and inoculation combinations (Figure 4.3). The impact of TiO_2 on *E. coli* reduction appeared to vary based on the inoculation method used. Seed-inoculated samples exhibited a significantly steeper slope under the TiO_2 treatment compared to the control ($P = 0.05$) (Table 4.2). The mean initial population for the TiO_2-treated seed-inoculated group was 8.77 log CFU/cm^2, and between 8.33 and 9.27 log CFU/cm^2 with 95% confidence, on the day of inoculation (i.e., day 0). The mean population at day 14 decreased to 7.20 log CFU/cm^2, between 6.27 and 8.01 log CFU/cm^2 with 95% confidence, resulting in a

reduction of 1.57 log CFU/cm². Conversely, the difference in the slopes between treated and control seems to be less conclusive for mat-inoculated samples ($P > 0.05$) (Table 4.2).

The *L. innocua* samples did not portray significant three-way interactions, although both two-way interactions (i.e., Treatment × Day and Sample Type × Day) were significant ($P \leq 0.05$) (Table 4.3). Thus, the slopes of log CFU/cm^2 versus time were compared between the different treatment and inoculation combinations (Figure 4.4). The slope observed in TiO_2-treated samples was steeper compared to the control ($P < 0.05$). Said differences between TiO_2-treated samples and the control were consistent regardless of the inoculation method (Table 4.4, Figure 4.4). More specifically, a mean reduction of 2.17 log CFU/cm^2 was observed for TiO_2-treated mat-inoculated samples. Moreover, the mean reduction was 1.91 log CFU/cm^2 for the TiO_2-treated seed-inoculated samples.

4.3.2. Physical and chemical analysis

The physical and chemical parameters analyzed in this study included the weight, pH, and color measurements (L, a, and b values) of the microgreen samples measured on days 0, 3, 7, and 14, and the height and titratable acidity measured on days 7 and 14 (Figure 4.5, 4.6). The analysis of these parameters showed that there were no significant differences between the control and the treatment ($P > 0.05$) for either inoculation method for the pH, height, weight, or the a* and L* color values (Table 4.5). There was a significant difference in titratable acidity between control and mat-inoculated samples with *L. innocua* ($P < 0.05$). Also, the b* color values of the mat-inoculated *L. innocua* and *E. coli* samples were statistically different from the control ($P < 0.05$) (Table 4.5).

4.4. Discussion

In this study, we evaluated the use of TiO_2 as an antimicrobial intervention to reduce *E. coli* and *Listeria* populations experimentally inoculated in a microgreen system. Since there is a growing demand for sustainable and eco-friendly strategies to mitigate microorganisms in the food industry (Arun et al., 2023; Elgohary et al., 2021), researchers have been exploring the use of TiO_2 as an antimicrobial agent due to its photocatalytic activity and its ability to generate ROS, which disrupt bacterial cell walls and ultimately lead to cell death (Laxma Reddy et al., 2017; Nadeem et al., 2018). Chorianopoulos et al. (2011) used titanium dioxide (TiO_2) treatments, with and without UV-A to reduce *Listeria monocytogenes* biofilm populations on stainless steel and glass surfaces. Their results showed that applying the TiO_2 treatments without UV-A did not reduce the *L. monocytogenes* biofilm population on either surface. Conversely, Chorianopoulos et al. (2011) reported a statistically significant reduction of approximately 2 logs when using a TiO_2 water dispersion system in combination with UV-A to disinfect surfaces. Similarly, in our study, we reported comparable reductions of *Listeria* populations (1.91 log CFU/cm^2 in mat-inoculated and 2.17 log CFU/cm^2 in seed-inoculated samples) in a microgreen tower integrated with a TiO_2 water disinfection system. Additionally, Zazouli et al. (2015) evaluated the effectiveness of TiO_2 nanoparticles (TiO_2-NPs) photocatalysts in removing *E. coli* from experimentally contaminated water with and without UV light. The results showed that increasing the TiO_2-NPs dose and contact time improved *E. coli* inactivation, with maximum removal rates reaching 100% in 40 minutes at a 0.8 g/L dose of TiO_2. In our study, we used a 0.5g/L TiO_2 concentration.

Foster et al. (2011) noted that while most studies indicate Gram-positive bacteria are generally more resistant to photocatalytic disinfection than Gram-negative bacteria, a few studies

suggest the opposite. Our research supports the latter finding, as we observed that the population of *L. innocua* showed a higher reduction of approximately 2 log CFU/cm^2, compared to a reduction of 1.57 log CFU/cm^2 for *E. coli*. The findings of our study also align with those reported by Bonetta et al. (2013), who evaluated the photoactivated antibacterial activity of TiO_2-coated surfaces against *E. coli*, *Staphylococcus aureus*, *Pseudomonas putida*, and *L. innocua*. Their study demonstrated that, while all bacterial species were effectively inactivated by the TiO_2-coated surfaces under UV light, *L. innocua* was the most susceptible to inactivation compared to the other bacteria (Han et al., 2016; Bonetta et al., 2013). In addition, Maness et al. (1999) investigated how the concentrations of *E. coli* cells and TiO_2 affect disinfection efficacy. They reported that while a concentration of 0.1 g/L TiO_2 was effective in reducing 92 to 98% of a 5-log *E. coli* population, this concentration was ineffective against suspensions of 8-log bacteria. They also found that increasing the TiO_2 dose to 0.5 or 1 g/L significantly improved killing efficiency at this higher cell concentration. Nonetheless, when the cell concentration reached 8.7 logs, the efficacy of 1 g/L TiO_2 decreased significantly.

Finally, in our study, we also compared two different routes of bacterial contamination: seeds and mats. In our study, we started with an initial microbial concentration of 8.2 to 8.7 logs, and a TiO_2 concentration of 0.5 g/L. We did not find significant differences in the mat-inoculated samples ($P > 0.05$) and only statistically significant results with the seed-inoculated samples ($P = 0.05$). This indicates that at these high concentrations of *E. coli*, the effectiveness of TiO_2 as a photocatalyst may be limited. Wright & Holden (2018) found that the route of inoculation did not significantly impact the population of *E. coli* or its colonization ability on broccoli microgreens. They reported that high *E. coli* populations were achieved in broccoli microgreens regardless of the mode of inoculation (inoculation of matting substrate or direct seed

contamination). In our study, we observed that the seed-inoculated samples treated with TiO_2 showed significantly lower *E. coli* populations ($P = 0.05$) compared to the mat-inoculated samples ($P > 0.05$). However, a slightly greater reduction in *L. innocua* populations was observed in TiO_2-treated mat-inoculated samples compared to seed-inoculated ones ($P < 0.05$), suggesting that the inoculation method may affect the antimicrobial efficacy of TiO_2.

Xiao et al. (2015) investigated the impact of various growth media on the proliferation of *E. coli* O157:H7 in radish microgreens from seed to harvest. They compared microgreens grown in a moss-based soil substitute, a hydroponic system, and a soil-grown system. Their findings suggested that hydroponically grown microgreens achieved a significant increase in *E. coli* population as compared to those grown in soil. Pathogen survival and growth could be attributed to the absence of competitive microflora in the irrigation water of hydroponic systems, unlike the germination mix used for soil. Additionally, the liquid plant growth media may facilitate the survival and proliferation of pathogens (Wright & Holden, 2018; Xiao et al., 2015). In our study, we used burlap as a soil substitute, which lacks the microflora present in the germination mix that could compete with the pathogens. This absence could likely have contributed to the lower microbial reduction observed in *E. coli* populations.

Finally**,** the physical and chemical parameters analyzed in this study, notably the weight, pH, height, and color measurements (L* and a* values) showed no significant differences between treated samples and controls. The only significant difference in the color measurements was observed in the b* value for the treated mat-inoculated samples with *L. innocua* and *E. coli* ($P < 0.05$). These findings highlight the safety of this disinfection method and suggest that it does not adversely affect microgreens. However, further research is needed to understand TiO_2's implications in scaled-up systems. Furthermore, TiO_2 was shown to have beneficial effects on

plant growth, such as boosting photosynthesis, reducing disease severity, and increasing yield (Chao et al., 2005). Owolade & Ogunleti (2008) reported that the use of TiO_2 lowers the occurrence of rice blast and tomato mold, leading to a 20% increase in grain weight due to its growth-promoting properties. In our study, we found a significant difference in the titratable acidity between control and treatment ($P < 0.05$) for *L. innocua* inoculated samples on day 14. The titratable acidity was measured based on the citric acid content, an organic acid present in arugula microgreens. The observed increase in titratable acidity is associated with enhanced photosynthesis, which is likely due to the elevated levels of citric acid (Parveen et al., 2020). TiO_2 may contribute to promoting photosynthesis, thereby leading to the production of compounds that further support plant health and growth. Additionally, while the pH did not show a statistically significant difference ($P > 0.05$), it is measured on a log scale, suggesting there may still be a practical scientific difference.

4.5. Conclusion

This study demonstrates the effectiveness of TiO_2 in reducing *E. coli* and *L. innocua* populations in microgreen systems, with a significant difference observed based on the inoculation method. Seed-inoculated microgreen samples treated with TiO_2 exhibited greater reduction as compared to the mat-inoculated samples when experimentally inoculated with *E. coli*. For *L. innocua*, both inoculation methods treated with TiO_2 resulted in a significant log reduction. Overall, physical and chemical analyses revealed no significant differences between control and treatment in the pH, height, weight, and the a* and L* color values. The only differences were observed in the titratable acidity between the control and the treated mat-inoculated samples with *L.innocua*, and the b* color value between control and mat-inoculated *L. innocua* and *E. coli* samples. This research suggests that TiO_2 is a promising antimicrobial agent

in microgreen systems. Nevertheless, further research is needed to explore the application of TiO_2 on a commercial scale, as there may be differences between laboratory conditions and those found in commercial facilities.

4.6. Acknowledgments

The authors would like to thank the Kansas Department of Agriculture Specialty Crop Block and the USDA - Specialty Crop Block Grant Program for funding this project.

4.7. Figures and Tables

Figure 4.1. Trays of Arugula microgreens cultivated on jute burlap in a controlled greenhouse environment.

Figure 4.2. Treatment system containing a TiO_2 tank and a UV lamp chamber.

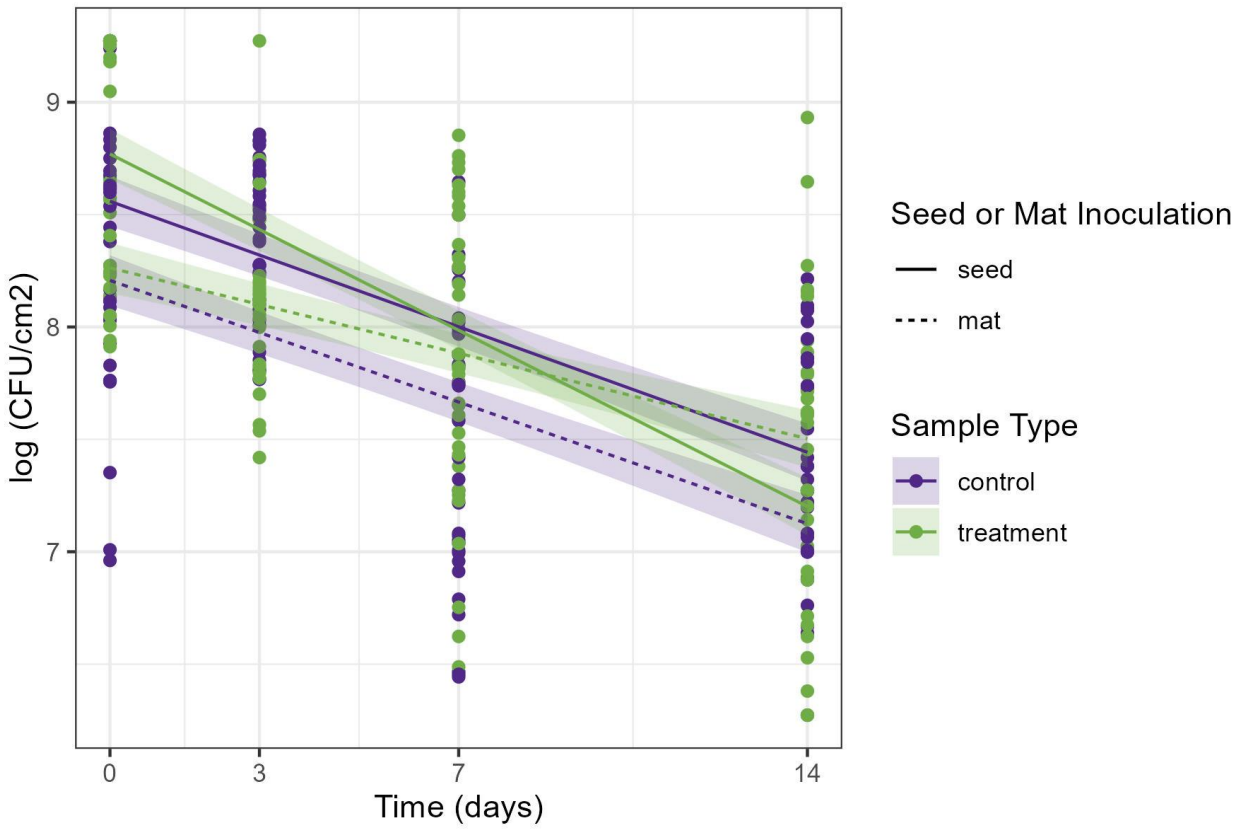

Figure 4.3. Log (CFU/cm²) of Rifampicin-resistant *E. coli* TVS 353 over 14 days across three trials, comparing mat (dashed line) and seed (solid line) inoculations, with control (purple) and treatment (green) in microgreen growing systems.

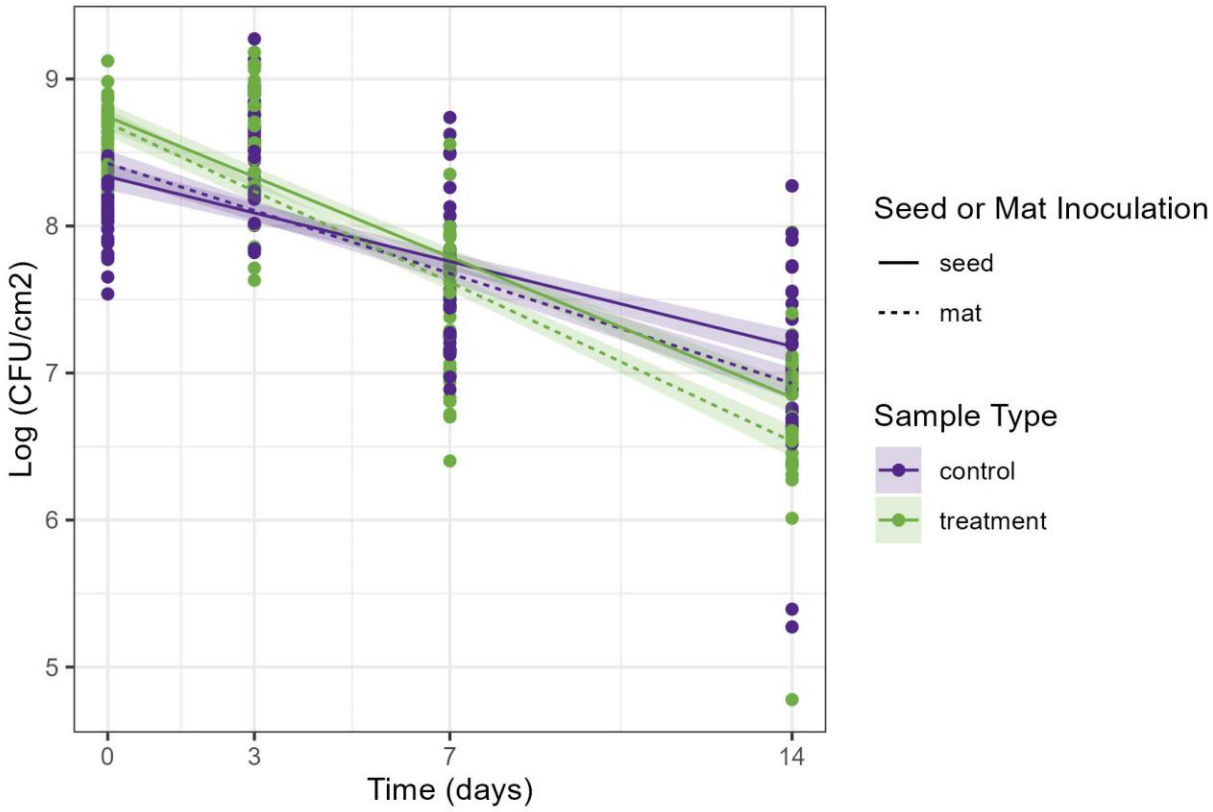

Figure 4.4. Log (CFU/cm^2) of Rifampicin-resistant *L. innocua* ATCC 33091 over 14 days across three trials, comparing mat (dashed line) and seed (solid line) inoculations, with control (purple) and treatment (green) in microgreen growing systems.

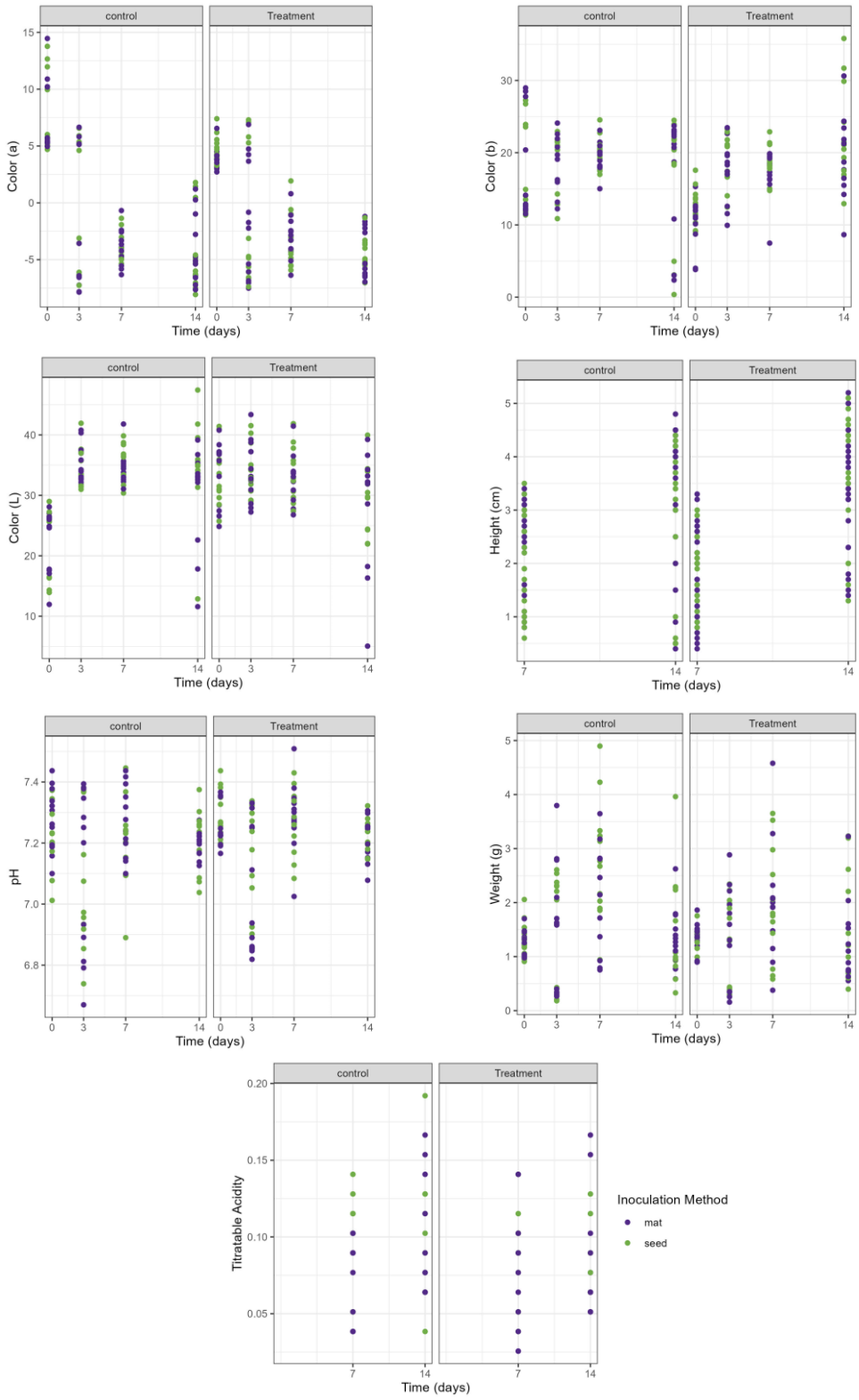

Figure 4.5. Physical and chemical parameters of microgreens inoculated with *E. coli* measured at intervals over a 14-day period comparing mat (purple) vs seed (green) and control vs treatment in microgreen growing systems.

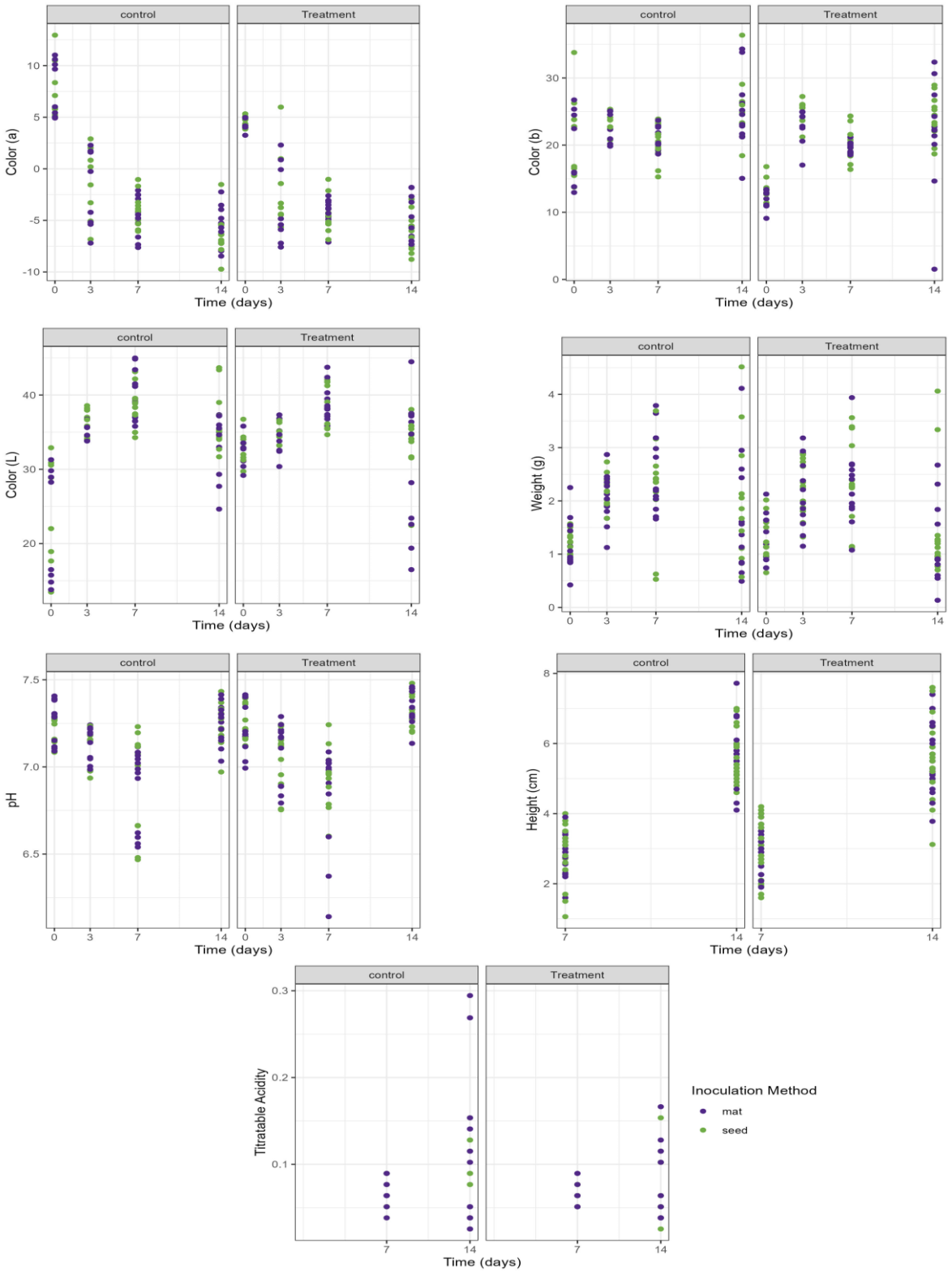

Figure 4.6. Physical and chemical parameters of microgreens inoculated with *L. innocua* measured at intervals over a 14-day period comparing mat (purple) vs seed (green) and control vs treatment in microgreen growing systems.

Table 4.1. Analysis of Variance (ANOVA) of the parameters affecting log CFU/cm^2 of *E. coli* over 14 days in microgreen growing systems.

Parameters	***P*-value**
Treatment	0.0883
Sample Type (seed or mat)	$P < 0.05$*
Day	$P < 0.05$*
Treatment × Sample Type	0.1513
Treatment × Day	0.6949
Sample Type × Day	0.0106*
Treatment × Sample Type × Day	0.0188*

Values marked with * indicate significance at $P < 0.05$

Table 4.2. Trend contrast estimates between treatment and control for seed and mat inoculation methods for *E. coli* over 14 days in microgreen growing systems.

Sample type	**Control - TiO_2**	***P* (|t| > t)**
Mat	-0.0231 ± 0.017	0.1674
Seed	0.0323 ±0.017	0.0536*

Values marked with ***** indicate significance at $P = 0.05$.

Table 4.3. Analysis of Variance (ANOVA) of the parameters affecting log (CFU/cm^2) of *L. innocua* over 14 days in microgreen growing systems.

Parameters	***P*-value**
Treatment	0.5433
Sample Type (seed or mat)	0.0781
Day	$P < 0.05$*
Treatment × Sample Type	0.4220
Treatment × Day	$P < 0.05$*
Sample Type × Day	0.0585*
Treatment × Sample Type × Day	0.7955

Values marked with * indicate significance at $P \leq 0.05$

Table 4.4. Trend contrast estimates between treatment and control for seed and mat inoculation methods for *L. innocua* over 14 days in microgreen growing systems.

Sample type	**Control – TiO_2**	***P* (|t| > t)**
Mat	0.0479 ± 0.016	0.0031*
Seed	0.0538 ±0.016	0.0009*

Values marked with * indicate significance at $P < 0.05$

Table 4.5. Contrast analysis of the physical and chemical parameters of microgreens over 14 days comparing seed vs mat inoculation and control vs treatment in microgreen growing systems.

Parameters	***L. innocua***		***E. coli***	
	$P(\mu_{control} \neq \mu_{TiO_2})$		$P(\mu_{control} \neq \mu_{TiO_2})$	
	Mat	**Seed**	**Mat**	**Seed**
Height	0.602	0.683	0.588	0.137
Weight	0.409	0.749	0.764	0.168
pH	0.777	0.700	0.558	0.148
Color L	0.641	0.731	0.278	0.498
Color a	0.372	0.273	0.638	0.470
Color b	0.004*	0.089	0.024*	0.963
Titratable acidity	0.044*	0.415	0.562	0.149

Values marked with * indicate significance at $P < 0.05$

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Chapter 5 - Overall Conclusions

The studies presented in this research offer valuable insights into the antimicrobial efficacy of TiO_2 as a water disinfectant agent. TiO_2 was used for its photocatalytic activity when activated by UV light, which led to the disruption of bacterial cell walls through the generation of reactive oxygen species (ROS).

Chapter 3 focused on investigating the antimicrobial activity of TiO_2 in controlled laboratory settings to identify the optimal time and concentration combinations for maximum efficacy. The results of this study showed that TiO_2, when activated by UV light, significantly reduced *E. coli* and *L. innocua* populations within 4 hours of treatment compared to the untreated controls ($P < 0.05$).

Chapter 4 further expanded this research by evaluating the antimicrobial efficacy of TiO_2 against *E. coli* and *L. innocua* in a microgreen system. Two inoculation methods were used, seed and mat inoculation, which were tested in both a control system and a treatment system, where TiO_2 was integrated into the water reservoir with daily treatment until harvest. *E. coli* and *L. innocua* populations were assessed across three trials, alongside microgreens' physical and chemical parameters, including weight, pH, color, height, and titratable acidity at different time points. The results demonstrated that TiO_2 effectively reduced microbial populations in microgreen systems, with its efficacy varying based on the inoculation method ($P < 0.05$). Furthermore, no significant differences in physical and chemical quality were observed between treated and control samples for the pH, height, weight, or the a* and L* color values ($P > 0.05$), except for a notable difference in titratable acidity between control and mat-inoculated samples with *L. innocua* and the b* color value between control and mat-inoculated *L. innocua* and *E. coli* samples ($P < 0.05$).

Various chemical agents have been used in the field to clean hydroponic systems and water reservoirs of these systems. However, these chemicals often pose environmental risks due to the generation of harmful byproducts and residues. In contrast, the findings from these studies emphasize TiO_2 as a promising agent, achieving significant microbial reduction in both *E. coli* and *L. innocua* populations without compromising the physical and chemical quality of the microgreens.