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Antimicrobial efficacy of nisin, oregano and ultrasound treatments against foodborne pathogens in ready-to-eat vegetables

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DECLARATION

I hereby declare that the work reported in this dissertation and submitted at the Department of Biotechnology and Food Science at Durban University of Technology for a Master's degree is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

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Student

As the candidate's supervisors we agree to the submission of this dissertation

Professor Oluwatosin Ademola Ijabadeniyi Date

As the candidate's supervisors we agree to the submission of this dissertation

DEDICATION

This dissertation is dedicated to my family and friends for their unwavering support and will to see me succeed.

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ABSTRACT

A marked increase in the prevalence of foodborne illnesses resulting in foodborne outbreaks globally have been noticed, primarily due to the contamination of foods with bacterial pathogens. Recently, there has been a growing interest in employing alternative decontamination methods against bacterial pathogens, as a result of problems associated with the commonly used chemical and thermal methods. Therefore, the current impetus is on employing decontamination techniques involving green technology (physical, biological) and hurdle technology. In this study, oregano, nisin and ultrasound treatments were employed on lettuce and cabbage at varying levels to elucidate their antimicrobial efficacy and subsequent effect on the physical and sensory properties of the vegetables.

Specifically, fresh cut lettuce and cabbage samples were inoculated with *Escherichia coli* O157:H7 and *Listeria monocytogenes* and were subjected to a series of combined treatment applications, of nisin, oregano essential oil and ultrasound. The Box Behnken, response surface methodology (RSM) technique was used in formulating the various combination treatments that would, in turn, demonstrate the synergistic capabilities of the three factors, nisin, oregano and ultrasound when combined. Results from the RSM were then used in the optimization of the combined treatment parameters by maximizing the microbial log reduction in both ready-to-eat vegetables. The physical properties studied on lettuce and cabbage subjected to combined treatments were colour and texture, and their structural damage was investigated using electrolyte leakage. Sensory properties were also analyzed on non-inoculated cabbage samples previously subjected to combined antimicrobial treatments.

The efficacy of nisin, oregano and ultrasound on the reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce studied using RSM/Box-Behnken model design, was found to be reliable ($p<0.05$). The most effective treatment on both pathogens was a combination of 771.2 IU/g nisin, 0.185% v/v oregano and 14.65 min ultrasound which showed log reductions of 3.43 and 9.20 CFU/mL for *E. coli* O157:H7 and *L. monocytogenes*, respectively. Lettuce treatment with the combined antimicrobial treatments resulted in no significant differences in textural properties, specifically hardness. However, mild colour changes and a slight increase in the electrolyte leakage rate was observed, though they were within the permissible limits.

The reduction of *E. coli* O157:H7 on cabbage was increased with the use of combined treatments of nisin, oregano and ultrasound. Hence, the combination (comprising 607.85 IU/g nisin, 0.20% v/v oregano and 14.98 min ultrasound) exhibited the highest log reduction of 3.66 CFU/mL. In the samples inoculated with *L. monocytogenes,* 731.25 IU/g nisin, 0.12% v/v oregano and 13.21 min ultrasound treatments were found to be the best combination exhibiting the highest log reduction of 8.27 CFU/mL. No significant colour and textural changes were observed between untreated and treated cabbage. However, a slight increase in the electrolyte leakage rate was observed after the application of the combined treatment. Sensory evaluation scores also had some factors that were slightly below par.

Overall, results from the study demonstrated that a combination of nisin, oregano and ultrasound, is a promising alternative to chemical treatments for the reduction of *E. coli* O157:H7 and *L. monocytogenes* as well as retaining the quality characteristics of fresh produce. Prospectively, other studies could explore the frontier field of artificial intelligence and machine learning, in the form of predictive microbiology and mathematical modelling, in the fresh produce industry. This would present a better understanding of risk assessment that is powered by the technological advantage of data analytics.

PUBLICATIONS AND CONFERENCES

Publications

Takundwa, B. A., Bhagwat, P., Pillai, S. & Ijabadeniyi, O. A. 2020. Antimicrobial efficacy of nisin, oregano and ultrasound against *Escherichia coli* O157: H7 and *Listeria monocytogenes* on lettuce. *LWT- Food Science and Technology*, 110522. https://doi.org/10.1016/j.lwt.2020.110522

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CHAPTER ONE

1 INTRODUCTION

Changes in human dietary choices have caused a global shift in the demand and consumption of healthy and nutritious food products in the form of fresh produce, fruits and vegetables (Colapinto *et al.*, 2018). Consequently, a significant increase in the production of fresh produce has been observed in the last two decades (Oladunjoye, 2017). According to the global data collection site, Statista (2020), between 1990 – 2018, fresh fruits experienced a production upsurge from 577 million metric tons (MMT) in 2000 to 868 MMT in 2018. Similarly, fresh vegetable production also increased from 682 to 1 089 MMT during the same period.

Fresh produce plays a significant role in human diets by providing nutrients of great importance, thereby preventing diseases to a greater extent. The functional properties found in fresh produce are presented in the form of phenolic compounds with antioxidant and free radical scavenging properties, such as anthocyanins, carotenoids, tocopherols and ascorbic acid (Qadri *et al.*, 2015, Traore *et al.*, 2020). Preservation of fresh produce in its raw state is more favourable for the effectiveness of its functional properties and its inherent tolerance to bacterial growth. Therefore, it is imperative to carefully monitor and maintain fresh produce quality, starting from the harvesting until the fresh produce reaches the final point, the consumer. However, present concerns in the fresh produce industry have highlighted the incidences of pathogen contamination throughout the production chain (Millan-Sango *et al.*, 2015, WHO 2018, CDC 2020).

Studies have shown the presence of *Escherichia coli* O157:H7 and *Listeria monocytogenes* as major contaminants in the fresh produce industry (Ozcan and Demirel Zorba, 2016). The USA has recorded a significant increase in the number of foodborne outbreaks linked to fresh produce consumption, with estimated figures of 14.8% in 1998 to 22.8% in 2007. From 2007 to 2011 in Europe, fresh produce was associated with 10% of the outbreaks, 35% of hospitalizations and 46% of deaths linked to foodborne illness. In Australia, the foodborne related outbreaks linked to fresh produce were reported to be 4% (Alegbeleye *et al.*, 2018). Statistics of foodborne outbreaks which have occurred in recent times report a case of the *E. coli* O157:H7, in the United Kingdom, linked to imported salad, as being responsible for 161 cases, 60 of them hospitalized and two confirmed deaths (Wadamori *et al.*, 2017). *L. monocytogenes* has also been found in salad vegetables and implicated in listeriosis cases, with high death rates of 19% so much that the Food and Drug Association (FDA) in the United States had to establish a zero-tolerance for *L. monocytogenes* in food (Żaczek *et al.*, 2015). The statistics highlighted above, calls to attention the importance of decontamination methods that can effectively control such food pathogens and in turn, reduce the prevalence of foodborne illnesses (Wadamori *et al.*, 2017).

Apart from illnesses, foodborne outbreaks have a negative impact, particularly to manufacturing industries; from the food recalls up to the loss in consumer confidence of certain food products. In a study of "Food recalls and warnings due to the presence of foodborne pathogens" conducted using information compiled by the US FDA, it was documented that fruits and vegetables had recall figures of 2.2% in 2013, 15.6% in 2012 and 2.6% in the previous year of 2017 (Paramithiotis *et al.*, 2017). The most common reason for the recalls was noted to be the presence of *L. monocytogenes*, *Salmonella* spp. and occasionally from *E. coli* strains, (Paramithiotis *et al.*, 2017).

A wide range of methods are applied as decontaminating techniques in the fresh produce industry, that is, chemical washings/spraying procedures, irradiative treatments and natural/biological processes (Goodburn and Wallace, 2013, Petri *et al.*, 2015). The industry mainly uses chemical washing methods, with chlorine sanitizing methods, the most dominantly used. Chlorine dioxide, bromine, iodine, trisodium phosphate, quaternary ammonium compounds, acids, hydrogen peroxide and ozonated water are also used (Goodburn and Wallace, 2013). The impetus for reducing the use of chemicals for fresh produce decontamination stems from the imminent possibility of water hyper chlorination. The resultant hyperchlorination then encourages the increase in concentration of chloramines and trihalomethanes, which are reported to have carcinogenic effects, thereby prompting the research of alternative methods (Brilhante São José and Dantas Vanetti, 2012, Millan-Sango *et al.*, 2016, Millan-Sango *et al.*, 2017). Chlorine-based compounds in high concentrations are corrosive and cause skin and respiratory tract irritation (Brilhante São José and Dantas Vanetti, 2012). The risks associated in using chlorine as a sole decontaminant and reports show that despite chlorine concentration, immersion time and pH, the typical log reduction for most studies are < 2 logs. This has brought about an interest in exploring alternative decontaminating techniques (Goodburn and Wallace, 2013).

Thermal technologies have been used for quite some time now; however, their ability to alter organoleptic properties and nutritional components of fresh produce has triggered the interest in the adoption of non-thermal processing techniques (Chemat *et al.*, 2011, Millan-Sango *et al.*, 2015, Chemat *et al.*, 2017). Ultrasound has emerged as a non-thermal process and an environmentally friendly/green technique which can be used as a decontamination tool for fresh produce (Chemat *et al.*, 2017). Using the cavitation principle, ultrasound can inactivate bacterial cells through their rupturing of bacterial cells, spores and enzymes denaturation from the released ultrasonic waves (Chemat *et al.*, 2011). It has been noted, however, that ultrasound alone as a decontamination method does not entirely inactivate bacterial spores (Sagong *et al.*, 2013). Therefore, it has been demonstrated in recent literature that combining ultrasound with other antimicrobials can significantly reduce bacteria levels in fresh produce (Sagong *et al.*, 2011, Millan-Sango *et al.*, 2015, Ozcan and Demirel Zorba, 2016). A process known as hurdle technology that combines various antimicrobial techniques for improved efficacy has gained interest and several studies of different hurdle methods have been reported to significantly reduce the microbial load in foods (Millan-Sango *et al.*, 2016, Ferrario and Guerrero, 2017, Traore *et al*., 2020).

Antimicrobials that are natural or extracted from natural sources are gaining much interest in the decontamination of fresh produce. Nisin has been reported as an antimicrobial peptide with antimicrobial activity against a range of foodborne pathogenic and spoilage microbes, some of which are, *Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus and Alicyclobacillus acidoterrestris* (de Oliveira Junior *et al.*, 2015). Nisin binds to the precursor of peptidoglycan and lipid II inhibiting cell wall biosynthesis, forming pores within the cell membrane, releasing essential ions and ultimately bacterial cell death. The antimicrobial activity of nisin is more significant in spores than vegetative cells due to their sporostatic potential (Khan and Oh, 2016). Oregano inactivates microbial load through its thymol and carvacrol, compounds that have the ability to disintegrate a microorganism's outer membrane (Cattelan *et al.*, 2018). Oregano and thyme are examples of such essential oils that can be used as antimicrobials due to their phenolic compounds responsible for bacterial inactivation, and they are being considered, generally recognized as safe (GRAS), (Burt, 2004). A combination of nisin at 500 IU/g and oregano at 0.6% (v/v) showed potent antimicrobial activity against *Salmonella enteritidis* (Govaris *et al.*, 2010).

This study, therefore, intends to assess the disinfecting efficacy of the combined use of ultrasound, an essential oil and nisin against two food pathogens, *viz*., *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Furthermore, to be able to establish the fitness of use of the combination treatments in the industry, further tests on vegetable colour, texture, electrolyte leakage, microstructure and sensory properties were conducted.

1.1 Problem statement

Decontamination methods that are currently used to minimize risks associated with foodborne outbreaks in fresh produce tend to pose challenges. The challenges come in the form of deterioration in organoleptic qualities (through the use of thermal treatments) and potential carcinogenic risks (through hyperchlorination due to excessive use of chlorine treatments) which necessitate the search for alternative decontamination methods. Therefore, this study seeks to investigate the efficacy of a non-thermal environmentally friendly treatment that effectively decontaminates and preserves the organoleptic properties of ready-to-eat vegetables.

1.2 Aim

To determine the combined impact of nisin, oregano and ultrasound treatments on microbial decontamination, physical properties, sensory properties and microstructure of ready-to-eat vegetables

1.3 Hypothesis

The application of combined treatments of nisin, oregano essential oil and ultrasound will result in a significant log reduction in the pathogens (*E. coli* O157:H7 and *L. monocytogenes*) to inactivation levels and subsequently maintain the physical and sensorial properties of ready-to-eat vegetables specifically lettuce and cabbage.

Hurdle technology use for instance the combination of ultrasound treatment and other antimicrobial methods (organic acids, essential oils, bacteriocins) have been reported to increase microbial log reduction significantly to inactivation levels (Sango *et al*., 2014; Ozcan and Demirel Zorba 2016). Conclusions from studies done have highlighted that minimum concentrations of antimicrobials do not significantly alter the structural and sensory components of the fresh produce (Burt 2004; Tiwari *et al.* 2009). Millan-Sango *et al.* (2015), reported no damages in the cross sections and surfaces of lettuce treated (using ultrasound and essential oils) samples.

1.4 Objectives

1. To investigate the antimicrobial efficacy of nisin, oregano and ultrasound treatments on pathogenic microorganisms (*E. coli* O157:H7 and *L. monocytogenes*) on ready-to-eat vegetables (lettuce and cabbage) using Response Surface Methodology.

2. To determine the effects of nisin, oregano and ultrasound treatments on the physical properties and quality of ready-to-eat vegetables (lettuce and cabbage).

3. To investigate the microstructure and sensory properties of ready-to-eat vegetables (lettuce and cabbage) after application of combined antimicrobial treatments.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 FRESH PRODUCE

2.1.1 Production and importance of fresh produce

There has been a relative increase in the production of fresh produce over the years. For example, a 9.4% increase in global production (1.74 billion tons) of fresh produce was observed in 2013 over the preceding year, which had 1.59 billion tons. This could be attributed mostly to the efforts by different governments to promote healthy eating through national strategic frameworks and to the globalization of the fresh produce industry (Alegbeleye *et al.*, 2018). This upward trend of fresh produce intake has been relative to the demand in eating healthy food and has been compensated across regions through a greater export volume (Yeni *et al.*, 2014). It is generally accepted that fresh produce is an excellent source of nutrients such as polyphenols, vitamins, dietary fibre, proteins, carbohydrates, and minerals. Another advantage of fruit and vegetables is the fact that they are known to reduce cholesterol and the risk of chronic diseases and congenital disabilities (McDaniel and Jadeja, 2019). They have also been recommended as one of the interventions to overcome obesity and other non-communicable diseases (WHO, 2019). Organic fruits and vegetables are an essential part of a healthy diet, and healthcare professionals are increasingly raising awareness of the benefits associated with fresh produce consumption (Archer *et al.*, 2015).

Statistics on global vegetable production have shown substantial growth across regional lines (Fig 2.1) and various vegetable types (Fig 2.2). According to a study by Olaimat and Holley, (2012), there was a reported $12.7 billion in fresh produce imports by the United States, while daily sales reached a staggering 6 million packages of cut produce. The annual consumption of fruits and vegetables in Canada increased by 56% by the year 2010. South Africa has also seen significant growth in the fresh produce industry between 1980-2007, with a reported increase of 18-26% in terms of output (Barrientos and Visser, 2013). Furthermore, South Africa produces 8.2 million tons of fruits and vegetables per year (Panda *et al.*, 2016). The global vegetable sector in comparison to the fruit sector has the largest portion in terms of worldwide fruit and vegetable markets and accounts for approximately 70% of its overall value (Żaczek *et al.*, 2015).

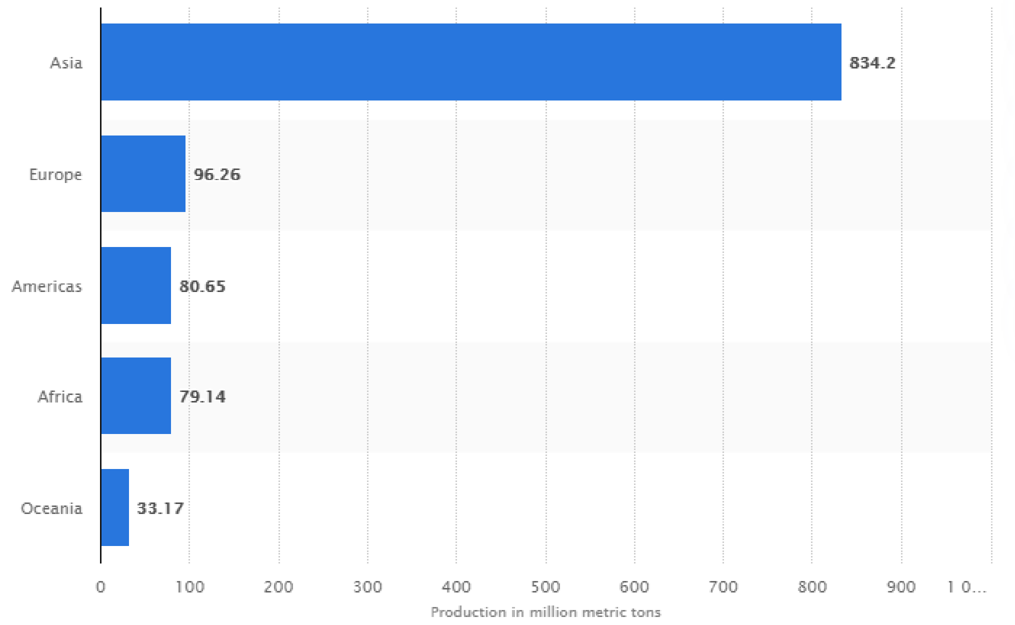

Figure 2. 1 Global vegetable production (2017) across different regions (Statista 2020)

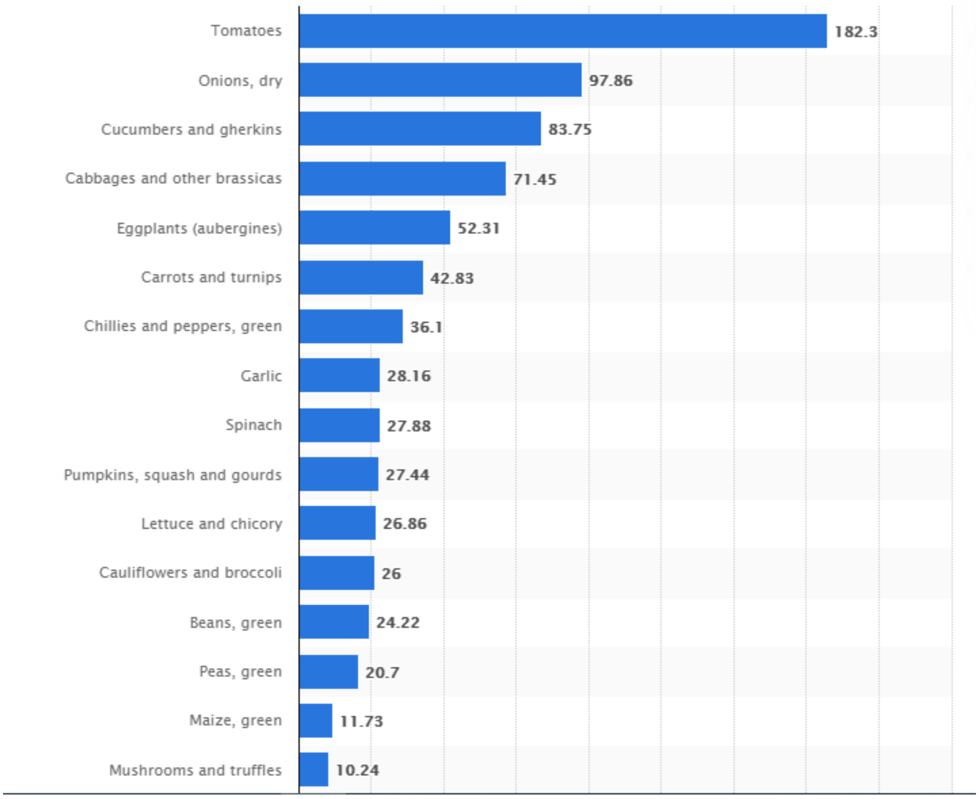

Figure 2. 2 Global vegetable production (2017) statistics based on types (Statista 2020).

Fresh-cut produce consists of peeled, shredded, sliced, trimmed and/or washed fruits and vegetables (Francis *et al.*, 2012). Conventional methods of preservation, such as drying, salting, are rarely extended to fresh-cut items because the emphasis is on preserving the beneficial functional properties, hence cannot undergo heavy processing (Qadri *et al.*, 2015). Therefore, minimal processing of fresh produce is relatively considered the gold standard when preserving fruits and vegetables. Consumers also have a natural preference to convenient 'on the go' foods, in response to their busy lifestyles. This is apparent from the increase in the use of fruits and vegetables in fast food chains, catering services, restaurants and hotels (McDaniel and Jadeja, 2019).

Notwithstanding the benefits of consuming raw fruits and vegetables, consistency and protection remain a concern, because these foods have been recognized as carriers for infectious disease transmission. During processing of fresh-cut products, the degradation of surface cells exposes the cytoplasm providing a rich nutrients source to microorganisms compared to intact products (Qadri *et al.*, 2015). In addition, high water activity, coupled with neutral to low acidic tissue pH found in most fresh produce varieties, promote rapid microbial development (Parish *et al.*, 2003). These conditions provide an ideal environment for various human pathogens and spoilage microorganisms for fresh cut products contamination, thus, causing their rapid degradation when compared to the whole fruit or vegetables.

2.1.2 Fresh produce involved in disease outbreaks

Table 2. 1: Recent foodborne outbreaks linked to fresh produce

Year	Country	Food involved	Pathogen	Cases/deaths	Reference
2020	USA	Pre-cut melon	*Salmonella* spp.	165/0	CDC (2020)
2020	USA	Clover sprouts	*E. coli*	39/0	CDC (2020)
2019	USA	Romaine lettuce	*E. coli* O157:H7	167/0	CDC (2020)
2018	Australia	Rock-melons (Cantaloupes)	*L. monocytogenes*	20/7	WHO (2018)
2018	European Union	Frozen corn/frozen vegetables	*L. monocytogenes*	47/9	ECDC (2018)
2017	USA	Leafy greens	*E. coli* O157:H7	25/1	CDC (2020)
2016	Australia	Rock melon	*Salmonella* spp.	97/0	(Wadamori *et al.*, 2017)
2016	United Kingdom	Imported salad	*E. coli* O157:H7	161/2	WHO (2018)
2016	Australia	Pre-packaged lettuce	*Salmonella* spp.	144/0	(Wadamori *et al.*, 2017)

The increase in fresh produce consumption is mainly attributed to changes in consumer trends as more people are becoming increasingly aware of the importance of essential nutrients in boosting their health and well-being. Food safety becomes a top priority in the food industry and public health sector, mainly because foodborne pathogens cause various illnesses and deaths across the world (Wadamori *et al.*, 2017).

In 2016, an outbreak of a rare strain of *E. coli* O157:H7 triggered a monitoring and surveillance program conducted by the Public Health England (PHE) in the United Kingdom (UK), as represented by Table 2.1. The causative agent for the outbreak which resulted in 161 cases with 60 hospitalizations and two deaths was mixed salad leaves (WHO 2018). Similarly, in the same year in Australia, two significant outbreaks associated with fresh produce were reported. Different types of *Salmonella* spp., Anatum and Hvittingfoss strains were responsible for 144 and 97 cases, respectively. Foods implicated were pre-packaged lettuce for *Salmonella* Anatum, and rockmelons for Hvittingfoss strain (Wadamori *et al*., 2017) respectively. A multistate outbreak of Shiga toxin-producing *E. coli* O157:H7 (STEC O157: H7) infections was investigated by the CDC and FDA in 2017 where twenty-five people from 15 states were infected with nine people hospitalized, including two kidney failures and one death. Reports from ill individuals suggested that the suspected cause of the outbreak in the United States and Canada were leafy greens and no recall was done since the life expectancy of leafy greens does not exceed a month (CDC 2020).

As of June 2018, a multi-country foodborne outbreak in five European nations, involving 47 confirmed cases and nine deaths due to listeriosis infection was tested. The epidemiological breakdown cited cases in Finland (23 cases), UK (11 cases), Sweden (7 cases), Denmark (4 cases), and Austria (2 cases). *L. monocytogenes* IVb, ST6 was the strain implicated in the outbreaks which spanned from 2015-2018. A freezing company in Hungary was identified as the source for cross-contamination after isolates that matched with the pathogenic strains were tracked back to the processing plant. Frozen corn and frozen vegetable mixes that included spinach and green beans were infected and were already in the food chain. Recalls and withdrawals of the implicated frozen vegetables were undertaken as part of the active response measures to combat the prevalence of the outbreak (ECDC 2018). A listeriosis outbreak that occurred in Australia in the year 2018 indicated that rock-melon (cantaloupe melons) from a single Australian grower were the cause of the epidemic. As is represented by Table 2.1, recalls were made and the Australian authorities advised their export partners, i.e., eight countries, to trace and halt the cantaloupes' distribution. There were 20 cases recorded in this outbreak, all of them resulting in hospitalizations, with seven confirmed deaths and one stillbirth (WHO 2018).

In a multistate outbreak reported in the USA in 2019, contaminated romaine lettuce from the Salinas Valley growing area in California was implicated and halted for sale. A total of 167 cases were recorded with *E. coli* O157:H7 being the strain responsible for the outbreak in 27 states. A total of 85 hospitalizations were registered, including 15 people who suffered kidney failure, a form of the hemolytic uremic syndrome (HUS) and no deaths were confirmed. Epidemiological, analytical, and trace back evidence were able to point out that romaine lettuce from California's Salinas Valley growing area was infected and was, therefore, the causative agent (CDC 2020). Recently in 2020, clover sprouts were implicated in an *E. coli* O103 outbreak, across six states in the USA. Epidemiological and laboratory investigations identified a specific chain of restaurants to have distributed the clover sprouts and were traced to a similar seed lot. A total of 39 cases of infection were reported with two hospitalizations and no deaths. The clover sprouts were consequently recalled as an active response against the spreading of the outbreak (CDC 2020). In the African context, not a lot of data has been collected on fresh produce linked foodborne outbreaks, which could be as a result of under-reporting or a lack of awareness of foodborne illnesses among the communities.

2.1.3 Lettuce

Figure 2. 3: Lettuce vegetable (*Lactuca sativa*).

Lettuce, a common vegetable in the human diet is a fat-free, low-calorie food that is valuable in providing vitamin A and folic acid to human nutrition. The presence of essential nutrients makes it preferable as an ingredient in salads providing cooling counterbalance because of its mild flavour. It is usually consumed as cold and raw, hence it undergoes none to minimal processing (Zhou, 2011).

Several foodborne outbreaks have been attributed to mixed salads and some linked to lettuce in particular. Recent cases documented involving lettuce were reported in Australia in the year 2016, 144 cases, attributed to the pathogen *Salmonella* spp. In 2014, 50 cases from *E. coli* were reported in the United Kingdom from sources of lettuce and cucumber (Wadamori *et al.*, 2017).

The United States also recorded a multistate outbreak of *E. coli* O157:H7 associated with consumption of shredded lettuce in restaurants in 2006, reporting 71 cases (53 hospitalized and 8 developed haemolytic-uremic syndrome) (CDC 2020). Strains with a similar genetic fingerprint were reported in Michigan, a total of 38 cases: 21 hospitalized and one developing haemolytic uremic syndrome (HUS). The outbreaks which occurred in the United States triggered a response by the Food and Drug Administration (FDA) in the form of a Lettuce Safety Initiative, aimed at addressing outbreaks associated with lettuce (Zhou, 2011).

2.1.4 Cabbage

Figure 2. 4: Cabbage vegetable (*Brassica oleraceae* var. *capitata* f. alba).

White cabbage (*Brassica oleraceae* var. *capitata* f. alba) is one of the most widely grown vegetables in the world (Traore *et al*., 2020). White cabbage has a significant place in various countries' popular cuisine, culture and is also commonly used in traditional medicine. White cabbage is a key source of phytonutrients in the human diet because of its availability on the local markets and general affordability. In addition to being used fresh in salads, cabbage can be boiled, stir-fried or eaten as a fermented food (Šamec *et al.*, 2017).

White cabbage is a cheap, but very nutritious food source that provides nutrients and phytochemicals that promote health (Barnejee *et al*., 2015). Phytochemicals have drawn much scientific interest and research has shown the presence of glucosinolates, phenolic compounds, carotenoids and several vitamins in cabbage. In addition to its nutritional properties, there is an increase in undergoing research centred on the phytochemicals, anti-cancer, antioxidant, anti-inflammatory and cardioprotective effects of cabbage (Šamec *et al.*, 2017). Interest in the role of free radical antioxidants in human health has compelled work in the fields of horticulture and food science to evaluate antioxidants in fruits and vegetables (Rokayya *et al.*, 2013). A few foodborne outbreaks have been directly linked with cabbage, either as shredded cabbage or in the form of coleslaw salad. The state of Minnesota reported an *E. coli* O111 outbreak which was linked to cabbage salad in 2014, where the number of cases recorded was fifteen in total and no deaths were recorded (Outbreak Database 2020). Another outbreak was recorded in a Mississippi prison in 2009, where cabbage contaminated with *Staphylococcus aureus* was implicated and there was a total of sixty-six cases with no deaths recorded (Outbreak Database 2020).

2.2 Pathogens associated with fresh produce

2.2.1 *Listeria monocytogenes*

L. monocytogenes is an anaerobic, facultative, Gram-positive bacterium that is rod-shaped, widely distributed in the environment. It is implicated for causing foodborne outbreaks in several food varieties, which include dairy, meat, sea and fresh produce (Carstens *et al*., 2019). This bacterial pathogen is able to survive temperature ranges from as low as 1.7ºC to relatively high temperatures of 45ºC; pH ranging from 4.7-9.2 and even high salinity conditions of up to 10% sodium chloride (Oladunjoye *et al*., 2017). Optimal growth conditions of *L. monocytogenes* are between 30-37ºC. The preceding attributes translate into its resistance spectrum enabling the bacterium to evade well-established food control techniques, such as acidification, lowering of water activity and cooling (Oladunjoye *et al.*, 2017). *L. monocytogenes* can also form biofilms on different surfaces, allowing more resistance to environmental stress, and contributing to surface disinfection and sanitation problems (Da Silva and De Martinis, 2013).

L. monocytogenes has at least 12 serotypes and is divided into genetic groups known as lineages. Lineage I consist primarily of serotypes 1/2b, 3b, 4b, 4d, and 4e and is more commonly implicated in listeriosis outbreaks. Lineage II includes serotypes 4a, 1/2c, 3a and 3c and is primarily responsible for sporadic cases of listeriosis. Lineages III and IV are uncommon in nature, with barely 3% of *L. monocytogenes* strains belonging to this group (Ragon *et al.*, 2008; Lomonaco *et al.*, 2015).

Consumption of contaminated food is the main route by which contamination with *L. monocytogenes* occurs. Infections through *L. monocytogenes* cause a condition known as listeriosis which is typically presented as sepsis, meningoencephalitis, as well as neurodegenerative disorders (Carstens *et al*., 2019). Listeriosis targets mostly immunocompromised people, such as pregnant women, the elderly, infants and patients with weak immune systems. Listeriosis is reported to have an incubation time of 1-70 days. Though incidences of listeriosis cases are considered relatively low, invasive listeriosis, however, has been responsible for mortality rates of 20%-30% with 1600 cases estimated per year in the United States (Bergholz *et al.*, 2016). The recent (2018) listeriosis outbreak in South Africa is the largest ever recorded in the world. About 1056 confirmed cases and 214 deaths were reported; raising the mortality rate of the disease to a staggering 20% (NCID, 2018). According to Hofmann *et al.* (2012), amongst the major foodborne outbreaks recorded, listeriosis has the highest rate of hospitalizations experienced, at 94% ahead of the 91.3% of *Vibrio vulnificus* and the 82.6% of *Clostridium botulinum*.

2.2.2 *Escherichia coli*

Escherichia coli is a gram-negative, facultatively anaerobic, non-spore-forming and rod-shaped bacterium. The *E. coli* cells are usually about two µm in length and 0.5 µm diameter, while their flagella can swim and are motile (Zhou, 2011). *E. coli* is commonly found in the lower intestine of warm-blooded organisms, food products, the environment and sometimes processing equipment. *E. coli* are coliforms and important faecal markers since they are associated with faecal matter and are found near water, soil and animals (Van Elsas *et al.*, 2011). Other strains of *E. coli* can proliferate at temperatures as low as 7ºC and as high as 46 ºC; optimal growth usually occurs at 37ºC, though some strains can grow at temperatures as high as 49ºC. *E. coli* cells can survive for minimal periods in the environment, processing equipment and food products. They are part of the Enterobacteriaceae family and are widely accepted as an ideal organism for faecal contamination (Zhou, 2011).

E. coli can also be classified based on serological characteristics and virulence properties with the diarrheagenic causing strains grouped into five major categories, Enteropathogenic, Enterotoxigenic, Enteroinvasive, Enterohemorrhagic and Enteroaggregative. *E. coli* genus has the most significant verotoxin (VT)-producing serotypes (VTEC O157). O157:H7 and O157: H-.VTEC O157 infections are usually lethal and have been implicated to have caused numerous foodborne disease outbreaks (Yeni *et al.*, 2016). VT-producing *E. coli* serotypes O157:H7 and O157: H- are characterized by their synthesis of toxins which may kill vero cells. These toxins are referred to as verotoxin, 'Shiga Like' Toxin (SLT), verocytotoxin or merely shigatoxin (ST) because they are closely related to shigatoxin, which is produced by the bacterium *Shigella dysenteria* type 1 (Wang *et al.*, 2012).

Verotoxin producing *E. coli* serotypes are communally referred to as VT-producing *E. coli* (VTEC). *E. coli* O157 is demonstrated by the identification of the O157 antigen. The serotypes most associated with disease cases of VTEC are referred to as enterohaemorrhagic *E. coli* (EHEC) (Yeni *et al.*, 2014). The term 'enterohaemorrhagic' refers to a typical symptom of VTEC O157 infection, known as haemorrhagic diarrhoea. Infection with one of the five *E. coli* pathotypes can cause diarrhoea. Nevertheless, the natural history of the disease varies and depends on the contributing pathotype of *E. coli* (Carstens *et al.*, 2019).

An infection with *E. coli* has an incubation period that varies from between 1 to 7 days. In some cases (0-10%), the disease may cause lethal complications in the form of acute renal failure and anaemia known as a haemolytic uremic syndrome (HUS). Enterohemorrhagic *E. coli* O157:H7 is particularly of interest to public health because it is the leading cause of haemolytic uremic syndrome (HUS) in the United States. Transmission generally occurs through a foodborne path, from human interaction, by consuming polluted water or by contact with animals (Yeni *et al.*, 2016). Symptoms can vary, including mild gastroenteritis, bloody diarrhoea, colitis haemorrhagic, HUS, and even death. Children seem to be more susceptible to HUS. An overall estimated cases of 63 513 have been linked to foodborne Shiga-toxin producing *E. coli* O157-related cases in the United States annually with a hospitalization rate of 46.2%, which is close to that of *Vibrio cholerae* rate at 43.1% (Carstens *et al.*, 2019).

Mitigation measures to control foodborne pathogens

2.3 Chemical methods

2.3.1 Chlorine

Chlorine has been the mostly utilized sanitizer in the fresh produce industry, with sodium hypochlorite (NaOCl) as the most common form of chlorine used for washing in the food industry. Hypochlorite acid (HOCl) and sodium hydroxide (NaOH) are the outputs of the sodium hypochlorite-water reaction which facilitate oxidative reactions (Tirpanalan *et al.*, 2011). Some compounds of combined chlorine, such as chloramines, are inaccessible to oxidative reactions and are thus not regarded as free chlorine. Although hypochlorous acid (HOCl) is more effective in terms of bactericidal activity than the hypochlorite ion (OCl-), when conditions are alkaline, the equilibrium shifts to the hypochlorite ion (OCl-). It is important to note that the percentages of HOCl and OCl at pH 7.0, which is the target pH for the fresh produce industry, are 78% and 22% respectively (Parish *et al.*, 2003, Betts and Everis, 2005). Although the concentration of HOCl increases as the pH decreases, the solution becomes corrosive at lower pH values for the food contact equipment. The commercial volume of chlorine applied in the fresh produce industry ranges between 50 and 200 ppm, with adjustable contact times between 1 and 10 minutes or even longer at chilling temperatures (Parish *et al.*, 2003, Goodburn and Wallace, 2013). However, many studies have reported that microbiological load reduction is achieved primarily in the first few minutes of treatment application and additional reduction when contact time is enhanced is insignificant (Tirpanalan *et al.*, 2011).

Chlorine remains one of the most widely used sanitizers due to its powerful oxidizing effect, being water-soluble, relative ease of application and its relatively low cost. There are many variables that can potentially affect the effectiveness of hypochlorite-based washing systems on fresh produce such as application, temperature, solution pH, washing time, and the amount of organic matter present (Parish *et al.*, 2003). There are also factors directly linked to the actual microbes that may affect chlorine-based disinfection, namely the initial bacterial load and number of microorganisms, the type of surface to be cleaned, the amount of disinfectant, the potential internalization of pathogens and the disinfectant's temperature and time of exposure (Gil *et al.*, 2009, Castro-Ibáñez *et al.*, 2017). Consequently, the inconsistency in the levels of log reduction among strains belonging to the same species that would have been exposed to the application of similar amounts of chlorine have been noticed. These inconsistences could be as a result of the disparities in the surface area of fresh produce that has been cut into pieces when processing, thereby altering the amount of free chlorine available in the solution. Chlorine can bind to organic material and form less efficient bound chlorine than free chlorine as a biocide (Castro-Ibáñez *et al.*, 2017). The effectiveness of the washing method will be decreased if the amount of organic material in the product increases (Gil and Selma, 2006). Chlorine-based systems for fresh produce disinfection have raised concern due to the production of by-products such as haloketones, halo-acetic acids, trihalomethanes, and their impact on the environment and human health (Gómez-López *et al.*, 2014). While adverse health effects on humans are not studied and described in detail, these by-products are recognized as carcinogenic (Meireles *et al.*, 2016).

Several studies on the efficacy of chlorine have been done, including that of Gómez-López *et al.* (2014), which focused on the minimum free chlorine and trihalomethane generated. Two types of tests were conducted to simulate a spinach wash tank from a fresh-cut industry. In the first, a wash tank containing clean water was continually filled with concentrated process wash water collected from spinach with high organic matter and inoculated with an *E. coli* O157:H7 mixture (5 log CFU/ml). During the test, a peristaltic pump dosed a chlorinated solution to the washing tank to adjust the concentration of free chlorine (FC) to 1 and 3 mg/l. In the second test, the washing tank contained process water from the start of the experiments and the concentration of FC was adjusted to 3 and 5 mg/l. During the washing of fresh-cut spinach 5 mg/l (up to a maximum of 7 mg/l) kept the wash water free of the pathogen for 1 hour, although trihalomethane levels > 1000 mg/l were produced. Results indicate a total residual of approximately 7 mg/l FC treatment is successful for inactivating *E. coli* O157:H7, under industrial conditions. Validation of the efficacy of the sanitizer using a dynamic system could facilitate the implementation of selected treatments in the food industry (Gómez-López *et al.*, 2014).

2.3.2 Chlorine dioxide

As a sanitizing agent, chlorine dioxide (ClO_2) reduces the microbial load through the disruption of protein synthesis and eventually the bacterial cell's membrane permeability (Betts *et al.*, 2005). It has been used for water treatment since 1944 and the FDA allowed the use of aqueous chlorine dioxide as a sanitizing agent for fruit and vegetables (Tirpanalan *et al.*, 2011). It is specified in the US that the amount permitted should not exceed five ppm as residual chlorine dioxide and that the application of chlorine dioxide ought to be preceded by rinsing with drinking water (Goodburn and Wallace, 2013). As with the HOCl solution, the reduction of the microbiological load and the effectiveness of chlorine dioxide hinges on the process factors applied.

Chlorine dioxide has several benefits over its chlorine counterpart, one of which is, its ability not be influenced by pH (Meireles *et al.*, 2016). Also, of note is the element that cross-reactivity with organic materials will be minimized. When reacting with ammonia, it does not form chloramines, and fewer organo-halogens are formed as ClO_2 reaction products as compared to chlorine and has a 2.5-fold higher oxidizing power than chlorine (Beuchat *et al.*, 2004). Chlorine dioxide, however, is unstable; therefore, it must be produced on-site, it can also be explosive at significant amounts and decomposes once it is subjected to higher temperatures (Parish *et al.*, 2003). Another drawback for the use of chlorine dioxide at 5 mg/L of gaseous chlorine dioxide has been observed to cause visual quality deterioration in a 14.5 min duration. Adverse changes in the sensory performance of lettuce were also recorded on the 3rd day of storage after 10.5 minutes of treatment with 1.4 mg/L chlorine dioxide (Tirpanalan *et al.*, 2011).

Hassenberg *et al.* (2017) studied the biocidal efficiency of ClO_2 and how it may be affected by variables such as pH, temperature and organic matter charges. He observed that successful technical implementations require information on the relationship between ClO_2 efficiency and factors that influence it. A comprehensive assessment of the influence of temperature on the efficacy of ClO_2 treatment (ClO_2 concentrations 0, 2, 4, 6, 8 and 10 mg/L) on *E. coli* (start load: 5 log CFU/mL) and the influence of organic matter load on the effective ClO_2 concentration in lettuce washing water was reported. Decreasing the temperature of the treatment water (from 15°C to 2°C) resulted in a reduction of *E. coli* counts. Microbes were significantly and completely inactivated at 15°C after 0.5 min at a concentration of 3 mg/L ClO_2.

Studies have shown that much higher concentrations of antimicrobial solutions are required to reduce the microbial load of fruits and vegetables significantly. Wisniewsky *et al.* (2000) recorded a reduction of only 2.5 logs in the *E. coli* O157:H7 population inoculated on apples following treatment with 80 ppm ClO_2. Pao *et al.* (2007) observed that a 5- log reduction of *S. enterica* inoculated on tomatoes, proceeding exposure to 20 ppm ClO_2 during 1 min. On lettuce, *L. monocytogenes* population reduced by 1.1 log cycle when five ppm aqueous ClO_2 was applied for 10 min (Zhang and Farber, 1996). Recent studies have shown that ultrasound combined with aqueous ClO_2 treatment can potentially enhance ClO_2's disinfectant effectiveness. Microbial populations of *Salmonella* and *E. coli* O157:H7 in apples were reduced to 2.5 and 4.3, respectively, after exposure with combined ultrasonic (170 kHz) and ClO_2 (20 – 40 ppm) treatments (Huang *et al.*, 2006). The application of gaseous ClO_2 in fresh goods have been observed to be more competitive and the potential for industrial applications appears to be higher. However, treatment success varies with the product type. Approximately 2 log reductions in mesophilic counts were reported when Gómez-López *et al.* (2007) applied 1.3 ppm ClO_2 gas for 30 s to carrots. ClO_2 has also shown effectiveness in destroying pathogens without tampering of the lettuce's visual quality. *E. coli*, *Salmonella* and *L. monocytogenes* count dropped by 3.4, 4.3 and 5.0 logs, respectively, in artificially inoculated lettuce after 30 min ClO_2 gas treatment at 4.3 ppm (Lee *et al.*, 2004). Sy *et al.* (2005) observed that treatment with 4.1 ppm ClO_2 gas caused the pathogenic log counts of carrots, cabbage and tomatoes to decrease between 3 and 6 log CFU/g. Nevertheless, only 1–2 log reductions in fresh-cut lettuce and onion were achieved and the procedure exhibited negative effects on the sensorial properties of the treated fresh produce.

Van Haute *et al.* (2017) reported that application of 5 mg/L of ClO_2 was able to inactivate the total plate count (TPC) to levels > 3 log reduction after 2 min exposure and to > 5 log reduction on *E. coli* within 3 min of exposure, on fresh-cut iceberg lettuce. At a ClO_2 concentration of 3 mg/L, *E. coli* was significantly and completely inactivated at 15ºC after 0.5 min (Jiang *et al.*, 2017). Bacterial reductions of 2.4, 2.3, and 2.1 log CFU/g were recorded against *E. coli*, *S. typhimurium* and *L. monocytogenes* respectively, at 400 ppm aerosolized ClO_2 on carrots (Cho *et al.*, 2017).

Fresh-cut coriander, one of the most commonly eaten leafy vegetables in China due to its versatility and taste, was subjected to chlorine dioxide treatments Jiang *et al.* (2017). The optimal conditions for chlorine dioxide treatment application on fresh-cut coriander were observed at a concentration of 60 mg/L, 10 min duration and a fresh-cut product: solution ratio of 1:8. The optimal chlorine dioxide treatment conditions were able to reduce the total number of bacterial aerobic colonies to 2.1 log CFU/g. Treatment with chlorine dioxide also successfully decreased the potentially pathogenic bacteria such as *Staphylococcus*, *Brevibacterium*, *Pseudomonas* and *Acinetobacter*, as well as other bacteria. As a result, treating fresh-cut coriander with chlorine dioxide under suitable conditions during processing or storage can successfully reduce the food safety risk. Such findings provide a valuable theoretical basis for fresh-cut product safety enhancements.

2.3.3 Ozone

Ozone is the oxygen element's chemically active triatomic allotrope that can be regenerated by ultraviolet radiation and corona discharge (Lone *et al.*, 2019). Ozone has a high propensity for facilitating oxidation-reduction reactions, that are responsible for the inactivation of contaminants by directly reacting as molecular ozone or by the derived free radicals. In 2001, FDA approved ozone as a generally recognized safe substance (GRAS) for industrial utilization as a decontaminant and sanitizer in the handling of food (Goodburn and Wallace, 2013). The application of ozone has been of commercial interest to the food industry because of its effectiveness in extending the shelf-life of fresh produce by inhibiting the growth of microorganisms and preventing decay on the fruit and vegetable surface (Meireles *et al*., 2016).

The antimicrobial effect of ozone emanates from oxidization of the thiol group of cysteine residues in bacterial enzymes that are involved in respiration resulting in the ultimate balance of a homeostatic environment (Deng *et al*., 2019). The strong oxidizing ability of ozone can also destroy the membrane of bacterial cells, consisting of various components such as polyunsaturated fatty acids, glycoproteins and glycolipids (Meireles *et al.*, 2016). Consequently, a chain reaction caused by ozone frequently leads to cell lysis due to subsequent cell contents leakage and rapid cell protein oxidation. Ozone breaks down into oxygen and does not create any by-products. It forms aldehydes, ketones, carboxylic acid which causes less regulatory concerns in the presence of organic matter. Ozone can decompose rapidly, leaving no organic residues and thus providing an alternative to chlorine-based washing solutions (Deng *et al.*, 2019). It is also proposed that the reuse and recycling of ozonated wastewater can be undertaken to reduce the industry's excessive water consumption (Garg *et al.*, 2016).

Many studies have analyzed the different methods of application to improve the efficacy of ozone decontamination. This includes bubbling ozone, low- and high-speed ozonation, gaseous ozone, aqueous ozone, ozonation combined with sonication etc. (Tirpanalan *et al.*, 2011). The most active method was identified to be the bubbling ozone across the different ozone application methods (Phaephiphat *et al.*, 2018). The solubility of ozone in water increases as the temperature decreases; therefore, ozone is more effective when microorganisms are inactivated at lower temperatures (Tirpanalan *et al.*, 2011).

Phaephiphat *et al.* (2018) studied the application of ozone microbubbles on sweet basil and reported log reductions of 2.8, 1.9, 4.2, and 4.2 log CFU/g on *S. Typhimurium* while the population of *E. coli* was reduced to 2.1, 1.0, 1.4, and 1.6 log CFU/g. In a study aimed at demonstrating the strength of gaseous ozone treatment against *E. coli* O157:H7 on spinach leaves, log reductions of up to 2.8 CFU/g were observed after treatment (Yesil *et al.*, 2017). Wang *et al.* (2019) showed that a combination of aqueous ozone with lactic acid (LA) produced an *E. coli* log reduction of 1.72 CFU/g at 2 mg/L and 1.70 CFU/g at 1 mg/L aqueous ozone on green lettuce. Aqueous ozone treatment (1.4 mg/L) decreased the total fresh-cut apple bacteria by 1.83–2.13 log CFU/g and increased the shelf-life to 10 days (Liu *et al.*, 2016).

Ozone is a potent oxidative agent which may cause physiological injury to the produce when certain levels are exceeded. Koseki and Isobe (2006) found that ozonated water above five ppm had damaged the lettuce leave surface texture and observed a rapid onset of browning on iceberg lettuce treated with ten ppm of ozonated water. In the same study however, treatments with three and five ppm of ozonated water were able to produce similar changes in the a* value to that of distilled water treatment. Song *et al.* (2000) investigated the treatment of onions using ozone during storage. Mould and bacterial counts decreased without any change in chemical composition and sensory quality. Shredded lettuce had reduced bacterial content in water bubbled with ozone gas (KIM *et al.*, 1999). Ozone was experimentally used to decontaminate whole black peppercorns and ground black pepper as a replacement for ethylene oxide (Zhao and Cranston, 1995).

No negative impact of ozone has generally been observed on vegetable nutritional content in the range of 1–5 ppm compared to traditional chlorine treatment. Koseki and Isobe, (2006) found that the lettuce ascorbic acid content was not affected by the sanitizing treatments, even at high concentrations of ozone (5 ppm) and chlorine (200 ppm) for a long exposure time (5 min). It has been stated that, at the end of 9 days of storage, the vitamin C content of low-dose (0,03 – 0,08) ozonated water treated fresh-cut celery was found to be significantly higher than that of untreated celery. In contrast, there was no difference between 0,18 ppm ozone treatment and vitamin C control treatment (Zhang *et al.*, 2005). The authors stated that vitamin C protecting ozone effect might be due to PPO activity inhibition (polyphenol oxidase) and tissue metabolism slowing down. Besides, studies have shown that the treatment with ozone does not result in a higher respiration rate compared to chlorine treatment in vegetables (Beltrán *et al.*, 2005, Zhang *et al.*, 2005).

2.3.4 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a cytotoxic species-forming oxidizer which ensures the formation of antimicrobial properties that may be either bactericidal or sporicidal, depending on the concentration, pH and temperature (Meireles *et al.*, 2016). Hydrogen peroxide can generate cytotoxic oxidizing agents such as hydroxyl radicals that oxidize microorganisms cell membranes, biomolecules and DNA (Parish *et al.*, 2003). The disinfectant, hydrogen peroxide, can be applied to food contact surfaces, packing material and aseptic filling. It has GRAS status and can also be used to disinfect the water and fresh produce (Tirpanalan *et al.*, 2011). Many studies have shown that hydrogen peroxide is effective in reducing the microbiological load on certain fresh products such as alfalfa sprouts, bell peppers, cucumber and cantaloupe without altering the sensory quality (Tirpanalan *et al.*, 2011). Based on the use of H_2O_2, however, the cross-contamination that can still occur in the washing water vegetables cannot be prevented as its decomposition is rapid and the kinetics of disinfection is relatively slow (Parish *et al.*, 2003). The main advantage of H_2O_2 is that it is dissolved into water and oxygen, so it does not leave essential residues, but the degree of degradation depends on the quantity of catalase enzyme (peroxidase) in the substance. Betts and Everis (2005) also revealed that another downside of applying H_2O_2 is the browning effects it can have on vegetables, particularly lettuce, which can be solved however by adding suitable anti-browning compounds like sodium (Sapers *et al.*, 2001; Meireles *et al.*, 2016).

Huang and Chen (2011) observed that minimally processed baby spinach infected with *E. coli* O157:H7 has been related to several outbreaks of foodborne diseases. A study was conducted to determine the effects of organic acids and hydrogen peroxide on the inactivation of *E. coli* O157:H7 on baby spinach alone and in binary combinations with or without mild heat (40 and 50°C). Leaves of baby spinach were dip-inoculated with *E. coli* O157:H7 at 6 log CFU/g level and stored for 24 h before treatment at 4°C. A log reduction of 1.5 log CFU/g was achieved after the treatment with a dosage of 2 x 10^4 ppm H_2O_2 was applied. Hydrogen peroxide at a higher concentration of 3 x 10^4 ppm managed to reduce the population of *E. coli* O157:H7 to 1.6 log CFU/g for baby spinach after exposure for 5 min (Huang *et al.*, 2012). Ukuku and Fett (2002) attained a log reduction in the range 2.0 – 3.5 log CFU/cm^2 when a high concentration of hydrogen peroxide was applied to *L. monocytogenes* inoculated melon surfaces.

2.3.5 Electrolysed oxidizing water (EOW)

Electrolysed oxidizing water (EOW) has proven to be a promising alternative technique of decontamination with a significant bactericidal impact, particularly as a valuable antimicrobial tool in the water sanitation and fresh produce industry. EOW, also known as activated water, is formed by electrolysis of a dilute salt solution (NaCl) passing through an electrolytic cell that contains a bipolar membrane separated by positively and negatively inert charged platinum electrodes (Ngnitcho *et al.*, 2017). A salt-saturated solution and current are passed through the chamber to create EOW, dissociating the solution into two different streams: EOW acid (AcEOW) and EOW alkaline (AlEOW). At the anode, the acid solution (pH between 2.5 and 3.5) consists of HCl, HOCl, Cl_2, OCl^-, and O_2 and has a high oxidation-reduction potential (ORP), between 1000 and 1200 mV (Meireles *et al.*, 2016). This solution is antimicrobial and has a chlorine-like mode of action (DNA mutations, cell protein disruption and enzyme disruption). Besides, the cell membrane may be damaged due to the solution's acidity and the action of hypochloric acid may be facilitated more effectively (Demirci and Bialka, 2011). The alkaline solution (pH between 10 and 11.5) that gathers by the cathode has a negative ORP and can be used as a detergent. Combination of both acid and alkaline solutions produces a much more stable neutral electrolyzed oxidizing water (NEOW), which is not so harsh on food-contact surfaces and can be used to decontaminate the fresh produce as it does not change the colour or appearance of the produce due to the solution's neutral pH (Cheng *et al.*, 2012). This process is environmentally friendly because it uses only salt and water to create the chemical solution and has been approved by the FDA at 200 ppm as the maximum concentration (Meireles *et al.*, 2016).

Afari *et al.* (2019) investigated the effects of electrolyzed oxidizing (EO) water treatment on the abilities of *L. monocytogenes* and *E. coli* O157:H7 to remain viable after treatment. Concentrations of inoculum (8, 6 and 4 log CFU/mL), EOs water at pH between 2,7 and 8,5 and two lettuce-to-EO water ratios (1:20 and 1:10 w/v) were evaluated for 5 min by exposing pathogens to EO water, followed by cultivation on non-selective media and evaluation of viability by cytometric flow and resuscitation attempts. Cultivability was maintained by treating pathogens with EO water at high initial populations, while lower populations lost cultivability at free concentrations of chlorine (FCC) above 1 mg/L. Ultraviolet absorbance at 254 nm (UV-254) was used as organic load indicator and VBNC cell formation was observed at 6 and 7.5 mg/L FCC of EO water samples with 0.059 and 0.102 UV-254 readings, respectively. Increased organic charge of EO water increased VBNC to induce FCC levels. Nevertheless, holding EO water residual chlorine above 9 mg/L stopped VBNC cells from forming and thus helped prevent cross-contamination during washing treatment of fresh produce.

The efficacy of low concentration electrolyzed water (LcEW) was investigated in order to inactivate spinach leaf pathogens as a convenient and safe alternative sanitizer and was compared with other sanitizers (Rahman *et al.*, 2010). Spinach leaves were immersed in deionized water (DIW), LcEW, heavy acid electrolyzed water (SAEW), aqueous ozone (AO), 1% citric acid (CA) and sodium hypochlorite solution (NaOCl) at room temperature for 3 minutes (23 ± 2°C) after *E. coli* O157:H7 and *L. monocytogenes*. The similar pattern of microbial reduction on spinach was evident for all pathogens with the washing of LcEW and SAEW. It was observed in the present study that LcEW inactivated at a maximum of 1.64 – 2.80 log CFU/g and DIW resulted in the lowest reduction, 0.31 – 0.95 log CFU/g of background or pathogenic microflora present on spinach leaves relative to the unwashed sample. The findings of this study suggest that LcEW and SAEW did not differ significantly ($P > 0.05$) in reducing background or pathogenic microflora on spinach and that LcEW could be a good sanitizer for washing vegetables without contamination, rather than using electrolyzed oxidizing (EO) water or SAEW (Rahman *et al.*, 2010).

2.4 Biological and biochemical methods

2.4.1 Phytochemicals

Plants can produce antimicrobial secondary metabolites (phytochemicals) against various microorganisms, including pathogens (Belletti *et al.*, 2010). Such metabolites are classified into various classes of chemicals, including essential oils, alkaloids, phenolics, polyphenolics, polyacetylene, peptides and lectins (Borges *et al.*, 2013). Due to their significant variation, phytochemicals' mode of action could be quite complex. The most common effect of phytochemicals includes enhancing the permeability of the cell membrane leading to intracellular compounds leakage (Tiwari *et al.*, 2009). Several of these phytochemicals, most notably essential oils, have been in the flavouring aspects of foods and have also gained access as antimicrobial agents in food production. A relatively significant number of these phytochemicals have GRAS status and have broad use in the food production industry (Meireles *et al.*, 2016).

Cossu *et al.* (2018) investigated synergistic interactions of food-grade phenolic acids (gallic and ferulic acid) and ultraviolet light (UV-A) to decontaminate fresh produce using fog to enhance the dispersion of phenolic acids on the surface of the vegetable. As test organisms, nonvirulent strains of *E. coli* O157:H7 and *L. innocua* were used, and spinach was chosen as a model fresh product. The synergistic combination of a phenolic acid accumulated in fog and UV-A light treatment achieved a reduction in the of up to 2 log CFU/cm^2. Furthermore, the procedure did not affect the texture but showed minimal effects on the colour of the spinach leaves. Ultimately, this technology can help create alternative methods to decontamination processes using compounds of the food-grade level.

Wang *et al.* (2019) studied the disinfection potential of lactic acid (LA) plus aqueous ozone (AO), an oxidizing sanitizer that does not result in secondary residue, on green lettuce. Concentrations of 1% LA (90s) plus 1 mg L^{-1} AO (30s) or 2 mg L^{-1} AO (30s) were used in contrast to the traditional method of washing with tap water. Microbial analysis showed that LA plus AO at 100 ppm chlorine (120s) or 1% LA (120s) plus tap water (30s) and 2 mg L^{-1} AO (150s), contributed to the most significant reduction of microbes (*E. coli* O157:H7, mesophilic bacterial counts, psychrophilic aerobic counts, moulds, and yeasts) when stored (0–5 days at 5°C). Analysis of consistency (colour, sensorial characteristics, leakage of electrolytes, polyphenolic material, and loss of weight) displayed that LA+AO did not cause additional quality loss when compared to treatment with tap water. Such results suggest that the efficacy potential of the proposed hurdle technology (LA+AO) to be utilized in the disinfection of fresh produce.

The use of nisin and citric acid as GRAS food ingredients which are often used as agents that lower microbial contamination in fresh-cut fruits and vegetables (Chen *et al.*, 2016). Assessment of parameters such as phenolic acid content, antioxidant activity, microbial log reduction and changes in colour were conducted after fresh-cut onions were treated with 50 $\mu g \cdot mL^{-1}$ nisin combined with 1% (w/v) citric acid (N+C). After 15 days of storage, total viable counts (TVC) were reported and the samples that were N+C treated remained significantly lower ($p < 0.05$) in comparison to the controls. Additionally, treatment with N+C led to an enhanced preservation of colour during storage total polyphenols and flavonoid content was enhanced. Thus, N+C may be applied as a suitable decontaminant for freshly cut onions (Chen *et al.*, 2016).

2.4.2 Bacteriophages

The bacteriophage or simply phage is a virus that infects bacteria and kills them. Phages are considered one of the earth's most plentiful and self-replicating biomaterials (Hagens and Loessner, 2010; Sillankorva *et al.*, 2012). Bacteriophages have been found to be natural antimicrobials that coexist with their specific bacterial hosts. Owing to its excellent qualities, the use of lytic bacteriophage in the food safety industry has gained attention in recent times. Bacteriophages provide the benefit of leaving uninfected food microbiota without inducing superinfection (Golkar *et al.*, 2014). The high self-replication rate of bacteriophage is also economical, since small quantities may be used. Besides, the abundant environmental availability of bacteriophages makes their isolation and cultivation relatively easy. The abundant availability of phage has contributed to its utilization in addressing food safety issues, including infection with common bacterial pathogens such as *Salmonella*, *Campylobacter*, *E. coli*, and *Listeria* (Schmelcher and Loessner, 2014). The increasing interest and appeal of phage biocontrol to enhance the challenges of food safety could be related to various factors. The key benefits of using lytic bacteriophages to kill unwanted bacteria are specificity, effective mode of action, availability and fewer effects on the product's organoleptic properties (Meireles *et al.*, 2016).

Snyder *et al.* (2016) determined the efficacy of phage treatment on two different types of fresh produce (cut green pepper and spinach leaves). The treatment rinsing time was either 2 or 5 min and holding temperature of treated products (4°C, 25°C or a combination of both temperatures). Phage suspension was applied to inoculated fresh produce, which was then kept for three days under different storage conditions. Optimized phage treatment decreased pathogenic *E. coli* populations by 2.4 – 3.0 log CFU/g on cut green pepper at 5 min rinse time and 3.4 – 3.5 log CFU/g on spinach leaves at 2 min rinse time, in 72 h. Much of the decline was attributed to the phage's antimicrobial action. The results indicated the abilities of bacteriophage in selectively controlling pathogens on fresh produce.

Perera *et al.* (2015) applied ListShield^{TM} treatment on inoculated lettuce, cheese, smoked salmon and frozen delicacies and managed to achieve a significant log reduction ($p < 0.05$) of *L. monocytogenes* by 91% (1.1 log), 82% (0.7 log), 90% (1.0 log), and 99% (2.2 log), respectively. The application of ListShield^{TM}, alone or in conjunction with an antioxidant/anti-browning solution on apple slices, resulted in a 93% (1.1 log) reduction of *E. coli* at temperatures of 4°C in a 24 h period. Application of ListShield^{TM} did not alter the organoleptic consistency of foods, as there were no noticeable variations in colour, taste, or appearance, thereby making lytic bacteriophage treatments environmentally friendly.

To monitor the reduction of *S. typhimurium* and *S. enteritidis* on lettuce, Spricigo *et al*. (2013) applied three separate lytic bacteriophages (UAB_Phi 20, UAB_Phi78 and UAB_Phi87). The treatment was conducted at room temperature for 60 min and the reduction achieved for *S. typhimurium* and *S. enteritidis* was 3.9 and 2.2 log CFU/g, respectively. Leverentz *et al.* (2003) tested Intralytix, Inc.'s phage mixtures LM-103 and LMP-102, which contained 14 and 6 lytic phages unique to *L. monocytogenes*, against serotypes primarily associated with listeriosis in humans. The listeriophageal mixture applied to slices of honeydew melon reduced *L.*

monocytogenes populations compared to control by 2.0 – 4.6 log cycles but had no effect on apple samples. Phage titers on melon slices were stable but declined rapidly on apple slices due to increased phage sensitivity to the more acidic environment (pH 4.4) of the apple slices compared to that of the melon slices (pH 5.8).

Another study by Oladunjoye *et al*. (2017) studied on the effects of a bacteriophage and sucrose monolaurate combination against *L. monocytogenes* inoculated on tomato and carrot samples. The study utilised the used of Artificial Neural Networks (ANN), to develop mathematical models that would optimize the best concentration treatments possible with the factors used. Bacteriophage treatment on the two fresh-cut produce samples showed reductions of <1 log and 2 log, for tomato and carrot, respectively. Adding sucrose monolaurate to the bacteriophage proved futile, as results at 100 and 250 ppm could not show an increased log reduction. Thereby showing that the use of bacteriophages in their singular form, is the most preferred method of application in this study.

2.4.3 Enzymes

Enzymes target mainly the extracellular polymer matrix that surrounds the biofilm cells which influences changes in the shape of the biofilm structure while making it more susceptible to destructive shear forces (Lequette *et al.*, 2010). Enzymes can directly attack biofilms that interfere with their growth, catalyze antimicrobial formation, interfere with quorum sensing occurrences, or even disrupt a mature biofilm (Thallinger *et al.*, 2013). Enzymes can, therefore, be an alternative to traditional chemical disinfectants in order to remove biofilms from fresh produce and/or abiotic surfaces (Meireles *et al.*, 2016). The use of proteases in pipelines and the removal of proteins from contact lenses are common applications of biofilm removal through enzyme application (Thallinger *et al.*, 2013). Like bacteriophages, enzyme application requires prolonged contact periods to be successful in regulating biofilm (Meireles *et al.*, 2016). The fact that the extracellular polymeric substances (EPS) are heterogeneous is another drawback on the use of enzymes for biofilm removal. The use of pure enzymes, therefore, does not guarantee the complete elimination of biofilm and should, therefore, be used as a mixture or combined with other treatments that are antimicrobial in nature. Most of these formulations are used to disinfect food-contact surfaces, however, the relatively high cost of enzymes can be a disadvantage (Augustin *et al.*, 2004; Thallinger *et al.*, 2013).

Balabanova *et al.* (2017) investigated the activity of nucleolytic enzymes that were extracted from marine bacteria. The recombinant alkaline enzyme phosphatase (CmAP) was applied against a biofilm formed by a cocktail of microbes, including *S. enterica*, *S. aureus*, *P. aeruginosa* and *B. subtilis* on pork sausages as the surface material. The concentration of CmAP for maximum effect exhibition on microbial growth and biofilms was 1.1 µg/ml with 2.5 units/ml activity, which decreased overall aerobic mesophilic and lactic acid counts in sausage shells by 3.5 log units for five days and 2.5 log units for six days, respectively. In a study carried out by Singh *et al.* (2015), it was shown that *Aspergillus clavatus* produced a mixture of amylases, proteases and pectinases that reduced *Pseudomonas aeruginosa* and *Bacillus subtilis* biofilms by 82% and 75%, respectively. Wang *et al.* (2016) elicited biofilm formation by a cocktail of seven *Salmonella* spp. strains isolated from meat process surfaces and poultry, that were grown on stainless steel surfaces. They reported that the resultant biofilm had been successfully removed through cellulase treatment followed by immersion in ceyltrimethyl ammonium bromide (CTAB). In comparison to proteinase K, dispase II, glucoside amylase and subtilisin, the enzyme cellulase was able to achieve higher cell reduction (85%, around 5.6 log CFU/cm2) in mature biofilm, though a significant number of residual biofilm cells remained.. Craigen *et al.* (2011) reported on the ability of α-amylase to minimize biofilms from *S. aureus* by 79%, which was however not successful in eliminating biofilms of *S. epidermidis*. Matrix formation inhibition was recorded at 82% while doses of 10, 20, and 100 µg/mL of α-amylases decreased *S. aureus* biofilms by 72%, 89% and 90%, respectively. At 5- and 30-min time intervals, *S. aureus* biofilms were reduced by 79% and 89%, respectively in the same study.

2.5 Physical methods

2.5.1 Ionizing irradiation

Ionizing irradiation has been suggested as an alternative decontamination method for fresh produce (Deng *et al.*, 2019). Several international authorities, like WHO and US FDA, have approved the use of ionizing irradiation for the removal of pathogens and unwanted spoilage microbes (FDA 2016). This treatment method entails exposure of food to ionizing radiation, such as gamma, electron beams or X-ray, to remove microbial contamination, to inhibit crop germination and to delay fruit and vegetable maturation, ensuring safety and extending the shelf life (Pinela and Ferreira, 2017). Amongst the various radiation sources, three are permitted to be used on foods. These include gamma rays (c-rays) from cobalt 60 or caesium 137, electrons produced from machine sources (e-beam), and X-rays formed when high-energy electrons strike a metal plate (Yang *et al.*, 2013, Deng *et al.*, 2019). Food irradiation exposes the food to ionizing radiation, which through radiolysis creates free radicals from the water. Water molecules lose an electron, producing hydroxyl radicals (OH) and H_2O_2 that interfere with nucleic acid bonds and cause damage to DNA (Tirpanalan *et al.*, 2011). Fresh and minimally processed fruit and vegetables require low-doses of irradiation and a maximum one kGy is approved for a shelf-life extension by approval bodies like the FDA and the EFSA (Tirpanalan *et al.*, 2011). Furthermore, all irradiated items must be branded with the RADURA logo, the international symbol for irradiated food and a comment "treated by irradiation" or "treated with irradiation" as transparency to the consumers (Pinela and Ferreira, 2017).

The effectiveness of sterilization can be improved when irradiation is applied at higher doses. Log reductions of 1.76, 2.13 and 3.93 log CFU/g, respectively, were reported when the aerobic mesophilic species on paprika were treated with c-ray at doses of 1, 5 and 10 kGy, correspondingly (Molnár *et al.*, 2018). In another study, Joshi *et al.* (2018) made use of the e-beam treatment, which was able to decrease log reduction of *Salmonella* Poona by 4.96 log CFU/g on cucumber slices, and the fruit quality (colour, pH, and water activity) did not deteriorate during a three-day refrigerated storage duration.

2.5.2 Ultraviolet (UV) treatment

Ultraviolet (UV) radiation can be utilized as a non-thermal physical treatment to decontaminate fresh or minimally processed fresh produce. UV is electromagnetic radiation of wavelengths ranging from 100 to 400 nm. It is subdivided into four different groups, namely, UV-A, UV-B, UV-C and UV vacuum (Gray 2014). UV-A has the longest wavelength from 315 to 400 nm, UV-B ranges from 280 to 315 nm, UV-C also known as germicidal from 200 to 280 nm and UV vacuum from 100 to 200 nm (Deng *et al.*, 2019). Ultraviolet radiation induces depolarization of the membrane and asymmetrical ionic flow at the cell level, which results in the formation of pyrimidine dimers DNA strands. Cell transcription and replication is hindered because of the DNA mutations, consequently resulting in the death of cells (Tirpanalan *et al.*, 2011). The highest germicidal activity is from the UV-C, since it can be absorbed by DNA, primarily at approximately 254 nm where maximum absorption levels are reached (Sinha and Häder, 2002). The UV-C radiation also inflicts significant damage to the integrity of the cytoplasmic membrane and cellular enzyme activity (Pinela and Ferreira, 2017). The maximal absorption aspect of the UV-C makes it appropriate for surface disinfection and non-thermal pasteurization of foods (Tirpanalan *et al.*, 2011). UV light can be applied either as continuous mode, using UV lamps, or as pulsed UV light (Condon *et al.*, 2014, Gray, 2014). UV lamps have a gas tube (xenon or krypton), mercury, and each side of the tube also has an electrode. As electric current is passed, the atoms of mercury become excited and UV light is emitted when the atoms return to their base state (Meireles *et al.*, 2016).

A study on the efficacy of UV-C was conducted by Graça *et al.* (2017), on *E. coli*, *L. innocua* and *Salmonella* spp. A range of doses was applied to the pear fruit, which included 2.5, 5, 7.5 and 10 kJm^{-2} across the varying test pathogens. Log reduction of 3.4 CFU/g was recorded after a dose of 7.5 kJm^{-2} was applied against *E. coli* inoculated pear. The doses 7.5 and 10 kJm^{-2} managed to reduce *L. innocua* on pear by 2.9 and 3.3 CFU/g log values, respectively. Log reduction on exposure to 7.5 and 10 kJm^{-2} doses was a uniform 2.4 log CFU/g for *Salmonella* in both applications (Graça *et al.*, 2017). In a similar study, where pear was the food matrix used, a UV-C treatment was applied on *E. coli*. Maximal log reductions of 3.7 and 3.1 CFU/g were obtained after application with 0-7.56 kJm^{-2} UV-C on intact and shredded pear surfaces, respectively (Syamaladevi *et al.*, 2013).

Doses of 2.5, 5, 7.5 and 10 kJm^{-2} were applied on mango samples inoculated with different *E. coli* strains and *Cronobacter sakazakii*. The overall range in log reductions reported was between 1.49 – 1.96 CFU/g on the *E. coli* inoculated samples. In contrast, the samples inoculated with *C. sakazakii* experienced reductions in the range of 2.09 – 2.72 log CFU/g (Santo *et al.*, 2018). George *et al.* (2015) conducted an experiment on the efficacy of UV when it is applied to mango in reference to aerobic bacteria, yeasts and moulds. The study focused on evaluating the degree to which a distance of only 15 cm in 0 – 60 min duration would result in log reduction. Maximal log reductions of 1.13 CFU/g for yeast and moulds and 1.07 CFU/g for aerobic bacteria were recorded after the experimental test was done. Tawema *et al.* (2016) focused on the efficacy of two doses of UV-C treatments, 5 and 10 kJm^{-2}, on cauliflower against *E. coli*, *L. monocytogenes* and yeast and moulds. A log reduction of 0.7 CFU/g was reported for *E. coli* when the five kJm^{-2} dose was used. In terms of *L. monocytogenes*, yeasts and moulds, two doses (5 and 10 kJm^{-2}) were applied and gave an equal 1 log reduction for both concentrations.

2.5.3 Pulsed light

The pulsed light (PL) instantly inactivates the pathogenic microorganisms on the surface of food materials using intense pulses of short duration and a wide spectrum rich in UV-C light that induces structural changes in the pathogens and thus prevents cell replication (Garg *et al.*, 2016). PL operates with xenon lamps capable of producing powerful and short-term "white light" broad-spectrum pulses, from 200 nm ultraviolet wavelengths to 1000 nm infrared wavelengths, with peak emissions of 400-500 nm. The power is magnified several times by storing and discharging energy in a high-power condenser over relatively long periods (fractions of a second) over a short period (millionths or thousandths of a second) creating several high energy pulses per second (Pinela and Ferreira, 2017). Photothermal and photophysical effects which are partially correlated with temporary overheating and continuous disruption caused by high-energy pulses may induce the disintegration of microbial cells (Keklik *et al.*, 2012). An immediate shutdown of metabolic activity, inactivation of intracellular esterase or PL-induced membrane disruption was demonstrated as a further inactivation mechanism (Krishnamurthy *et al.*, 2010).

Due to its rapid delivery of more powerful energy, PL has advantages of high efficacy compared to the continuous irradiation method (Rowan, 2019). PL's antimicrobial potential is

4–6 fold greater than continuous UV light (Pataro *et al.*, 2015; Deng *et al.*, 2019). It may also minimize the adverse effects of nutritional and organoleptic qualities on food, as there is no significant rise in temperature during treatment (Garg *et al.*, 2016). Significant and immediate inactivation of microbials in short-term applications, lack of residual compounds and incredible versatility are also some of the advantages of this treatment. Furthermore, evidence shows that PL kills yeast through a multi-hit or mechanistic process that affects the permeability of the cell membrane along with the stability and functionality of DNA and macromolecule, depending on the applied dose (Rowan *et al.*, 2015).

Mukhopadhyay *et al.* (2019) focused on investigating the ability of PL to inactivate *E. coli* O157:H7 in spinach samples. The study was able to establish reductions ranging between 1.7 – 3.4 CFU/g after treatment was applied. PL configurations in this study were set at a dose of 1.27 Jcm^{-2}, at three pulses/ s in the duration of 1 – 30 s. Agüero *et al.* (2016) also did research work on spinach to determine the efficacy of PL on two pathogens, *L. innocua* and *E. coli*. The authors applied two pulses of 0.3 ms/ pulse, using a dose of 4 on the spinach samples. Log reduction was reported for *L. innocua* and *E. coli* as 1.85 and 1.72 CFU/g, respectively. Another study sought to evaluate the efficacy of a range of PL doses, 7.5 – 10 kJm^{-2}. The fresh produce under investigation were melon samples which were exposed to the bacteria *Cronobacter sakazakii*. Reductions were observed in the range of 1.8 – 2.4 log CFU/g (Santo *et al.*, 2016).

Pataro *et al.* (2015) investigated the physicochemical quality of tomatoes after exposing them to pulsed light treatment. Lycopene, total carotenoid, phenolic compounds, and antioxidant activity of PL-treated tomatoes increased to 6.2, 2.5, 1.3, and 1.5 times, respectively, relative to the untreated samples. In another study carried out by Huang and Chen (2014) which sought to show the impact of PL on the blueberry fruit. Impressive log reductions greater than 5.8 log

CFU/g of *E. coli* O157:H7 were observed on blueberry after treatment with a dose of 1.27 Jcm^{-2} PL.

2.6 Hurdle technology in fresh produce

Hurdle technology is an approach premised on the idea of combining several antimicrobial techniques that might not be entirely sufficient alone, but combined, can offer a robust method in mitigating risk and eliminating pathogens in food products (Leistner, 2000). A number of options, each different to the other, can make a hurdle method, such as, chemical, physical, physiochemical, and microbial hurdles. Hurdle technology presents a number of opportunities in the fresh produce industry in the improvement and enhancement of food preservation and safety. One such advantage is, the ability of combination methods to overpower the tendency of microbes building up resistance against conventional preservation techniques (Khan *et al.*, 2017). Hurdle technology targets different sites and mechanisms within the bacterial cells, through their synergism, rendering the microbes inactive when combined strategically. Hurdle technology avoids the severity of single hurdle use, in high intensity that is unsafe to the environment (Rahman, 2015). One of the reasons why hurdle technology gained interest was, health and environmental consciousness, which probed the use of lower concentrations of antimicrobials, therefore preventing excess chemical residues and undesirable side effects (Ganguly, 2013). Lower concentrations inversely influence other economic aspects in the food industry, like lowering production costs and saving energy. Use of hurdle technology also encourages the use of natural preservatives that would otherwise be ineffective in the singular form but is enhanced when combined with other technologies, be it physical, physicochemical, chemical and biological (Singh and Shalini, 2016).

One of the primary purposes of food preservation is increasing food products' shelf life, hence combined methods offer stability and safety of food products for a much longer time. Through intelligently setting up hurdles that target factors such as water activity, the activity of enzyme systems, pH etc., the food products are kept stable for as much as possible and can maintain stability even without refrigeration (Leistner, 2000). Another advantage for using hurdle technology, particularly in foods that are often preferred minimally processed, as in the case of fruits and vegetables, is that there is a minimal alteration of the fresh produce's nutritional components and organoleptic qualities (Singh and Shalini, 2016). Hurdle technology is also highly versatile across a wide range of food products, with more than 60 combinations and counting, making it suitable for application in modern and classic food processing. One could make combinations that are either very specific or ones that are broad, depending on the microbes that need reduction. Hurdle technology also allows for "scientific creativity", whereby food scientists can combine the principles and mode of action of their antimicrobials and develop novel combination of decontamination methods (Khan *et al.*, 2017). The concept of hurdle technology fits well with the growing consumer trends for minimally processed foods, as in the case of fruits and vegetables. A broad spectrum of choices, in the form of biological, physical and chemical factors, and their intensities can be individually adjusted to make hurdles that fit the purpose of use (Pinela and Ferreira, 2017).

2.6.1 Use of hurdle technology in fresh produce

The conventional methods of preserving fresh produce, fruits and vegetables, have mostly included the use of a single preservation step, applied at a high level which more often than not, causes noticeable organoleptic changes to the food products (Singh and Shalini, 2016). Due to the changes in consumer preference on the methods used for preservation of fresh produce (fruits and vegetables), in a ploy to decontaminate fresh produce in an environmentally friendly manner that also preserves organoleptic qualities, hurdle technology is being investigated. Intelligently combining antimicrobial methods in an industry were minimally processed fresh produce is preferred, has gained interest and research is now leaning towards finding combined alternatives.

Millan-Sango *et al.* (2015) studied the efficacy of combining ultrasound and essential oils as a decontamination method against *E. coli* in romaine lettuce. The two factors employed were ultrasound and oregano essential oil, used at different time intervals and concentrations, respectively. Concentrations of oregano EO (0.010% v/v, 0.014% v/v, 0.018% v/v, 0.022% v/v and 0.025% v/v) were then combined with continuous and pulsed mode ultrasound. Results showed that a combination of ultrasound and 0.025% v/v oregano essential oil, resulted in microbial reduction and inactivation of *E. coli* by 4.7 ± 0.15 logs in pulsed mode, while 0.025% v/v EO combined with continuous mode ultrasound, showed a reduction below the limit of detection. Singular treatments of ultrasound and oregano EO, was only able to reduce the level of bacteria by ≤2 logs. By drawing on the concept of hurdle technology, Millan-Sango *et al.* (2015) were able to show that there is a synergetic effect of the two antimicrobial agents, ultrasound and oregano EO, in the reduction and inactivation of *E. coli* on the surface of lettuce, as compared to their singular treatments. Furthermore, the structural and organoleptic quality parameters of lettuce showed no significant differences with control after combined treatment.

Ozcan and Demirel Zorba (2016) investigated the antimicrobial activities of EOs (cinnamon and lemon) and ultrasound, *in vitro* and food applications. A salad mixture of lettuce, parsley and dill was used and subjected to *L. monocytogenes* inoculation. Growth inhibition in EOs (cinnamon and lemon) was tested using the agar disk diffusion method and showed results ranging between 13-21 mm inhibition zone diameters. For the minimum inhibitory concentrations (MIC), cinnamon EO showed higher antimicrobial activity (2.00 ± 0.01) than lemon EO with the activity of 0.75 ± 0.29. Lemon EO could not be used for a combined test as just 1% resulted in unacceptable changes in organoleptic qualities. Therefore, combined treatment was done, with ultrasound and 2% cinnamon EO, and produced results of only 0.85 log CFU/g inhibition on day 1, gradually increasing and equalling the control by day 9. Conclusively, Ozcan and Demirel Zorba (2016), do not provide a promising alternative for fresh produce decontamination.

Petri *et al.* (2015) evaluated the use of combined disinfection methods against *E. coli* on fresh-cut vegetables, lettuce and carrots. Pressure was applied in the form of vacuum and positive pressure, and both were combined with peroxyacetic acid (PAA: 100 mg/L), chlorine water (CW), chlorine dioxide (ClO_2: 2 mg/L) and tap water (W). Highest reduction of *E. coli* was observed in the wash water, better than in lettuce and carrots. The combinations only managed a microbial reduction of between 1-3 log units in lettuce and carrots. In terms of the physical quality of the carrots, no significant changes were detected in any of the treatments conducted. Lettuce, however, showed an increase in porosity, translucency and damage to plant tissue. After combining the average antimicrobial activity with the detrimental effects on the organoleptic parameters, it can be concluded that this decontamination strategy did not produce a promising prospect as a decontamination strategy for fresh produce (Petri *et al.*, 2015).

Cattelan *et al.* (2018) evaluated the combined effects of Oregano EOs and salt concentrations against *E. coli* in salad dressing using a mathematical model. Results obtained showed the interaction between oregano EO and salt, and their combined effect in *E. coli* reduction. The highest reduction in the *E. coli* count (≤1 log) was observed when oregano EO concentration range 0.4 to 0.5% was combined with a salt concentration range of 1.1 to 1.2%. The same reduction of (≤1 log) was also recorded when the oregano EO concentration range of 0.1 to 0.2% was combined with a salt concentration range of 1.3 to 1.4%. The combinations of oregano EO and salt exerted an antibacterial effect on *E. coli* when they were used in concentrations that were inversely proportional and can thus be used as a promising decontamination technology.

In a study done by São José and Vanetti (2015) on the effectiveness of combined ultrasound with chemical sanitizers for the decontamination of watercress, parsley and strawberries, assessment on the physicochemical parameters and natural combined microbiota were done. Ultrasound treatment, in combination with chlorinated water, hydrogen peroxide, peracetic acid and chlorine dioxide, contributed to a more significant reduction in microbial contaminants compared with treatments in which only sanitizer solutions were used. The highest reduction of 4.1 log CFU/g in aerobic mesophiles and 4.0 log CFU/g in yeast and mould population was observed after the combined treatment of ultrasound and 40 mg/L peracetic acid, was applied. Although there was a greater reduction in combined treatments, some of the chemical sanitizers used in the study led to changes in colour and firmness, for example, sanitization treatments used on watercress such as hydrogen peroxide, chlorine dioxide, ultrasound in association with sodium dichloroisocyanurate, peracetic acid and chlorine dioxide caused it to be blackened.

Huang *et al.* (2018) investigated the decontamination efficacy and cross-contamination of assisted ultrasound, a combination of ultrasound with other sanitizers/surfactants. Ultrasound was combined with 0.1% Tween-20 and 0.1% SDS against *E. coli*, *L. innocua* and *P. fluorescens* in lettuce. The results showed both the combined methods and ultrasound alone, to have no significant differences in log reduction, both showing bacterial reduction between ≥ 2 log and < 3 log in *E. coli* and *L. innocua*. As for *P. fluorescens*, however, bacteria adhered more strongly to the lettuce leaves and ultrasound alone could only reduce bacterial load up to 1 log. The combined treatment managed to increase log reduction up to 2 logs for *P.*

fluorescens, indicating an increased efficacy against *P. fluorescens* by approximately 1 log. It was concluded that combined treatment/ assisted ultrasound enhanced removal of bacteria significantly only in particular strains, and not significantly in others. The combined treatment had little effect on the colour changes on the lettuce. It was also evaluated that the quality attributes tested, did not show significant changes on lettuce after treatments.

Huang and Chen (2019) studied the combined use of pulsed light (PL) exposure and H_2O_2 against *Salmonella* on grape tomatoes. The study aimed to evaluate the disinfecting efficacy of PL-H_2O_2 on the grape tomatoes and inactivation of residual bacteria in the wash water. Results of the sanitation of grape tomatoes by PL-H_2O_2 treatment, in the pilot-scale, showed log reduction of > 5.69 log CFU/g (spot inoculation) and 3.05 log CFU/g (dip inoculation). While the results of residual *Salmonella* in wash water were < 2 log CFU/ml for both, using spot inoculation. In a more industrialized scale, the reduction results on grape tomatoes were reported as follows; spot inoculation- 300g (> 4.77 log CFU/g), 1000g (> 4.99 log CFU/g), 2000g (5.43 log CFU/g) and dip inoculation- 300g (2.90 log CFU/g), 1000g (2.92 log CFU/g), 2000g (> 2.80 log CFU/g). The results for residual *Salmonella* in wash water were as follows; spot inoculation- 300g (< 2.67 log CFU/ml), 1000g (< 5.33 log CFU/ml), 2000g (< 2 log CFU/ml). PL-H_2O_2 in this study proved to be a powerful sanitizer in both the grape tomato and wash water. This combined method is environmentally friendly and since pulsed light warms the water slightly, they are fewer air pockets inside the tomato fruit, thereby decreasing absorption of moisture that may cause spoilage and contamination.

Fröhling *et al.* (2018) studied the effectiveness of plasma-treated water (PTW) in decontaminating microbes on lettuce. Activating water through the cold plasma method in order to inactivate microorganisms, has gained interest in the fresh produce industry. The highest reduction of total viable count (TVC) in this study were observed at 1.8 log CFU/g, after application of the plasma-treated water. Experiments ran for seven days, and they were no significant differences in log reduction between the control and the treated product. The microbial log reduction results that were low could point to the broad microflora that could foster and encourage growth and resistance between themselves. Even though microbial profiling was conducted using MALDI-ToF MS, there was a substantial microbial diversity and high variations were revealed in the microbial community, some of which could not be identified. Quality parameters remained more or less the same after treatment and no significant changes were reported.

A study done by Cao *et al.* (2017) has focused on the evaluation of PL treatments on the inactivation of *Salmonella* on blueberries and its impact on shelf life and quality attributes. Separate experiments were conducted between water-assisted PL and dry PL treatments to evaluate the efficacy of the two methods. The highest microbial reduction in the study was obtained from the combined technology of water-assisted PL treatment, recording *Salmonella* reduction levels of 4.4 log and 0.9 for spot inoculation and dip inoculation, respectively. Agitation of water in water-assisted PL allows for physical washing of the *Salmonella* cells, into the water, which has a greater chance of inactivation in water. Dry PL alone recorded a microbial reduction of 0.9 log and 0.6 log for spot and dip inoculation, respectively. In terms of other quality attributes, that is, firmness, colour, weight loss and health benefit-compounds (antioxidants, anthocyanins, phenolics), water-assisted PL treatment presented minimal to no significant effects to the blueberries. However, in terms of shelf life, since blueberries have a short shelf life when adequately ripe, none of the PL treatments were able to improve the longevity of blueberries in storage even after a relative inactivation of some yeasts and moulds.

2.7 Antimicrobial techniques to be employed

2.7.1 Ultrasound

Ultrasound has gained interest as a decontamination method due to its ultrasonic waves that can rupture bacterial cells, denature enzymes and ultimately modify the metabolism of cells, disrupting their cellular and functional components (Mukhopadhyay and Ukuku, 2018). Furthermore, ultrasound is an environmentally friendly decontamination technique with low energy consumption, a relatively high degree of safety, and also preserves the organoleptic characteristics of fresh produce (Chemat *et al.*, 2011). The ability of ultrasound to inactivate bacteria is attributed to two phenomena: cavitation and sonolysis. Cavitation starts with the generation of bubbles that grow and enlarge their size until such a point as they can no longer withstand the growing pressure (Sango *et al.*, 2014). The bubbles then implode, causing the liquid molecules to collide, generating a high temperature (approximately 5000ºC) and pressure (approximately 50 MPa) (Mukhopadhyay and Ramaswamy, 2012). The high temperatures and pressures that would have been created from the bubbles generate hydrogen atoms, hydroxyl radicals and thereby result in the phenomenon known as sonolysis (Chemat *et al.*, 2011, Millan-Sango *et al.*, 2016). Therefore, apart from the physical impact which is responsible for the death of microbial cells, the initiating reactive species also leads to the inactivation of microbes and the energy released helps to enter food surfaces that are otherwise problematic to access using other methods of sanitization (Pinela and Ferreira, 2017).

Applications of ultrasound technology are relevant for a various of processes in food production. Among these processes, it has shown potential as an antimicrobial and enzymatic control agent and has been seen to be applicable in solid, liquid and gas systems in the food industry (Mukhopadhyay and Ukuku, 2018). Studies have reported that ultrasound treatment requires very high intensities to inactivate bacteria, which in turn has an adverse effect on the organoleptic and physical characteristics of fresh produce. Therefore it has been reported that ultrasound alone is not effective as a sole decontamination technology and therefore suggestions of assisting ultrasound with other antimicrobials as a prospective hurdle kind of technology are being tested (Millan-Sango *et al.*, 2017). A study by Alenyorege *et al.* (2020) assessed the microbial quality and physicochemical parameters of fresh-cut Chinese cabbage samples exposed to dual-frequency and triple-frequency ultrasound washing. Total bacteria counts were reduced to 3.1 logs, while yeast and moulds were significantly decreased by 2.8 logs. Kilicli *et al.* (2019) focused on improving the microbial quality of lettuce by combining ultrasound and low-intensity electrical current. The maximum inactivation rates were determined with log reductions of 2.89, > 3, and 2.98, reported for mesophilic bacteria, coliforms and yeasts and moulds, respectively. Another study that focused on assisted ultrasound, where ultrasound treatment was combined with surfactants and sanitizers to reduce the inoculated lettuce samples of *E. coli*, *L. innocua*, and *P. fluorescens* by 2,61, 2,23, and 1,10 log CFU/cm^2, respectively (Huang *et al.*, 2018).

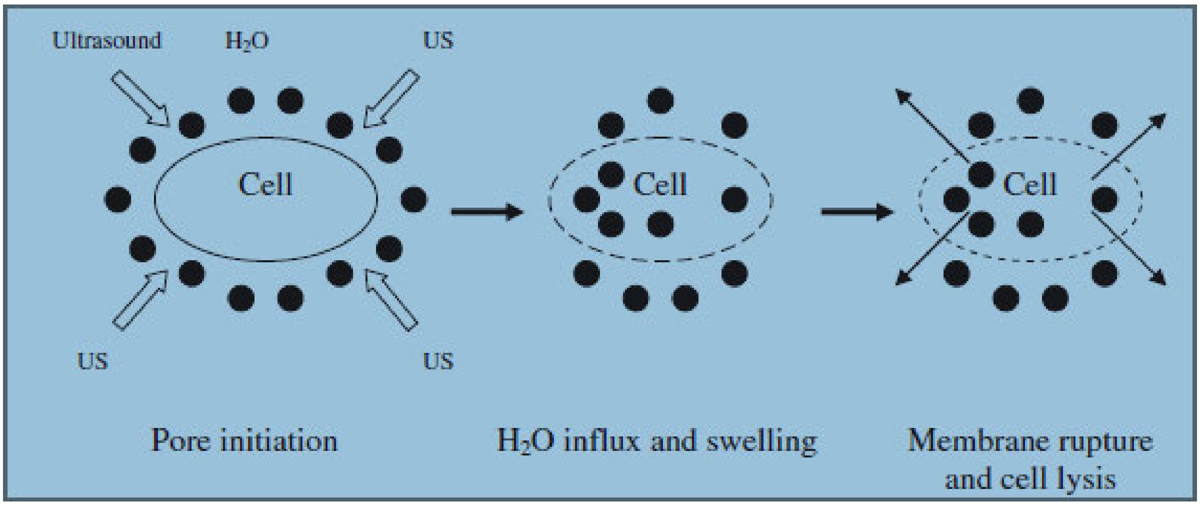

Figure 2. 5. Mechanism of ultrasound-induced cell damage (Chemat *et al.,* 2011)

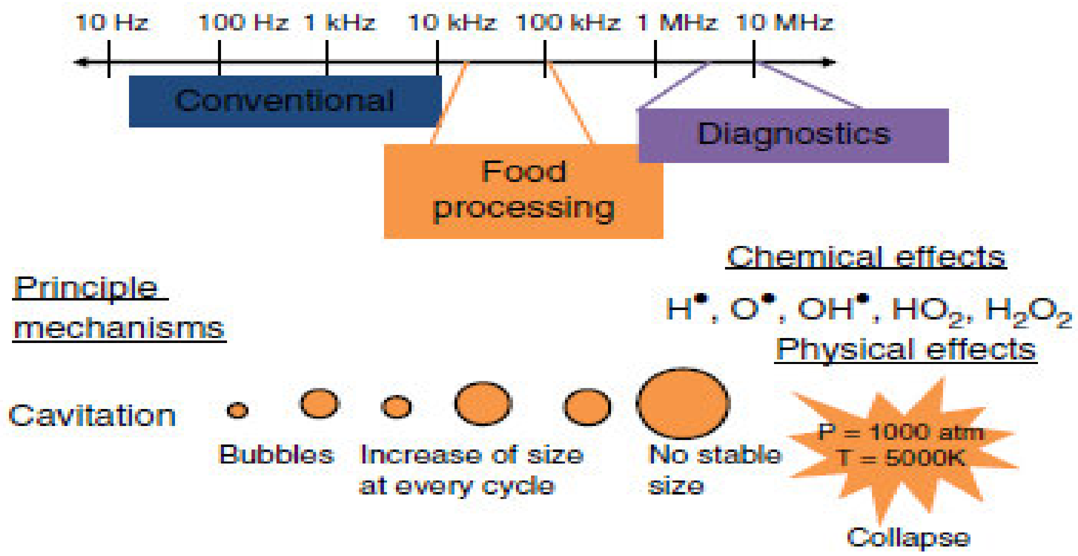

Figure 2. 6. Ultrasound antimicrobial mechanism of action (Sango *et al*., 2014)

2.7.2 Nisin

Nisin is a bacteriocin that is derived naturally from *Lactococcus lactis* subsp. lactis and has been adopted since the 1950s as a food preservative with GRAS status (Khan and Oh, 2016). Nisin is an antimicrobial peptide that is composed of 34 amino acids with 13 of them involved in post-translational modifications (Hasper *et al.*, 2004). Nisin expresses antimicrobial actions through binding to the precursor of peptidoglycan and lipid II thereby inhibiting cell wall biosynthesis, forming pores within the cell membrane, that result in the release of essential ions and, eventually cell death or inhibition of outgrowing germinated bacterial spores (de Oliveira Junior *et al.*, 2015; Khan and Oh, 2016).

Nisin has different limits across the globe, for instance, France, Britain and Australia have no maximum limit, while the United States permits not more than 10000 IU/g. Russia has a limit of 8000 IU/g, while Argentina, Italy and Mexico have a limit of 500 IU/g for their processed foods (Gharsalloui *et al.*, 2016). Of note, however, is the fact that nisin is more effective in

Gram-positive bacteria as Gram-negative bacteria pose resistance through blocking access to the cytoplasmic membrane. To a similar effect, dormant spores seem to protect themselves from nisin action (Modugno *et al.*, 2018). Therefore due to this relatively narrow antibacterial spectrum, suggestions of combining nisin with other technologies are being investigated to effectively inactivate a broad spectrum of bacteria (Liao *et al.*, 2018).

Ukuku *et al.* (2019) evaluated the efficacy of the single and combined effects of nisin along with cold plasma treatment on apples. The study was targeted at eliminating the pathogen *L. monocytogenes* obtained from accessible sources of general consumers, in this case, the farm and the market. The application of the combined nisin and cold plasma at different nisin treatment times 3 min and 1 h, managed to reduce *L. monocytogenes* on apples by 2.5 and 4.6 log CFU/g, respectively. Another study investigating the efficacy of nisin on the reduction of *L. monocytogenes* on fresh lettuce was conducted over five days. The nisin applied onto lettuce was able to retard *L. monocytogenes* growth successfully and inhibit it by more than 90% (McManamon *et al.*, 2019).

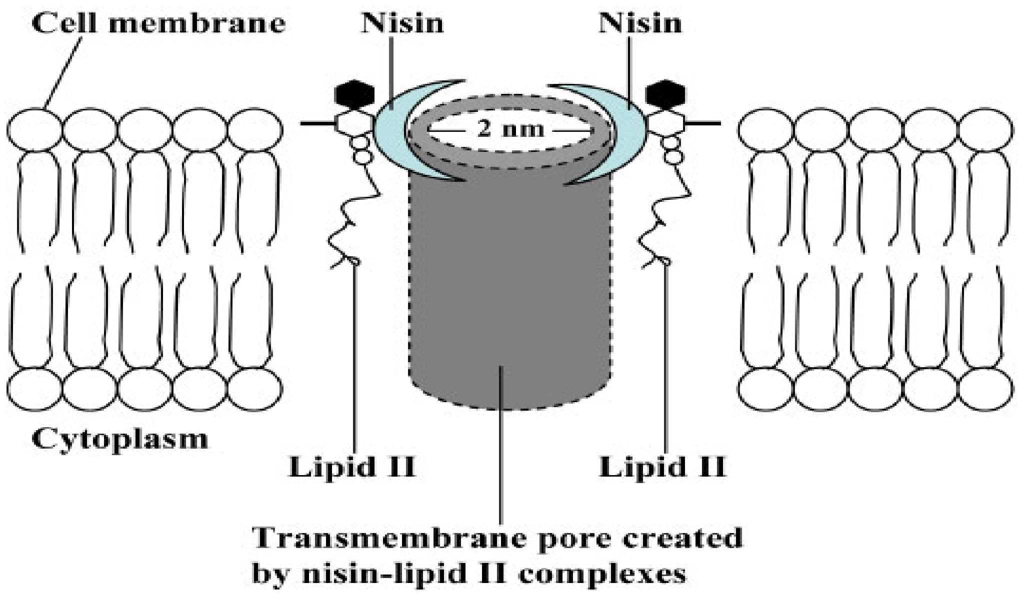

Figure 2. 7. Nisin mechanism of action and pore formation (Punyauppa-path *et al.*, 2015)

2.7.3 Oregano essential oil

Studies have reported on oregano essential oil as one of the most effective natural antimicrobial and antioxidant agents. Two phenolic compounds, namely carvacrol and thymol, constitute about 75-85% of oregano essential oil and are reported to be the primary sources of the antimicrobial and functional properties (Rodriguez-Garcia *et al.*, 2016). Antimicrobial activity of the two phenolic compounds is linked to the lipophilic character that causes an attraction between them and the cell membrane structures. Carvacrol, a hydrophobic phenolic compound, is widely identified as a critical component of oregano oil with well-documented inhibitory activity against bacteria, fungi and yeasts (Rubilar *et al.*, 2013). Storage of carvacrol in the cell membrane and its ability to expel protons and bond with hydrogen can lead to the conformation of the membrane, ultimately leading to cell death. While categorized as GRAS by the USFDA, oregano essential oil has also shown antioxidant activity and shows potential in improving the shelf-life and safety of fresh produce (Cattelan *et al.*, 2018).

The application of oregano essential oil follows a series of events, namely, membrane expansion, increase in fluidity and permeability, disturbing embedded proteins, altering ion transportation process and inhibiting respiration (Lorenzo *et al.*, 2014). Oregano essential oil has demonstrated antimicrobial activity against a broad spectrum of bacterial species. In Gram-negative bacteria, thymol and carvacrol facilitate the disintegration of the outer membrane, triggering a release of lipopolysaccharide components, thereby increasing the permeability of adenosine triphosphate in the cytoplasmic membrane and thus changing the cell's passive permeability (Guarda *et al.*, 2011). Gram-positive bacteria have been reported to show greater sensitivity to the application of oregano essential oil due to the propensity of hydrophobic phenolic compounds to the cell membrane (Rodriguez-Garcia *et al.*, 2016). Oregano has also been investigated in terms of antimicrobial synergy when it is combined with other technologies and the results have been promising.

A study was conducted by Dávila-Rodríguez *et al.* (2019) to assess the inhibitory activity of 3 different essential oils, namely oregano, rosemary and cinnamon, in the form of nano-emulsions. Oregano essential oil proved the most potent of the nano-emulsions after exposure to the bacteria under evaluation. A log reduction of 5 CFU/g for both *E. coli* and *L. monocytogenes* was observed on inoculated celery within a period <60 min. Bhargava *et al.* (2015) investigated the efficacy comparison between two oregano oil nano-emulsion concentrations (0.05 and 0.1%) on inoculated lettuce. Log reduction at 0.05% nano-emulsion concentration was recorded at 3.44, 2.31, and 3.05 log CFU/g in *L. monocytogenes*, S. *typhimurium* and *E. coli* O157: H7, respectively. The 0.1 % treatment was reduced to 3.57, 3.26, and 3.35 log CFU/g reductions on the same bacteria, respectively.

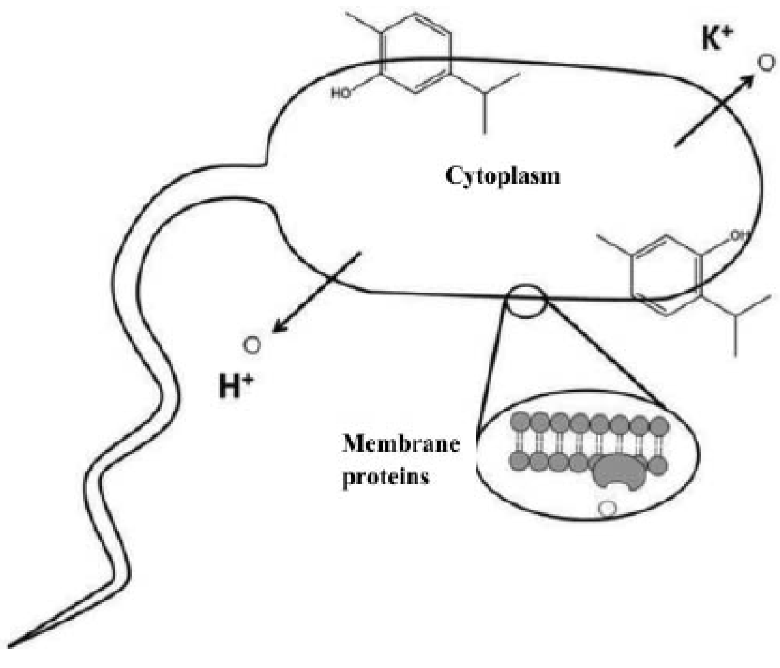

Figure 2. 8. The antimicrobial mechanism of carvacrol and thymol and changing of the bacterial cell's permeability, through disintegration of the outer membrane and consequent release of cytoplasmic constituents (Rodriguez-Garcia *et al.*, 2016)

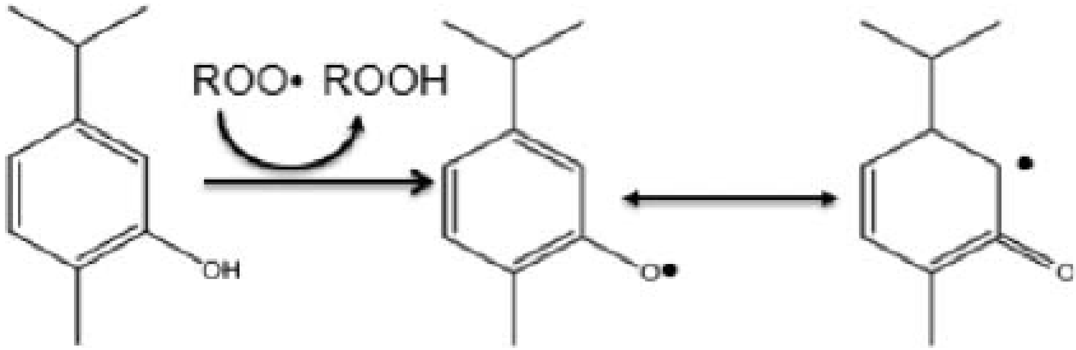

Figure 2. 9. Carvacrol's free radical scavenging mechanism (Rodriguez-Garcia *et al*., 2016)

CHAPTER THREE

3 Antimicrobial efficacy of nisin, oregano and ultrasound against *Escherichia coli* O157:H7 and *Listeria monocytogenes* on lettuce

Abstract

Ready-to-eat vegetables such as lettuce are prone to bacterial contamination, predominantly from *Escherichia coli* O157:H7 and *Listeria monocytogenes*, the leading causes of foodborne illnesses globally. Chlorine is widely used as a decontaminant in the fresh produce industry; however, potential carcinogenic risks limit its usage. As an alternative, green methods and hurdle technology are gaining interest. In this chapter, the efficacy of a combination of nisin, oregano and ultrasound on the reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce was studied using response surface methodology/Box-Behnken model design and was found to be reliable ($p<0.05$). A combination of 771.2 IU/g nisin, 0.185% v/v oregano and 14.65 min ultrasound was the most effective treatment on both pathogens, showing log reductions of 3.43 and 9.20 CFU/mL for *E. coli* O157:H7 and *L. monocytogenes*, respectively. The treated lettuce samples did not show any significant differences in textural properties; however, mild colour changes and a slight increase in the electrolyte leakage rate was observed, within permissible limits. Interestingly this is the first report on the combination of nisin, oregano and ultrasound, as a promising alternative to chemical treatments for the reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce, without compromising its appearance and quality.

Keywords: Foodborne pathogen; *Escherichia coli* O157:H7; *Listeria monocytogenes*; Efficacy; Hurdle technology

3.1 Introduction

There has been a shift in dietary trends globally in the last two decades, as consumers are progressively concerned about health and nutrition, leading to increased consumption of fresh produce (Colapinto *et al*., 2018). Consequently, a relative increase in foodborne illnesses linked to fresh produce consumption has been noticed. Studies have shown the presence of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in fresh produce and a marked increase in the prevalence of foodborne outbreaks and illnesses have been linked to these organisms (Ozcan and Demirel Zorba, 2016). The National Institute for Communicable Diseases (NICD, 2018) reported a severe outbreak of *L. monocytogenes* in South Africa (1056 cases, 214 deaths), while the World Health Organization (WHO, 2018) recorded an outbreak of *Salmonella typhimurium* in the United States (265 cases, 1 death). In the United Kingdom in 2016, an outbreak of *E. coli* (161 cases, 2 deaths) was reported by the Centre for Disease Control and Prevention (CDC, 2018). This highlights the severity of fresh produce associated foodborne illnesses and the dire need for decontamination methods that can effectively control the prevalence of foodborne pathogens and in turn eliminate such outbreaks (Wadamori *et al*., 2017).

Various decontamination methods are used for fresh produce, the most common being, thermal and chlorination techniques (Goodburn and Wallace, 2013; Petri *et al*., 2015). However, both these techniques have associated problems such as deterioration in organoleptic qualities and potential carcinogenic risks, respectively. Therefore, the current impetus is on using non-thermal/non-chemical methods to decontaminate and preserve the properties of fresh produce (Millan-Sango *et al*., 2017). Combined methods, also known as hurdle technology, have gained interest and studies have shown its success in controlling microbial growth while maintaining sensory quality and nutritional properties of ready-to-eat vegetables (Ozcan and Demirel Zorba, 2016). Non-thermal methods such as the use of nisin, oregano oil and ultrasound have shown potential as antimicrobials, when applied separately (Bhargava *et al*., 2015; Li *et al*., 2016). Furthermore, a combination of ultrasound and oregano essential oil against *Salmonella* on lettuce showed a log reduction of 3.08 (Millan-Sango *et al*., 2016), while the combination of nisin at 500 IU/g and oregano at 0.6% v/v showed strong antimicrobial activity against *S. enteritidis* (Govaris *et al*., 2010). However, the combined effect of nisin, oregano oil and ultrasound have not been evaluated previously and could provide better results synergistically. Therefore, the present study has focused on the impact of combined treatments of nisin, oregano and ultrasound on the microbial decontamination and physical properties of lettuce using response surface methodology (RSM), as an optimization tool.

3.2 Materials and methods

3.2.1 Strains and preparation of inocula

E. coli O157:H7 ATCC 43888 and *L. monocytogenes* ATCC 7644 were procured from Merck, South Africa. *E. coli* O157:H7 and *L. monocytogenes* were revived in tryptic soy broth (TSB) and brain heart infusion broth (BHI), respectively. Both the cultures were incubated (Incotherm Labotec, South Africa) at 37ºC for 18 h and cells were harvested by centrifugation (Eppendorf 5810R, Germany) (Sagong *et al*., 2011). The resultant pellets were suspended in phosphate-buffered saline (PBS) to get a final bacterial count of 10^9 CFU/mL using the McFarland standard as reference (Oladunjoye *et al*., 2017).

3.2.2 Preparation of lettuce

Fresh lettuce was procured from a local supermarket in Berea, Durban; the outer leaves were discarded and the inner leaves were washed with distilled water to eliminate any debris or dirt on the produce. Excess water was blotted out and the lettuce samples were cut into 4×4 cm pieces and disinfected in 70% ethanol for 5 min and left to dry in the Vivid Air-2004 laminar flow cabinet (Zhou, 2011).

3.2.3 Inoculation of lettuce

Lettuce samples were inoculated on both sides separately with *E. coli* O157:H7 or *L. monocytogenes*, through spot inoculation (10^9 CFU/mL) using a modified method by Sagong *et al*. (2013). Lettuce samples were placed separately on sterile aluminium foil and the bacterial suspension was applied onto the leaf surface. The inoculated samples were left to dry for 1 h in a laminar flow cabinet (Vivid Air-2004, South Africa) for proper bacterial attachment.

3.2.4 Experimental design: response surface method

The effective concentrations of nisin, oregano and the duration of ultrasound treatment against *E. coli* O157:H7 and *L. monocytogenes* on lettuce were optimized by response surface methodology (RSM) using Box Behnken model. The independent variables were nisin, oregano and ultrasound, and the levels of these three factors were determined based on literature (Govaris *et al*., 2010; Millan-Sango *et al*., 2015) and the preliminary studies carried out in our lab. The three variables, nisin, oregano and ultrasound, were designated as A, B and C, respectively (Table 3.1) and the response was measured as log reduction of inoculated bacteria. Table 3.2 outlines the experimental design with the coded levels generated by Design-Expert® version 11 software (StatEase Inc, Minneapolis, USA).

For predicting the optimal point, a second-order polynomial model was fitted to correlate the relationship between the independent variables and the response (log reduction). The equation for the three factors is stated as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_1{}^2 A^2 + \beta_2{}^2 B^2 + \beta_3{}^2 C^2 \quad (1)$$

where Y is the predicted response; β_0 is model constant; A, B, and C are independent variables; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are interactive coefficients; and β_1^2, β_2^2, and β_3^2 are the quadratic coefficients. Response surface graphs were obtained using multiple regression analysis, which illustrates the effect of variables individually as well as in combination.

Table 3. 1: Experimental range and levels of the independent variables

Factors		**Coded Levels**		
		-1	**0**	**+1**
Nisin (IU/g)	A	400	600	800
Oregano (% v/v)	B	0.05	0.125	0.2
Ultrasound (min)	C	5	10	15

Ultrasound specifications (Frequency- 50 kHz, Power- 600 W)

3.2.5 Combined antimicrobial treatments

Ultrasonication was carried out in an ultrasound water bath (Scientech- 705, South Africa) set at a uniform frequency of 50 kHz, power of 600 W and time variations of 5, 10 and 15 minutes. The temperature was kept constant at 25ºC during each treatment. All washing steps were carried out in 50 mL centrifuge tubes containing 2 g lettuce sample, immersed in a mixture of nisin and oregano solution (20 ml), and microbial analysis was performed immediately (Alenyorege *et al.*, 2019).

3.2.6 Microbial analysis

Untreated and treated samples (2 g) were transferred to stomacher bags containing PBS (30 mL) and macerated for 10 minutes in a stomacher blender 400 (Seward, UK). Serial dilutions of macerated lettuce samples were made and inoculated on plate count agar (PCA) plates and incubated at 37ºC. The *E. coli* O157:H7 and *L. monocytogenes* plates were incubated for 24 h and 48 h, respectively (Millan-Sango *et al*., 2016).

3.2.7 Colour analysis

The colour of the treated and untreated lettuce samples was quantified according to the method of Chang *et al*. (2017), using Color flex EZ 0840 (Hunter colourimeter; USA). To determine the colour, coordinates of L*, a* and b* values that indicate colour lightness, redness, and yellowness of the sample, respectively, were tested. The equation, $\Delta E = [(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2]$ and $C = (a^{*2} + b)^{1/2}$ was used to determine the colour differences. The discolouration was measured from different areas on the samples through random selection and all experiments were conducted in triplicate.

3.2.8 Texture analysis

Textural changes of treated and untreated lettuce samples were evaluated with a Shimadzu EZ Texture Analyzer EZ-LX/E2-SX series (Kyoto, Japan) with a blade set probe. Measurement conditions were pre-test speed: 5 mm/s, test speed: 2 mm/s, and post-test speed: 10 mm/s. A sample was placed on the platform and the blade was forced through the leaf. The blade probe was set at a height of 70 mm from the bottom of the platform plunger and moved downward with a speed of 2.0 mm/s. The maximum force was recorded using the Texture Expert software (Texture Technology Corp, Massachusetts, USA.) and the hardness value was calculated from force vs. time curves according to Chang *et al.* (2017).

3.2.9 Electrolyte leakage

The electrolyte leakage rate (ELR) was measured according to the protocol of Millan-Sango *et al.* (2016). Treated and untreated lettuce samples (4 x 4 cm) were placed in a Duran bottle containing 200 mL distilled water and shaken gently. The electrical conductivity of the solution was measured at 1 min (C1) and 60 min (C60) of incubation using a conductivity meter (MetrohHM 644 conductometer, Switzerland). The samples were then autoclaved at 121ºC for 15 min, and the total conductivity (CT) of the solution was measured after cooling. ELR was calculated using the following equation:

$$\mathrm{ELR} = \frac{(\mathrm{C60} \times \mathrm{C1})}{\mathrm{CT}} \times 100$$

3.2.10 Statistical analysis

All the experiments were performed in triplicate. Analysis of variance (ANOVA) was used to analyze the data and Fischer's Least Significant Differences Test was used to compare means ($p < 0.05$).

3.3 Results and discussion

3.3.1 Effectiveness of combined treatments on the reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce

This study was performed to compare the effectiveness of different combinations of nisin, oregano and ultrasound in reducing the numbers of *E. coli* O157:H7 and *L. monocytogenes* on fresh lettuce. Different combinations of treatments showed an increasing trend in log reduction where the lower and middle concentrations of the tested factors could not significantly reduce the numbers of *E. coli* O157:H7 and *L. monocytogenes* (Table 3.2).

A combined treatment of 600 IU/g nisin, 0.2% v/v oregano and 15 min ultrasound provided the highest log reduction of 3.15 CFU/mL against *E. coli* O157:H7 followed by the combination of 800 IU/g nisin, 0.2% v/v oregano and 10 min ultrasound which showed 3.03 CFU/mL log reduction. In both the combinations that showed the highest log reductions, oregano concentration was at the higher end (0.2% v/v), while nisin concentration and ultrasound treatment were varying from middle to higher range. The synergism from the combined treatments could be attributed to two main phenomena, the acidic pH of the antimicrobial solutions and the droplet sizes generated during ultrasonication (Millan-Sango *et al*., 2015). The deduction suggests, if the essential oil is undissociated, it binds better to the hydrophobic regions of the bacterial cell membrane proteins and lipid membranes, thereby making dissolution much easier to occur. This acidification is facilitated by both nisin and oregano essential oil, which have a lower pH when combined, thereby resulting in a better synergistic action against *E. coli* O157:H7 (Millan-Sango *et al*., 2016). The inhibitory properties of ultrasound could be associated with its ability to generate the droplet sizes of the antimicrobial solution with some reports suggesting that smaller particles could enhance the biological activity of antimicrobials (Salvia-Trujillo *et al*., 2015). Ultrasound through cavitation and sonolysis also provides a synergistic effect when combined with nisin and oregano by reducing the number of *E. coli* O157:H7 on lettuce. Similarly, oregano oil has been well-documented for its antimicrobial and antioxidant activity against a broad spectrum of bacteria (Hernández González *et al*., 2017). This is primarily due to its phenolic hydrophobic compounds, carvacrol and thymol (Guarda *et al*., 2011). Millan-Sango *et al*. (2015) achieved a 2.65 log reduction of *E. coli* O157:H7 when a combination of ultrasound and oregano was used on lettuce, which corroborates the findings of our study. Irrespective of nisin's inefficiency to act against Gram-negative bacteria; in this instance, nisin essentially contributed to lowering the pH thereby making the combination treatment more robust.

For *L. monocytogenes*, the highest log reduction of 8.59 CFU/mL was achieved with the combination treatment of 800 IU/g nisin, 0.125% v/v oregano and 15 min ultrasound (Table 3.2). The second highest log reduction of 8.2 CFU/mL was observed at the combination treatment of 800 IU/g nisin, 0.2% v/v oregano and 10 min ultrasound. In both these instances with high log reduction, the combinations had nisin concentration at the higher end (800 IU/g) with varying oregano concentration and ultrasound treatment from middle to higher range. Such high log reduction values observed, represent the strong synergistic activity of the combined antimicrobial treatments. Since both nisin and oregano target the bacterial cytoplasmic membrane, an enhanced antilisterial activity was to be expected, corroborating the additive antimicrobial activity against Gram-positive bacteria by Govaris *et al*. (2010). Ultrasound treatment also improved the activity of the antimicrobials through an increased cell porosity and a positive influence on particle size which has been supported by other studies as well (São José *et al*., 2014, Huang *et al*., 2018). Therefore, a combination of nisin, oregano and ultrasound treatments work synergistically and could offer a more robust antimicrobial method.

Table 3. 2: Experimental design used for RSM with three independent variables and showing the observed log reduction of *E. coli* O157:H7and *L. monocytogenes* on lettuce. The actual values are the average of triplicate determinations.

	A	B	C	*E. coli* O157:H7		*L. monocytogenes*	
Run	Nisin IU/g	Oregano % v/v	Ultrasound Min	Actual Log reduction CFU/mL	Predicted Log reduction CFU/mL	Actual Log reduction CFU/mL	Predicted Log reduction CFU/mL
1	0	-1	-1	1.68	1.59	3.83	3.82
2	-1	+1	0	2.85	2.86	3.93	4.19
3	0	0	0	2.61	2.77	3.95	3.89
4	+1	0	-1	1.88	1.98	5.84	6.11
5	-1	-1	0	1.8	1.87	2.61	2.90
6	+1	-1	0	1.67	1.66	7.95	7.69
7	0	0	0	2.85	2.77	3.9	3.89
8	0	+1	+1	3.15	3.24	6.29	6.30
9	0	0	0	2.73	2.77	4.2	3.89
10	-1	0	+1	2.64	2.54	3.84	3.57
11	+1	+1	0	3.03	2.96	8.2	7.91
12	-1	0	-1	2.44	2.46	3.18	2.90
13	0	0	0	2.83	2.77	3.46	3.89
14	0	+1	-1	2.21	2.19	4.54	4.56
15	0	-1	+1	1.53	1.55	5.54	5.52
16	0	0	0	2.85	2.77	3.95	3.89
17	+1	0	+1	2.93	2.91	8.59	8.87

From Table 3.2, it can be deduced that with the increasing levels of nisin concentration, oregano concentration and ultrasound treatment time, a linear increase in the log reduction of *E. coli* O157:H7 and *L. monocytogenes* was observed. The variances of the model for the reduction of

E. coli O157:H7 and *L. monocytogenes* on lettuce were also studied (Table 3.3). The quadratic regression model p-values for *E. coli* O157:H7 and *L. monocytogenes* were both <0.0001, indicating that the models were significant ($p < 0.05$) and adequate to describe the relationship between the factors under investigation along with their responses. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. There was a high regression coefficient (R^2) of 0.98 for both *E. coli* O157:H7 and *L. monocytogenes*, which indicated that the model had a high probability of representing the entire sample. For *E. coli* O157:H7, the predicted R^2 of 0.82 is in reasonable agreement with the adjusted R^2 of 0.96; i.e. the difference is less than 0.2. Similarly, for *L. monocytogenes,* the predicted R^2 of 0.81 is in reasonable agreement with the adjusted R^2 of 0.96. Adequate precision measures the signal to noise ratio, and a ratio greater than 4 is desirable. The ratios of 19.25 and 21.78 for *E. coli* O157:H7 and *L. monocytogenes* respectively, indicate adequate signals, thereby making this model sufficient in navigating the design space. Thus, it becomes relatively easy to predict the log reduction in a population of *E. coli* O157:H7 and *L. monocytogenes*.

Table 3. 3: Analysis of variance for the reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce.

	***E. coli* O157:H7**					***L. monocytogenes***				
Source	Sum of Squares	df	Mean Square	F-value	p-value	Sum of Squares	Df	Mean Square	F-value	p-value
Model	4.58	9	0.5092	39.07	< 0.0001	53.03	9	5.89	46.08	< 0.0001
A-Nisin	0.0061	1	0.0061	0.4643	0.5175	36.21	1	36.21	283.17	< 0.0001
B-Oregano	2.6	1	2.6	199.46	< 0.0001	1.15	1	1.15	8.97	0.0201
C-Ultrasound	0.5202	1	0.5202	39.92	0.0004	5.9	1	5.9	46.14	0.0003
AB	0.024	1	0.024	1.84	0.2167	0.2862	1	0.2862	2.24	0.1783
AC	0.1806	1	0.1806	13.86	0.0074	1.09	1	1.09	8.54	0.0223
BC	0.297	1	0.297	22.79	0.0020	0.0004	1	0.0004	0.0031	0.9570
A^2	0.0119	1	0.0119	0.9162	0.3704	4.61	1	4.61	36.06	0.0005
B^2	0.6184	1	0.6184	47.46	0.0002	2.27	1	2.27	17.74	0.0040
C^2	0.2595	1	0.2595	19.91	0.0029	0.757	1	0.757	5.92	0.0452
Residual	0.0912	7	0.013			0.8951	7	0.1279		
Lack of Fit	0.0477	3	0.0159	1.46	0.3513	0.6068	3	0.2023	2.81	0.1721
Pure Error	0.0435	4	0.0109			0.2883	4	0.0721		
Corrected Total	4.67	16				53.93	16			
R^2	0.98					0.98				
Adjusted R^2	0.96					0.96				
Predicted R^2	0.82					0.81				

The polynomial model for log reduction (Y) was regressed by considering the significant terms in the following equations:

E. coli O157:H7: $Y = 2.77 - 0.0275A + 0.5700B + 0.2550C + 0.0775AB + 0.2125AC + 0.2725BC - 0.0533A^2 - 0.3833B^2 - 0.2482C^2$

(3)

L. monocytogenes: $Y = 3.89 + 2.13A + 0.3788B + 0.8588C - 0.2675AB + 0.5225AC + 0.0100BC + 1.05A^2 + 0.7340B^2 + 0.4240C^2$

(4)

3.3.2 Interaction of variables

Responses of the log reduction of *E. coli* O157:H7 in lettuce are depicted in the form of 3-D response surface plots and 2-D contour plots (Figure 3.1). The plots represent the responses of two other variables when one is fixed and coded at 0 levels. The three variables of interest in this study were nisin, oregano and ultrasound. Oregano showed a strong positive interaction on microbial log reductions along with the gradual increase in ultrasound duration, deeming both of them as most significant factors for the log reduction of *E. coli* O157:H7 in lettuce (Figure 3.1 a_3-b_3). These findings were supported by previous reports, where assisted ultrasound and ultrasound combined with oregano showed enhanced antimicrobial activity (Millan-Sango *et al*., 2015, Liao *et al*., 2018). However, nisin was the least significant factor compared to ultrasound and oregano regardless of an increase in its concentration, as evident by the representative non-significant p-value which was 0.5175. This result confirms how Gram-negative bacteria is typically resistant to nisin due to its inability to penetrate the outer membrane and consequently the cytoplasmic membrane of the bacterial cells (Modugno *et al*., 2018).

The interactive effects between variables understudy on the log reductions of *L. monocytogenes* are illustrated in Figure 3.2. Nisin and ultrasound exhibited a stronger positive interaction with enhanced log reductions of *L. monocytogenes* in comparison to oregano, which was borderline significant having p-value of 0.0201, making the other two factors the most significant (Figure 3.2 a_2-b_2). Nisin has been reported to facilitate pore formation on the bacterial cell membrane, particularly on Gram-positive bacteria (de Arauz *et al*., 2009). This association with anionic lipids on the cytoplasmic membrane contributes to the formation of pores, causing an efflux of adenosine triphosphate (ATP), amino acids, pre-accumulated rubidium, or the collapse of essential ion gradients and ultimately leading to cell death (Abdollahzadeh *et al*., 2014, Tong *et al*., 2014, Li *et al*., 2016).

An increasing trend in the log reduction was observed in both *E. coli* O157:H7 and *L. monocytogenes* with increasing levels of the independent variables studied. Further increase in concentrations might yield more promising results with elevation in the log reduction values, however, it could in turn adversely affect the physical and sensorial attributes of the lettuce.

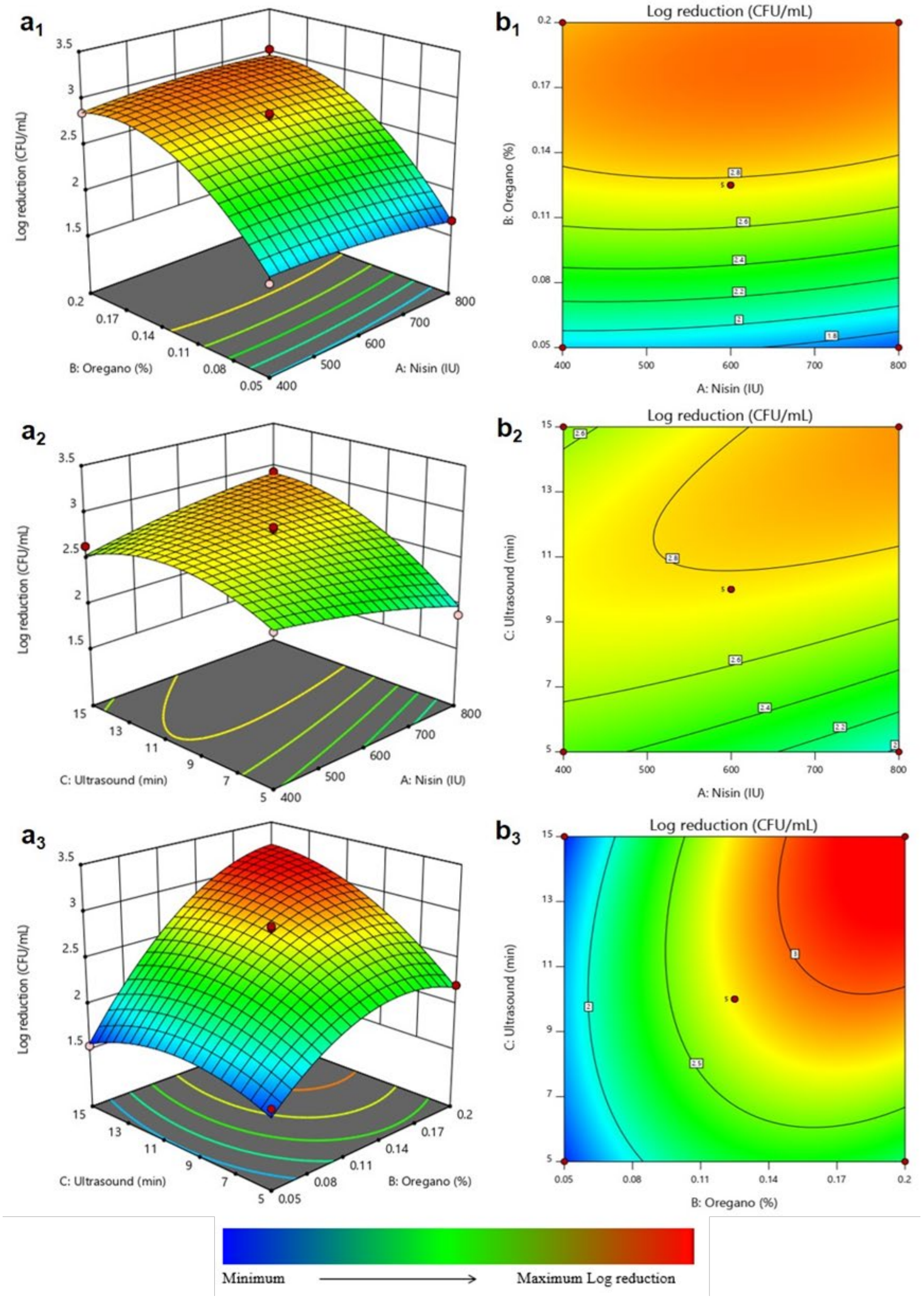

Figure 3. 1: 3D response surface plots (a) and 2-D contour plots (b) of log reduction through combined effects of nisin, oregano and ultrasound, on lettuce against *E. coli*: O157:H7 Interactions between (a_1b_1) oregano and nisin; (a_2b_2) ultrasound and nisin; (a_3b_3) ultrasound and oregano and their effects on log reduction.

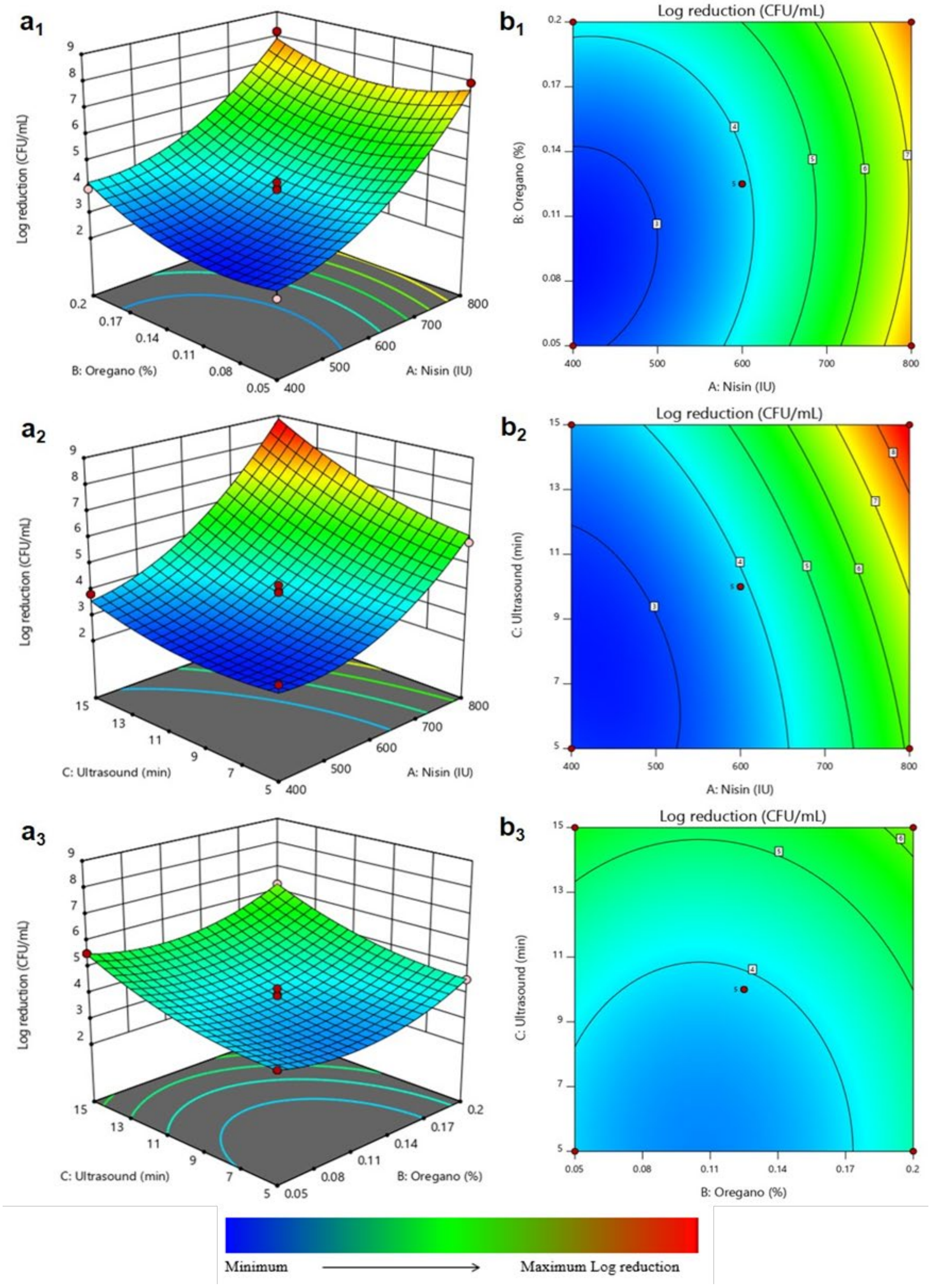

Figure 3. 2: 3D response surface plots (a) and 2-D contour plots (b) of log reduction through combined effects of nisin, oregano and ultrasound, on lettuce against *L. monocytogenes*: Interactions between (a_1b_1) oregano and nisin; (a_2b_2) ultrasound and nisin; (a_3b_3) ultrasound and oregano and their effects on log reduction.

3.3.3 Validation of the experimental model

After analyzing the model parameters, a maximum log reduction of 3.47 CFU/mL could be achieved against *E. coli* O157:H7, when nisin, oregano and ultrasound were set at an optimized condition of 800 IU/g, 0.2% v/v and 15 min, respectively. A prediction of log reduction greater than 9 CFU/mL was observed for *L. monocytogenes* in the suggested combinations, hence, to validate the results, lettuce samples were inoculated with 10^{10} CFU/mL *L. monocytogenes* in further experiments. Subsequently for *L. monocytogenes*, a maximum log reduction of 9.32 CFU/mL was achieved when nisin, oregano and ultrasound were set at an optimized level of 797.45 IU/g, 0.197% v/v and 14.97 min, respectively. These results were relatively close to the model-predicted values of 3.45 CFU/mL and 9.60 CFU/mL for *E. coli* O157:H7 and *L. monocytogenes*, respectively. The close correlation observed between the experimental and predicted values validated the model. Furthermore, one combinational treatment (771.2 IU/g nisin, 0.185% v/v oregano and 14.65 min ultrasound) simultaneously presented excellent log reductions against both the organisms, giving a log reduction of 3.43 and 9.20 CFU/mL for *E. coli* O157:H7 and *L. monocytogenes*, respectively. Therefore, the combination of nisin, oregano and ultrasound presents a strong antimicrobial decontamination technique. Subsequently, to determine the practical applicability, the extrapolated optimized solutions and one of the previous combinations with the optimum log reduction against *E. coli* O157:H7 and *L. monocytogenes* (Table 3.2 run 11) were used to determine the impact on the physical (colour and texture) and structural (electrolyte leakage) properties of lettuce.

3.3.4 The effect of combined treatment on the colour of lettuce

The treatment of lettuce with the combination of nisin, oregano and ultrasound showed only marginal colour differences between the untreated control and the treated samples (Table 3.4).

Colour differences are grouped in separate ranges that indicate extent by which variations can be observed, with values 0 - 3 representing an acceptable range, 3.1 – 6 present a reasonably significant range and large colour differences are perceived at values exceeding 6.1–12 (Klug *et al*., 2016). The values obtained in our study fall within the range of acceptable colour differences as postulated by Chen and Mujumdar (2009). However, the lightness of the lettuce samples showed a marginal increase of up to 4 units. This could be possibly due to the solution becoming slightly acidic during treatments with nisin and oregano oil having low pH and could have initiated some chlorophyll degradation (Huang and Nitin, 2017). The colour difference could also be due to the heterogeneous composition of lettuce samples. Alenyorege *et al*. (2020) in another study reported variations in total colour, after the application of ultrasonic treatments on Chinese cabbage. They further pointed out that low differences observed in the overall colour results could not easily be detected during sensory evaluations, hence there were no wide variations in perceptual effects on the Chinese cabbage. Also similar to our studies, Sagong *et al*. (2011) and Millan-Sango *et al*. (2015) did not find any significant changes in colour properties of lettuce after combining ultrasound with organic acids and essential oils.

Table 3. 4: Colorimetric parameters of lettuce as a result of exposure to different concentrations of antimicrobial combinations.

	L*	a*	b*
Control	54.19 ± 1.68^{a}	-4.51 ± 0.62^{b}	17.82±1.28
800 IU/g, 0.2% v/v, 10 min	55.82 ± 1.38^{ab}	-3.89 ± 0.28^{a}	18.92±1.25
771.2 IU/g, 0.185% v/v, 14.65 min	56.31 ± 1.95^{b}	-3.74 ± 0.90^{a}	19.03±1.50
797.45 IU/g, 0.1974% v/v, 14.97 min	56.60 ± 1.65^{b}	-3.40 ± 0.69^{a}	19.87±1.77
800 IU/g, 0.2% v/v, 15 min	56.21 ± 1.80^{b}	-3.88 ± 0.54^{a}	19.88±1.83

Control was treated with distilled water. Values are represented as mean ± SD (n=3). Mean values with different letter superscripts in a column are significantly different $p<0.05$, with annotations: (L^*) representing lightness, (a^*) red-green and (b^*) yellow-blue. The order of the treatment combinations is nisin (IU/g), oregano (% v/v) and ultrasound (min), respectively.

3.3.5 The effect of combined treatments on the texture of lettuce

The texture (hardness) parameter of ready to eat vegetables is a physical attribute which portrays an important measure of quality. Hence, texture analysis on lettuce was conducted following the treatments with nisin, oregano and ultrasound or with distilled water (control). There were no significant differences in overall texture (hardness) values between control and sample A. which confirms that the texture of lettuce was not affected by the application of one of the combined antimicrobial treatments tested (Fig. 3). Similar results were observed for ultrasound-based treatments on different vegetables which substantiates this finding (Sagong *et al*., 2011; Irazoqui *et al*., 2019). However, a relative decrease in hardness was observed for the rest of the treated samples, exposed to the optimized combination treatments. Significant decrease in the hardness clearly suggests that ultrasound duration has a significant impact on the texture of treated lettuce. Longer ultrasound exposure (14.65 – 15 min) showed a greater reduction in lettuce texture than the treatment at 10 min. A study by Alenyorege *et al*. (2019) on ultrasound treatment applied to Chinese cabbage, resulted in the weakening of the cell wall, softening of leaves through the formation of microchannels and ultimately impacting the firmness of the samples tested. Similar results were observed for ultrasound-mediated softening of different vegetables corroborating the findings of our study (Salgado *et al*., 2014; Huang *et al*., 2018).

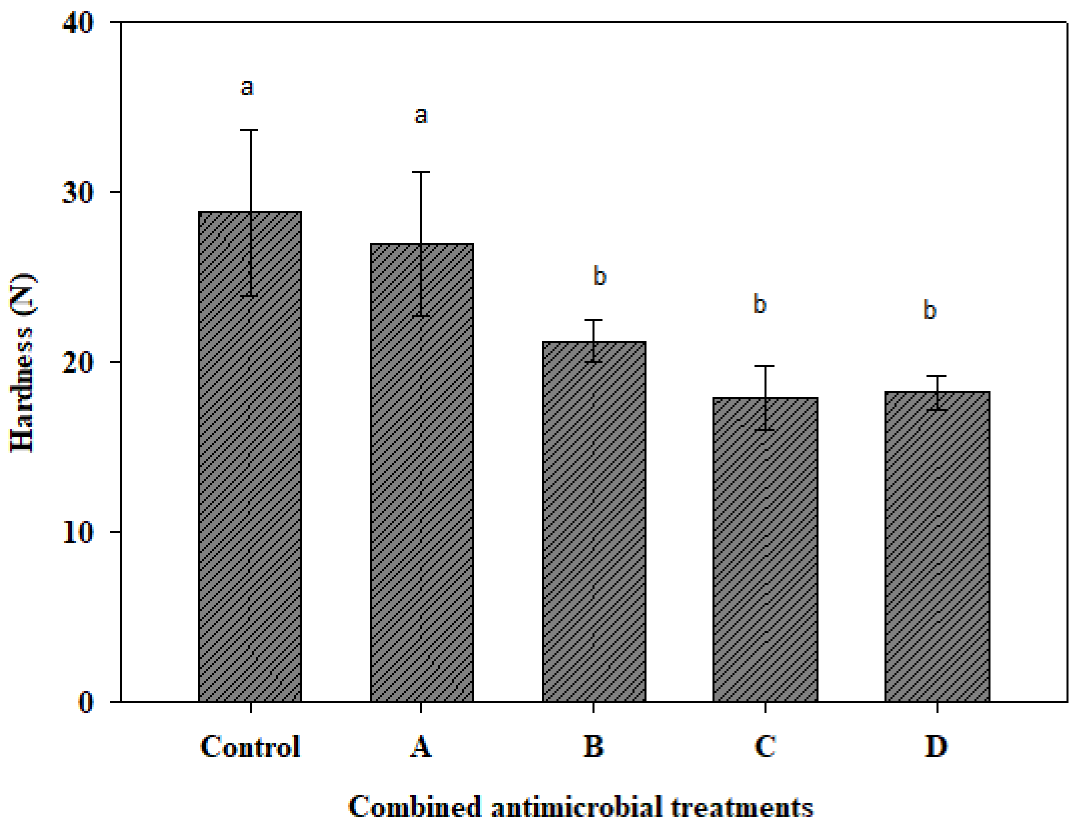

Figure 3. 3: The effect of a combined antimicrobial treatment on lettuce hardness. Control was treated with distilled water. **A**. 600 IU/g, 0.2% v/v, 15 min **B**. 800 IU/g, 0.125% v/v, 15 min and **C.** 800 IU/g, 0.2% v/v, 10 min. Values are represented as mean ± SD (n=3). The mean values with different letter superscripts are significantly different ($p < 0.05$).

3.3.6 Electrolyte leakage rate of lettuce

The extent of tissue injury in ready-to-eat vegetables is revealed through relative electrolyte leakage which is a measure of the penetrability of the leaf's cell membrane. The electrolyte leakage rate (ELR) in lettuce samples without any treatment was 13.48±0.72, and a relative increase in the electrolyte leakage rate was noted after the combination treatment (Table 5). The addition of ultrasound and oregano essential oil to the surface of lettuce has previously been shown to increase the electrolyte leakage rate notably (Millan-Sango *et al.*, 2016). This effect can be evident in this study as the increase in oregano concentration from 0.185% v/v to

0.2 % v/v, appears to have an effect on ELR, with the 16.55 ELR value (0.185% v/v), being fairly lower in comparison to the 18.22 - 18.58 ELR from the high-end oregano concentration range (0.197% v/v - 0.2% v/v). Additionally, cavitation induced stress and micro-streaming activity from ultrasonic waves could have triggered structural changes in the vegetable tissues, including cell wall and protopectin collapse, leading to an increased ionic movement and ultimately a compromise in membrane strength (Alenyorege *et al.*, 2019). Considering that most of the optimal treatments presented in this study had ultrasound exposure at the higher end, it could be possible that an increase in ultrasound time could have had an effect on lettuce ELR. In retrospect, however, increase in the electrolyte leakage can be associated with the presence of essential oils on the lettuce surface after the treatment application rather than with the disruption to the biological structure of the lettuce (Millan-Sango *et al.*, 2016).

Table 3. 5: Electrolyte leakage rate of lettuce samples after treatment with nisin, oregano and ultrasound at different concentration combinations.

	Relative electrolyte leakage
Control	13.48^{a}±0.72
800 IU/g, 0.2% v/v, 10 min	18.22^{c}±1.37
771.2 IU/g, 0.185% v/v, 14.65 min	16.55^{b}±1.61
797.45 IU/g, 0.1974% v/v, 14.97 min	18.58^{c}±0.88
800 IU/g, 0.2% v/v, 15 min	18.57^{c}±1.42

Control was treated with distilled water. Values are means ± SD (n=3). Means followed by different letters in a column are significantly different at p<0.05. The order of the treatment combinations being nisin (IU/g), oregano (% v/v) and ultrasound (min).

3.4 Conclusions

In this study, the synergistic effects of three variables, nisin, oregano and ultrasound on the inactivation of *E. coli* O157:H7 and *L. monocytogenes* on lettuce was observed. The use of a nisin (771.2 IU/g), oregano (0.185% v/v) and ultrasound (14.65 min) combination treatment showed a most significant impact on the log reduction of *E. coli* O157:H7 and *L. monocytogenes*. It is noteworthy that the optimized combination is very effective against *L. monocytogenes* which suggests that it could be extended to other fresh produce which has been earlier reported for *L. monocytogenes* outbreaks. A major benefit of microbial decontamination using the present study is its potential use in the food industry due to its antimicrobial compounds that come from natural materials which are generally recognized as safe (GRAS). The combination treatment also had minimal effects on the physical characteristics of lettuce and could be used effectively as an environmentally friendly alternative to other conventional methods. For potential scaling up of this combined treatment method, further tests on organoleptic properties and feasibility studies in an industrial environment using a larger sampling size of lettuce could be conducted.

CHAPTER FOUR

4 Antimicrobial efficacy of nisin, oregano and ultrasound against *Escherichia coli* O157:H7 and *Listeria monocytogenes* on cabbage

Abstract

The effect of combined nisin, oregano and ultrasound treatments on the reduction of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on raw cabbage was investigated using Box-Behnken response surface design. The optimized treatment comprising 607.85 IU/g nisin, 0.20% v/v oregano and 14.98 min ultrasound showed highest log reduction of *E. coli* O157:H7 (3.66 CFU/mL) on cabbage. Whereas, in the case of *L. monocytogenes*, an optimal combination of 731.25 IU/g nisin, 0.12% v/v oregano and 13.21 min ultrasound resulted in the highest log reduction of 8.27 CFU/mL. SEM images showed the effectiveness of the combination treatments against studied pathogens. The physical properties (colour and texture) of cabbage were analysed after treatment, while the structural damage was determined by electrolyte leakage analysis. There were no colour and textural changes observed between untreated and treated cabbage. The electrolyte leakage rate increased slightly after application with the combination treatments, and the sensory scores were observed to have some factors that were below par. The results demonstrate that a combination of nisin, oregano and ultrasound, is a promising alternative for the reduction of *E. coli* O157:H7 and *L. monocytogenes* as well as retaining the quality characteristics of fresh produce.

Keywords: hurdle technology, antimicrobial efficacy, *E. coli* O157:H7, *L. monocytogenes*, cabbage

4.1 Introduction

Cabbage (*Brassica oleracea* var *capitata*) belongs to the brassica family of leafy green vegetables and cultivated worldwide as an annual crop. It is commonly produced globally mainly because of the increasing preference by farmers, customer tastes, affordable prices, and local market availability (Samec *et al*., 2017). Cabbage is consumed as raw, fermented, boiled, stir-fried, or used typically in salads like coleslaw (Jovanovic *et al*., 2016). Cabbage is a rich source of phenolics, glucosinolates, vitamins and minerals and its nutritional value is well known (Jovanovic *et al*., 2016). Cabbage is an excellent source of carotenoids, vitamin C, essential source of dietary fibre and an excellent source of manganese. It is also a good source of polyphenols and anthocyanins, which are critical nutrients and are antioxidant and anti-inflammatory (Samec *et al*., 2017). The abundance of antioxidants and the presence of glucosinolates in cabbage are partly responsible for its benefits in cancer prevention and cardiovascular disease safety (Jovanovic *et al*., 2016). Through several interactions at diverse places, fresh produce like cabbage undergoes a series of processing steps. It includes, harvesting, sorting, packing, transportation and distribution, before the product reaches the retail stores. There is a high probability for contamination with pathogens at each of these steps (Lee *et al*., 2014). Cabbage is also widely marketed in shredded form as a minimally processed (MP) product due to the convenience and fresh characteristics associated with it (Banerjee *et al*., 2015).

Use of chemical preservatives, most commonly chlorine, as decontamination methods in the fresh-cut vegetable industry has been the application of choice for some time now. This has mainly been due to the ease of application, broad antimicrobial spectrum and low cost associated with chemical preservatives (Inatsu *et al*., 2017). Consumers have been probing for a reduction in the use of synthesised preservatives that are of chemical origin, for more natural, environmentally friendly alternatives (Banerjee *et al*., 2015). In this context, natural-based antimicrobials have been getting increased attention as a result of their biodegradability and being eco-friendly. Among them, essential oils, bacteriocins, bacteriolytic enzymes are more prominent (Pisoschi *et al*., 2018).

Natural products like oregano essential oil and nisin have shown potential in the decontamination and inhibition of various pathogens in the food industry (Calo *et al*., 2015; Modugno *et al*., 2018). Oregano essential oil has demonstrated antimicrobial activity against a broad spectrum of bacterial species and is categorised as Generally Recognized As Safe (GRAS) (Cattelan *et al*., 2018). Oregano essential oil acts in a series of steps in the disruption of bacterial cells which include membrane expansion, increasing fluidity and permeability, disturbing embedded proteins, altering ion transportation process and inhibiting respiration (Cristani *et al*., 2007). The inner portions of vegetable leaves are usually difficult to access when decontaminating fresh produce (Gao *et al*. 2014). Hence ultrasound, a non-chemical and non-thermal decontamination method, has been gaining attraction through its antimicrobial efficacy potential which utilizes mechanisms of cavitation and sonolysis (Sango *et al*., 2014). The amount of energy released by collapsing of bubbles, generates reactions between hydrogen atoms and bacterial DNA hydroxyl radicals, consequently leaving internalised pathogens inactivated (Traore *et al*., 2020). Several studies have combined different antimicrobial methods to formulate a more vigorous decontamination treatment. A study by Inatsu *et al*. (2017) reported that the combination treatment of sodium hypochlorite and organic acids enhanced log reduction of *L. monocytogenes* by 3 CFU/g on shredded cabbage and bean sprouts. Another research which focused on the antimicrobial efficacy and preservation of the physical properties of cabbage showed reductions of *E. coli* O157:H7 at 3.93 log CFU/g and

Salmonella at 3.84 log CFU/g after a combined ultrasound and ozone treatment, while maintaining the colour and vitamin C of cabbage (Traore *et al*., 2020).

The present study has focused on evaluating the antimicrobial efficacy of a combination of nisin, oregano and ultrasound treatment on the reduction of *E. coli* O157:H7 and *L. monocytogenes* and assessment of its impact on the physical properties of cabbage.

4.2 Materials and methods

4.2.1 Preparation of materials

Food grade nisin was purchased from Merck (South Africa) and *Origanum vulgare* essential oil (Carvacrol min 70%) was procured from Nautica Organics (South Africa). Fresh cabbage was purchased from a local supermarket in Durban and the damaged leaves on the outside were discarded and the remaining samples were washed with distilled water to remove any debris or dirt on the leaves. Blotting paper was used for drying the selected cabbage samples, which were cut into 4×4 cm and disinfected in 70% ethanol for 5 minutes (Sow *et al*., 2017, Traore *et al*., 2020).

4.2.2 Strains and preparation of inocula

Strains, *L. monocytogenes* ATCC 7644 and *E. coli* O157:H7 ATCC 43888 obtained from Merck, South Africa were used in this study, for Gram-negative and Gram-positive bacteria, respectively. Inocula preparation was similar to the one described in chapter 3.2.1.

4.2.3 Inoculation of cabbage

After surface disinfection of the samples, the spot inoculation method was employed for bacterial inoculation with *E. coli* O157:H7 and *L. monocytogenes*. Leaf slices were placed on sterilised foil, and a sterile serological pipette was used to prudently drop 100 µL of appropriately diluted bacterial culture onto each leaf surface at different locations. The inoculated samples were then kept at room temperature in a laminar flow cabinet for 60 min to allow the inoculum to dry effectively. Dried leaves were turned over for bacterial exposure, and the same procedure was replicated on the flipped side (Traore *et al.*, 2020). Cabbage leaves were then subjected to treatment subsequent to inoculation and cell attachment, as described below.

4.2.4 Experimental design: response surface method

The effects of nisin, oregano and ultrasound on the reduction of *E. coli* O157:H7 and *L. monocytogenes* on cabbage were modelled by response surface methodology (RSM) using Box Behnken design. The independent variables were nisin (400-800 IU/g), oregano (0.05-0.2% v/v) and ultrasound (5 -15 min). The levels of the three factors were determined from the literature. Log reduction was the dependent variable for the study. The three variables, nisin, oregano and ultrasound, were designated as A, B and C, respectively (Table 4.1). The outline of the experimental design (17 runs) with the coded levels was generated by the statistical software Design-Expert version 11 (StatEase Inc., U.S.A.). Each experimental run was prepared in a randomised order. The system behaviour was described by a quadratic polynomial model regression as given below:

$Y = \beta 0 + \Sigma^3_{k=1} \beta i\, Xi + \Sigma^3_{k=1} \beta ii\, Xi\, Xi + \varepsilon$ ....Equation (1)

Where Y is the response variable, β0 is a constant; βi and βii are the linear and interactive coefficients, respectively; Xi is the level of concentration of the factors, and ε is the random error. The optimization objective was to maximise the log reduction.

Table 4. 1: Experimental range and levels of the independent variables.

Factors		Coded Levels		
		-1	**0**	**+1**
Nisin (IU/g)	A	400	600	800
Oregano (% v/v)	B	0.05	0.125	0.2
Ultrasound (min)	C	5	10	15

Ultrasound specifications (Frequency- 50 kHz, Power- 600 W)

4.2.5 Combined antimicrobial treatments

Ultrasound treatments were performed in an ultrasound water bath (Scientech Model 705, South Africa), with a sample ratio of 2 g to 20 mL of combined nisin-oregano solution. Time intervals of 5,10 and 15 minutes were applied to the ultrasound water bath at a uniform frequency of 50 kHz and Power of 600 W. During the treatment time, the temperature was kept constant at 25ºC in order to maintain the physical properties of the leaves intact. The inoculated cabbage samples (2 g) were placed in sterile 50 mL centrifuge tubes and immersed in 20 ml nisin-oregano solution before undergoing microbial analysis thereafter (Alenyorege *et al*., 2019, Traore *et al*., 2020).

4.2.6 Microbial analysis

Untreated and treated samples (2 g) were transferred into the stomacher bag containing 30 mL of PBS and macerated for 10 minutes in a stomacher blender (Seward 400, UK). Serial dilutions of macerated cabbage samples were prepared and inoculated on plate count agar (PCA) plates. Incubation was performed for all the samples at 37ºC for 24 h with respect to *E. coli* O157:H7 and 24-48 h in the case of *L. monocytogenes*. Bacterial log reduction levels were determined after application of the combined nisin, oregano and ultrasound concentrations (Millan-Sango *et al.*, 2016).

4.2.7 Colour analysis

The colour of the cabbage samples was quantified using a colourimeter (Color flex EZ 0840, Hunter Lab, USA), according to Chang *et al.* (2017) method. In order to determine the level of discolouration, mean values of L*, a*, and b* coordinates which indicate colour lightness, redness, and yellowness, respectively, were evaluated. Discolouration of the samples was estimated from different areas on the cabbage.

4.2.8 Texture analysis

Changes in lettuce and cabbage leaf texture were evaluated using a Shimadzu EZ Texture Analyzer EZ-LX/E2-SX series (Kyoto, Japan) with a blade set probe. The maximum force was recorded using Texture Expert software (version 1.22, Texture Technology Corp.). The maximum force (hardness) value was calculated from force vs. time curves, according to Chang *et al.* (2017). All experiments were performed in triplicates, with independently prepared samples.

4.2.9 Electrolyte leakage

The electrolyte leakage rate (ELR) was measured following the protocol described by Millan-Sango *et al.* (2016). Cabbage samples (4 x 4 cm) were treated with the combined nisin, oregano and ultrasound treatments, as described previously. After the treatment process, the samples were placed in a Duran bottle containing 200 mL of distilled water and shaken gently. The electrical conductivity of the solution was measured at 1 min (C1) and 60 min (C60) of incubation using a conductivity meter (MetrohHM 644 conductometer, Switzerland). The samples were then autoclaved at 121ºC for 15 min, and the total conductivity (CT) of the solution was measured after cooling. ELR was calculated using the following equation:

$$\mathrm{ELR} = \frac{(\mathrm{C60} \times \mathrm{C1})}{\mathrm{CT}} \times 100$$

4.2.10 Sensory evaluation and analysis

In order to evaluate the effect of the combined treatments on the consumer acceptability and sensory quality of cabbage, a multivariate comparison test, on appearance, aroma, flavour, texture, and overall liking was conducted (Duarte *et al*., 2018). The samples used for this analysis were not inoculated, for safety reasons. The sensorial panel was comprised of 20 individuals, who provided their consent and were interested in adjudicating between the control and treated samples. All panellists belonged to the Department of Biotechnology and Food Science, Durban University of Technology, and had prior experiences in undertaking the sensory analysis of other food products. To eliminate bias, the session was held in an atmosphere with fewer interruptions, minimal noise, less movement, regulated temperature, and controlled odours. Freshly cut cabbage samples were served in white plates which were numbered randomly to the sensory adjudicators (Banerjee *et al*., 2015). After the consumption of a single sample, panellists were provided with water to rinse their palates. The parameters under investigation were appearance, aroma, flavour, texture, and overall liking (Park *et al*., 2017). A nine-point hedonic scale was used to assess the overall impression of the control and treated samples (Barnejee *et al*., 2015).

4.2.11 Scanning electron microscopy (SEM)

Effectiveness of antimicrobial treatments on the removal and disruption of microbial cells and structural damage of cabbage was analysed by scanning electron microscopy (SEM). Cabbage samples were inoculated with *E. coli* O157:H7 and *L. monocytogenes* and were then separated into the untreated control and treated samples. To fix and bind the bacteria, samples were immersed in a solution of 2.5 % v/v of glutaraldehyde in 0.1M of imidazole for 16-20h. Prior to being dehydrated in ethanol for 10 min, the samples were washed with imidazole buffer (0.1 M). The specimens were cleaned, fitted with double-sided carbon tape and sealed with an ultra-thin layer of gold. The samples were retained in a desiccator for further usage to prevent any reabsorption of moisture. The images were captured at magnifications of 500 × and 5000 × and 10000 × using SEM (ZEISS ULTRA PLUS FEG-SEM, Zeiss, Germany) according to a method by Millan-Sango *et al.* (2015).

4.2.12 Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA), to analyze the disparities between the treatments after application. All experiments were conducted in triplicate and Fischer's Least Significant Differences Test was used to compare means ($p < 0.05$).

4.3 Results and discussion

4.3.1 The effect of combined antimicrobial treatments on the reduction of *E. coli* O157:H7 and *L. monocytogenes* on cabbage

This study was performed to evaluate the efficacy of different combinations of nisin, oregano and ultrasound on the log reduction of *E. coli* O157:H7 and *L. monocytogenes* in cabbage. The highest log reduction of 3.66 CFU/mL was observed for *E. coli* O157:H7 when cabbage was exposed to a combination treatment of 600 IU/g nisin, 0.2% v/v oregano and 15 min ultrasound. The second highest log reduction was recorded at the combination treatment, 400 IU/g nisin, 0.125% v/v oregano and 15 min ultrasound, which had a log reduction of 3.53 CFU/mL. Ultrasound duration appears in both instances to be the most dominant factor, after showing the best log reduction results at the higher end,15 min, exposure. A trend in the different oregano concentrations seems to suggest how the efficacy of the treatments increased during combination, from the middle to higher range, as evident from the log reduction values. Oregano appears to exhibit better antimicrobial properties against *E. coli* O157:H7 with an increased ultrasound duration, thereby forming a more robust synergy. Nisin, however, seems to exhibit mild to low antimicrobial properties, during combination treatment. This is evident in instances where the lowest nisin concentration (400 IU) in combination with middle to higher ranges of oregano and ultrasound, showed higher reduction values as compared to when nisin concentration is in the higher end (800 IU). Nisin might therefore be showing antagonism, the opposite of synergism when applied against *E. coli* O157:H7. The resistance of Gram-negative bacteria, such as *E. coli* O157:H7 to nisin activity is well established in several studies, due to the blockage of access into the cytoplasmic membrane by the bacterium's outer membrane (Zhang *et al*., 2014; Modugno *et al*., 2018). Similar observations, where nisin did not show any additive function in the inhibition of *E. coli* to an ultrasound and nisin combination in milk and nutrient broth, have been reported (Wang *et al*., 2018).

A comparative analysis into the different combined treatments applied to *L. monocytogenes* inoculated cabbage is illustrated in Table 4.2. Combination treatments significantly reduced the bacterial log counts across a wide range ($p > 0.05$). The highest log reduction of 8.23 CFU/ml was achieved at a combination treatment of 600 IU/g nisin, 0.2% v/v oregano and 15 min ultrasound, followed by 800 IU/g nisin, 0.2% v/v oregano and 10 min, which attained an 8.15 CFU/ml reduction. One common factor in both the treatments is the concentration of oregano, which, at the higher end of 0.2 % (v/v) managed to influence the efficacy of the treatments positively. It can also be observed that if two factors are applied in their highest forms of concentration with a middle range concentration for the remaining factor, high log reduction values can be realised. As represented in Table 4.2, when nisin concentration was at the mid-level range (600 IU/g) combined with oregano and ultrasound time at their highest range end. A similar trend was observed when ultrasound time was reduced to the middle range (10 min) and combining with nisin and oregano at their highest concentrations, resulted in significant log reduction. The inhibition capacity exhibited by the combined nisin, oregano and ultrasound treatments on *L. monocytogenes* inoculated cabbage, demonstrates the strong synergism between these three factors.

The mechanisms involved in the bacterial inactivation for both *E. coli* O157:H7 and *L. monocytogenes* are directly linked to the inhibitory actions of the individual factors involved, and the type of vegetable under investigation, in this case, cabbage. Ultrasound targets the cell membrane through cavitation and sonolysis, a mode of action that cause esterase inactivation, resulting in cellular death (Traore *et al*., 2020). Micro-mechanical shocks emanating from localised high-pressure regions may materially detach some bacterial cells whilst making surface attachment to the cabbage on other cells weaker (Forghani and Oh, 2013). The formation of reactive species as free radicals can occur during the cavitation cycle, which can cause damage to the microbial DNA and trigger oxidation reactions, which ultimately contributes to microorganism inactivation (Duarte *et al*., 2018). The synergistic properties of nisin in combination with several other antimicrobials, like oregano essential oil, carvacrol, limonene and even thermo-sonication have been well reported (Zhang *et al*., 2014, Liao *et al*., 2018). Nisin is regarded to be effective when applied to Gram-positive and spore-forming bacteria, hence the apparent inhibitory activity against *L. monocytogenes* in this study. The influence of nisin, oregano and ultrasound synergy can also be attributed to the fact that nisin can boost the inhibitory action of oregano essential oil by increasing the number and size of pores formed in cell membranes both of which will lead to a more robust antimicrobial treatment (de Oliveira *et al*., 2015). This can be observed in similar studies done on the effect of nisin and essential oil (Rohani *et al*., 2011; Rahnama *et al.*, 2012). Through the expansion of the membrane, increased fluidity and permeability, destruction of embedded proteins, alteration of ion transport and inhibition of respiration, oregano essential oil exhibits antibacterial activity (Calo *et al*., 2015). In combination with ultrasound and nisin, oregano shows synergistic properties and a broad antimicrobial spectrum, as is noted in several other studies (Govaris *et al*., 2010, Millan Sango *et al*., 2016).

Table 4. 2: Experimental design used for RSM with three independent variables and showing the observed log reduction of *E. coli* O157:H7 and *L. monocytogenes* on cabbage. The actual values are the average of triplicate determinations.

	A	B	C	*E. coli* O157:H7		*L. monocytogenes*	
Run	Nisin IU/g	Oregano % v/v	Ultrasound Min	Actual Log reduction CFU/mL	Predicted Log reduction CFU/mL	Actual Log reduction CFU/mL	Predicted Log reduction CFU/mL
1	0	-1	-1	2.29	2.27	2.98	3.09
2	-1	+1	0	2.84	2.81	7.52	7.74
3	0	0	0	2.66	2.67	7.71	7.51
4	+1	0	-1	2.37	2.36	7.71	8.23
5	-1	-1	0	2.50	2.54	2.82	2.86
6	+1	-1	0	2.56	2.59	7.71	7.59
7	0	0	0	2.61	2.67	7.87	7.81
8	0	+1	+1	3.66	3.68	8.23	8.12
9	0	0	0	2.66	2.67	7.71	7.81
10	-1	0	+1	3.53	3.55	7.52	7.51
11	+1	+1	0	2.80	2.76	8.15	8.11
12	-1	0	-1	2.22	2.20	3.4	3.25
13	0	0	0	2.71	2.67	7.87	7.81
14	0	+1	-1	2.30	2.36	7.71	7.74
15	0	-1	+1	3.39	3.33	7.52	7.49
16	0	0	0	2.72	2.67	7.87	7.81
17	+1	0	+1	3.37	3.39	8.08	8.23

The results obtained were applied to a second-order polynomial regression equation to determine the optimal conditions for the independent variables. Coded values were used to describe the predicted regression coefficients against calculated values in the form of an equation. The level of log reduction is a function of the different factors applied (nisin, oregano and ultrasound), which were provided as model equations, developed using statistically significant regression coefficients ($p < 0.05$).

The polynomial model for log reduction (Y) was regressed by considering the critical terms in the following equations:

E. coli O157:H7: $Y = 2.67 + 0.0013A + 0.1075B + 0.5963C - 0.025AB - 0.0775AC + 0.065BC - 0.0173A^2 + 0.0202B^2 + 0.2178C^2$ (3)

L. monocytogenes: $Y = 7.81 + 1.30A + 1.320B + 1.190C - 1.070AB + 0.9375AC - 1.000BC - 0.5943A^2 - 0.6618B^2 - 0.5342C^2$ (4)

Analysis of variance (ANOVA) was used to check the significance of coefficients and the adequacy of the quadratic equations were expressed in terms of p and F values (Table 4.3). Both the p values for *E. coli* O157:H7 and *L. monocytogenes* were <0.001, indicating a highly significant quadratic regression model. The model F- values of 112.28 and 321.92 for *E. coli* O157:H7 and *L. monocytogenes*, respectively, demonstrates the significance of the model and only a 0.01% chance that noise could produce an F- value this large. There were high regression coefficients (R^2) of 0.99 for *E. coli* O157:H7 and 0.99 for *L. monocytogenes*, which indicated that the model had a high probability of representing the entire sample. A difference of less than 0.2 implies a reasonable agreement between the predicted R^2 and adjusted R^2. Therefore, *E. coli* O157:H7 at predicted R^2 of 0.93 and adjusted R^2 of 0.98, and *L. monocytogenes* at predicted R^2 of 0.97 and adjusted R^2 of 0.99 expressed a good correlation in determining the quality of fit. The ratios of 34.41 and 50.33 for *E. coli* O157:H7 and *L. monocytogenes*, respectively, indicate adequate signals, as any value greater than 4 when measuring adequate precision, infers the ability of the model to navigate the design space.

Table 4. 3: Analysis of variance for the reduction of *E. coli* O157:H7 and *L. monocytogenes* on cabbage.

	E. coli O157:H7					*L. monocytogenes*				
Source	Sum of Squares	Df	Mean Square	F-value	p-value	Sum of Squares	Df	Mean Square	F-value	p-value
Model	3.18	9	0.3538	112.28	< 0.0001	56.04	9	6.23	321.92	< 0.0001
A-Nisin	0	1	0	0.004	0.9515	13.49	1	13.49	697.65	< 0.0001
B-Oregano	0.0924	1	0.0924	29.34	0.001	13.99	1	13.99	723.4	< 0.0001
C-Ultrasound	2.84	1	2.84	902.69	< 0.0001	11.4	1	11.4	589.4	< 0.0001
AB	0.0025	1	0.0025	0.7935	0.4026	4.54	1	4.54	234.56	< 0.0001
AC	0.024	1	0.024	7.63	0.028	3.52	1	3.52	181.76	< 0.0001
BC	0.0169	1	0.0169	5.36	0.0537	4.04	1	4.04	208.88	< 0.0001
A^2	0.0013	1	0.0013	0.3977	0.5483	1.49	1	1.49	76.87	< 0.0001
B^2	0.0017	1	0.0017	0.548	0.4832	1.84	1	1.84	95.33	< 0.0001
C^2	0.1996	1	0.1996	63.36	< 0.0001	1.2	1	1.2	62.13	0.0001
Residual	0.0221	7	0.0032			0.1354	7	0.0193		
Lack of Fit	0.0142	3	0.0047	2.4	0.2086	0.1047	3	0.0349	4.54	0.0889
Pure Error	0.0079	4	0.002			0.0307	4	0.0077		
Corrected Total	3.21	16				56.17	16			
R^2	0.99					0.99				
Adjusted R^2	0.98					0.99				
Predicted R^2	0.93					0.97				

4.3.2 Interaction of variables

The significant or insignificant interactions between the respective variables tested were calculated, taking into account 2-D contour plots, and 3-D response surface plots. The model responses are mapped against two experimental variables, where one factor is fixed and coded at 0 levels. The three variables investigated in this study were nisin, oregano and ultrasound. To dec interactions between two variables by using 2-D contour plots and 3-D response surface plots is a concise and facile process that facilitates the obtaining of the optimum levels.

The maximum log reduction of 3.66 CFU/ml for *E. coli* O157:H7 could be obtained when ultrasound time was at its highest coded value of 15 min. The positive effect of ultrasound time on overall log reduction is evident through the linear correlation between the two, factor and response, as an increase in ultrasound time is concurrent with the rise in log reduction. A strong synergistic interaction between oregano and ultrasound treatment was revealed, when the most effective treatment had both factors in the higher end of 0.2 % (v/v) and 15 min, respectively. From the middle to a higher range, oregano appears to be more active and aids in inducing the best possible positive interaction against *E. coli* O157:H7 when in combination with ultrasound treatment. Similar studies with positive effects of ultrasound and/or oregano have previously been reported, where increased levels of log reduction on inoculated cabbage were observed (Duarte *et al*., 2018, Traore *et al*., 2020). Nisin, however, did not have any positive interaction with any of the two factors (oregano and ultrasound), instead appears to invoke a contrasting behaviour. A slight antagonistic effect was evident relative to the increase in nisin concentration, as was highlighted in treatments that had nisin at the higher end, in terms of concentration. Similar studies that reported the ineffectiveness of nisin in combinations when applied to inoculated fresh produce corroborate the findings of this study (de Oliveira *et al*., 2015; Liao *et al*., 2018). The interactions between nisin and oregano were observed to be insignificant for log reduction against *E. coli* O157:H7. The 3-D plots and 2-D contour plots indicate how an increase in the concentration for both factors (nisin and oregano), did not enhance bacterial log reduction, when an interactive analysis between the two was performed.

In terms of *L. monocytogenes*, positive interactions between nisin, oregano and ultrasound were observed (Figure 2). Several of the combined treatments applied to cabbage were able to reduce the log reduction of both bacteria considerably. Interactive synergies were achieved for all the different treatments in combination, showing a linear increase with every upsurge in individual concentration. Enhanced antimicrobial effects are a testament of the well documented bactericidal abilities of nisin, oregano and ultrasound (Bhargava *et al*., 2015, Millan-Sango *et al*., 2015, Ukuku *et al*., 2019). Another reason why the treatments were particularly effective when applied to cabbage could be due to the morphology of its leaves. Cabbage has a relatively even and smoother surface, through its waxy cuticle, that facilitates easier detachment of inoculated bacteria (Srey *et al*., 2014). This would suggest, a physical treatment method like ultrasound would significantly initiate bacterial cell detachment, thereby enhancing the effects posed by complimentary antimicrobials like nisin and oregano. The results obtained in this study show that increasing concentrations of nisin, oregano and ultrasound on *L. monocytogenes* inoculated cabbage, achieves synergistic effects and ultimately a more significant log reduction. It is also conceivable that the combined antimicrobial treatments have a more pronounced effect on *L. monocytogenes*, as compared to *E. coli* O157:H7 due to the poor attachment of *L. monocytogenes* to cabbage surfaces as reported by Srey *et al*. (2014). Other studies have also highlighted on the relative resistance of *E. coli* to hurdle treatments, citing how Gram-negative bacteria more readily form lipopolysaccharides on surfaces, thereby the lowering the penetration potential of antimicrobials (Forghani and Oh, 2013; Traore *et al*., 2020).

4.3.3 Validation of the experimental model

Using the selected optimal conditions with the desirability of 1, the suitability of the established mathematical model equations for predicting optimum response values was validated using a numerical and graphical optimization technique. The processed model parameters managed to achieve a maximum log reduction of 3.66 CFU/mL for *E. coli* O157:H7 when nisin, oregano and ultrasound were set at an optimal solution of 607.85 IU/g, 0.20% (v/v) and 14.98 min, respectively. In reference to *L. monocytogenes*, a maximum log reduction of 8.27 CFU/mL was observed at an optimized solution of 731.25 IU/g, 0.12% (v/v) and 13.21 min, respectively. The results were in close proximity with the model's predicted values which were 3.67 CFU/mL for *E. coli* O157:H7 and 8.55 CFU/mL for *L. monocytogenes*, respectively. The observed close correlation between the experimental and predicted values is able to validate the model. Furthermore, in consideration of the functionality of the actual treatment process and its subsequent effects on the cabbage leaf, further tests (physical and structural) on the impact of applying the antimicrobial treatments were undertaken.

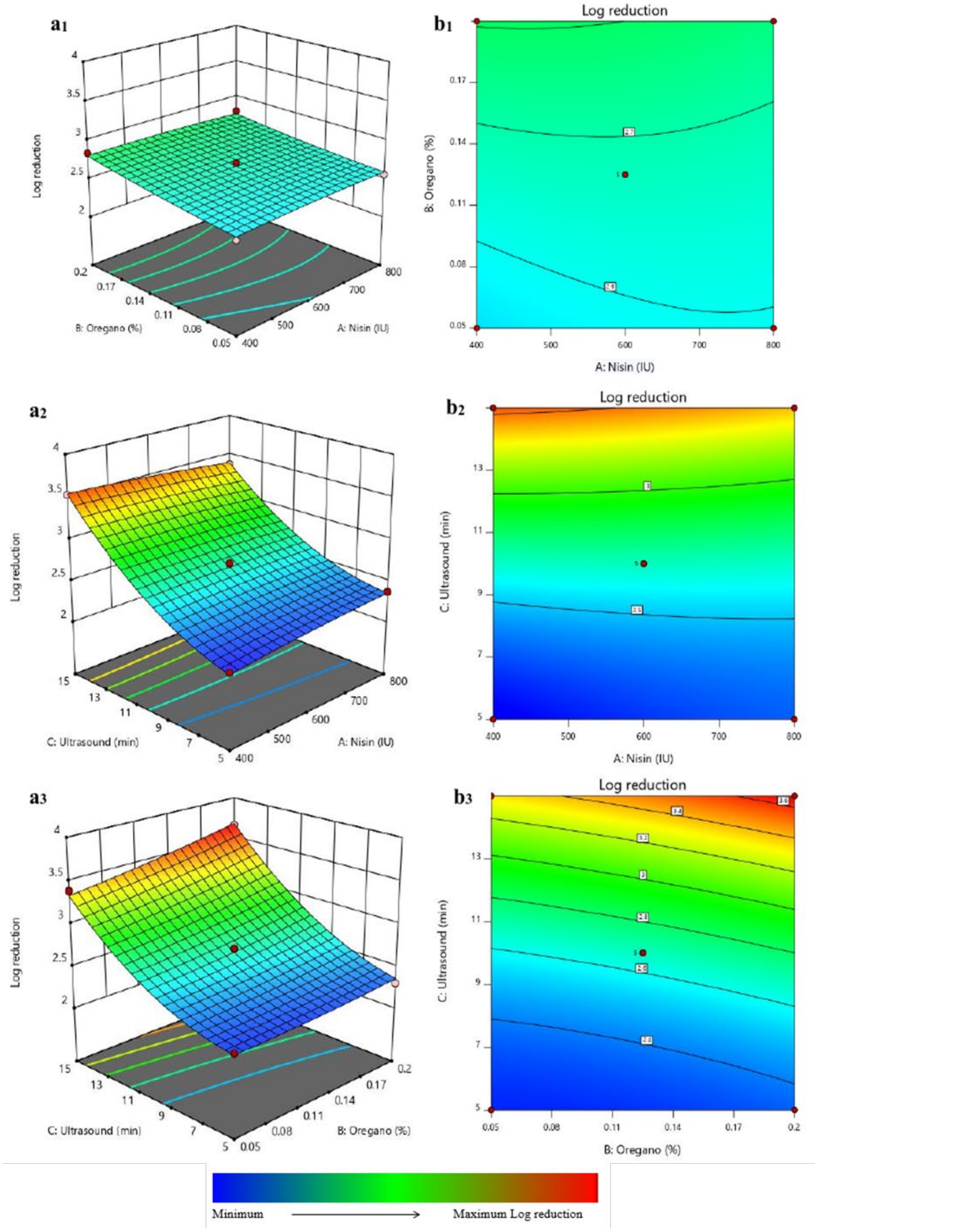

Figure 4. 1: 3D response surface plots (a) and 2-D contour plots (b) of log reduction through combined effects of nisin, oregano and ultrasound, on cabbage against *E. coli* O157:H7: Interactions between (a_1b_1) oregano and nisin; (a_2b_2) ultrasound and nisin; (a_3b_3) ultrasound and oregano and their effects on log reduction.

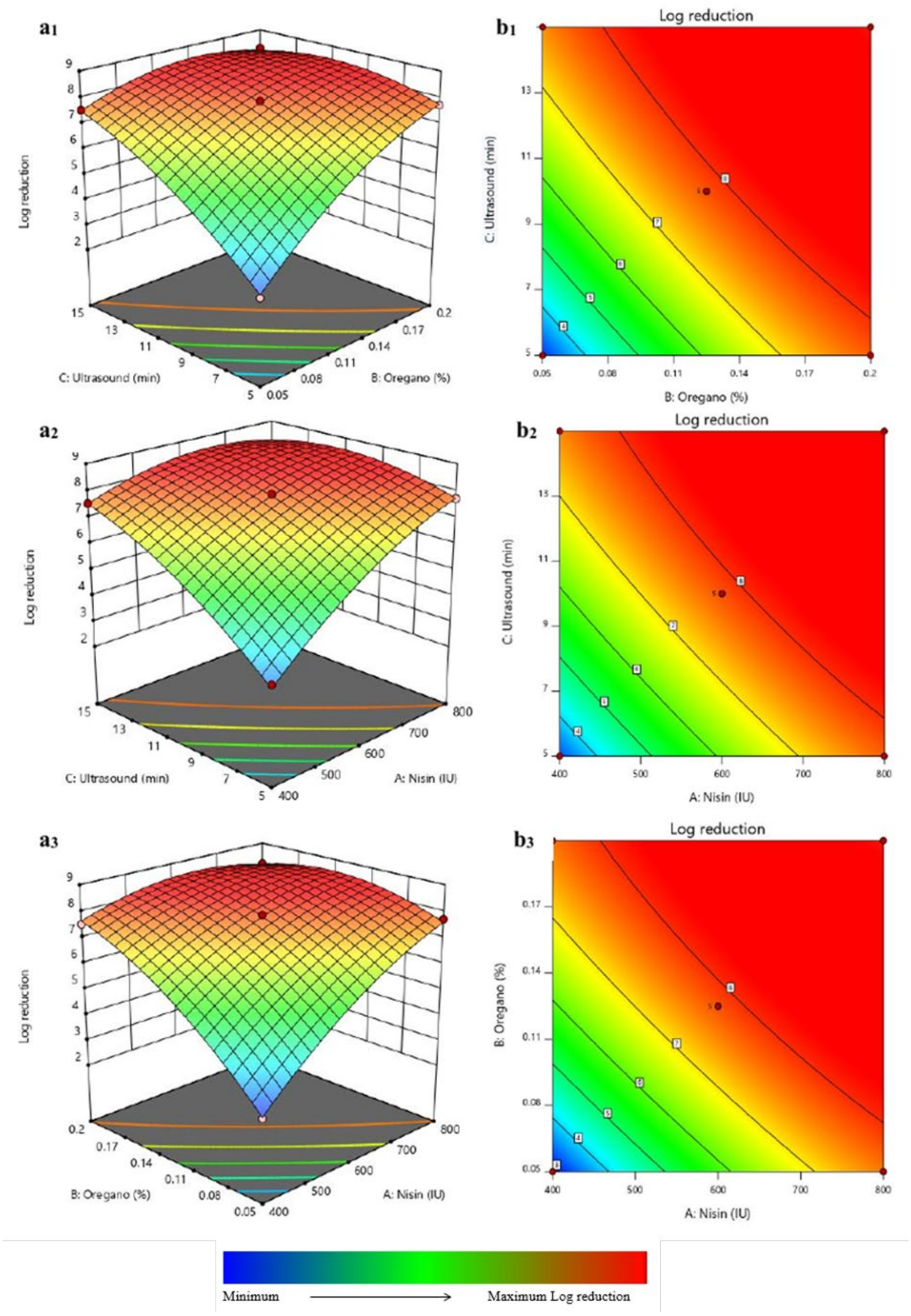

Figure 4. 2: 3D response surface plots (a) and 2-D contour plots (b) of log reduction through combined effects of nisin, oregano and ultrasound, on cabbage against *L. monocytogenes*: Interactions between (a_1b_1) oregano and nisin; (a_2b_2) ultrasound and nisin; (a_3b_3) ultrasound and oregano and their effects on log reduction.

4.3.4 The effect of combined treatment on the colour of cabbage

The treatment of cabbage with a combination of nisin, oregano and ultrasound showed minimal differences between the control and the treated cabbage samples (Table 4.4). The minimal differences in colour of cabbage are much more likely due to their heterogeneity and are all in the acceptable range in terms of colour differences (Klug *et al*., 2016). Differences in colour are divided into different ranges that reflect the degree to which variations can be found, with values 0-3 indicating an admissible range, values 3.1-6 showing a relatively reasonable range, and the broad colour differences are observed at values greater than 6.1-12 (Chen and Mujumdar, 2009). These results could be attributed to the ability of combined treatments to exhibit antimicrobial properties whilst preserving the physical properties of the vegetables. Altering the colour profile of leafy vegetables like cabbage negatively influences its overall appearance; therefore, any treatment that effectively decontaminates vegetables while preserving colour properties is more ideal. A study by Srey *et al*. (2014) reported that there were no significant changes in colour properties when combined ultrasound treatments were applied to fresh cabbage. Another study conducted by Traore *et al*. (2020), where combined ultrasound and ozone treatments were applied to cabbage, differences in terms of sample net colour were not observed. Other studies have also reported slight to significant differences in terms of colour. One such report is that of Alenyorege *et al*. (2020), where variations in the total colour of Chinese cabbage after exposure to multifrequency ultrasound treatments were reported. Alenyorege *et al*. (2020), argued that such low differences in overall colour were not easily detectable; hence there were no perceptual defects noticeable on the actual cabbage leaves.

Table 4. 4: Colorimetric parameters of cabbage as a result of the exposure to different concentrations of antimicrobial combinations.

	L*	a*	b*
Control	66.94±1.32^{a}	-3.26±0.44	15.22±1.28^{b}
400 IU/g, 0.125% v/v, 15 min	70.22±1.08^{bc}	-2.84±1.28	12.82±1.60^{ab}
600 IU/g, 0.2% v/v,15 min	68.82±1.90^{ab}	-2.30±0.65	10.81±1.41^{a}
800 IU/g, 0.125% v/v, 15 min	71.46±1.15^{c}	-2.42±0.28	11.47±1.79^{ac}
800 IU/g, 0.2% v/v, 10 min	67.50±1.44^{a}	-2.94±0.57	13.90±1.21^{bc}

Control was treated with distilled water. Values are represented as mean ± SD (n=3). Mean values with different letter subscripts in a column are significantly different p<0.05, with annotations: (L^*) representing lightness, (a^*) red-green and (b^*) yellow-blue. The order of the treatment combinations is nisin (IU/g), oregano (% v/v) and ultrasound (min), respectively.

4.3.5 The effect of combined treatments on the texture (hardness) of cabbage

The texture (hardness) parameters of cabbage following treatments with water (control) and the different combination treatments of nisin, oregano and ultrasound are presented as a measure of quality. The hardness values after treatment are shown in Figure 4.3. There were no significant differences ($p > 0.05$) between the control and the treated samples after comparing the differences in sample texture before and after treatment. These findings indicate that the application of combined treatments, did not exhibit any adverse effects, or destabilise the cabbage's leaf tissue integrity. Similar observations were made in other studies on the preservation of cabbage texture after different treatment applications. One such study is that of Srey *et al*. (2014), where a combined method of ultrasonication, ultra-violet C and cold plasma did not adversely affect the texture of cabbage and lettuce. In another study by Barnejee *et al*. (2015), allyl isothiocyanate was applied to shredded cabbage with no significant changes observed after treatment. Choi *et al*. (2019) reported on the preservation of textural properties on shredded Chinese cabbage after exposure to plasma-activated water and mild heat treatments. Such positive results of texture consistency after treatment exposure imply that the combined treatments under investigation can be used as potent antimicrobial methods since they are able to preserve texture quality. However, to have a balanced viewpoint, some reports have reported on the negative impact of treatment applications to cabbage samples. A study of ultrasound application on Chinese cabbage by Alenyorege *et al*. (2019) reported a weakening of the cell wall, which negatively impacted the sample firmness and prompted softening of the leaves under investigation.

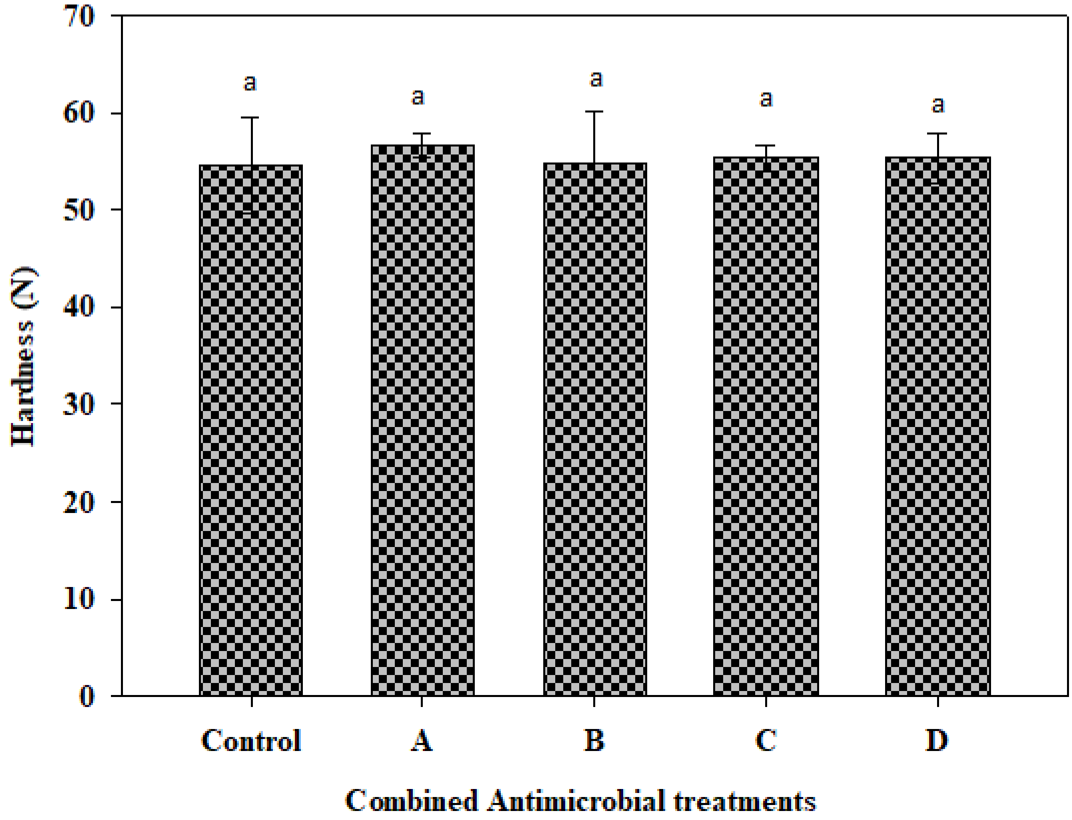

Figure 4. 3: The effect of combined antimicrobial treatments on cabbage hardness. Control was treated with distilled water. **A**. 400 IU/g, 0.125% v/v, 15 min, **B**. 600 IU/g, 0.2% v/v,15 min, **C.** 800 IU/g, 0.125% v/v, 15 min and **D**. 800 IU/g, 0.2% v/v, 10 min. Values are represented as mean ± SD (n=3). The letter a represents no significant differences between samples and control ($p < 0.05$).

4.3.6 Electrolyte leakage rate of cabbage

The penetrability of the membrane was stated as the relative electrolyte leakage of the cell membrane, which reveals the extent to which the cabbage's leaf tissue has been damaged. The electrolyte leakage rate (ELR) of the cabbage samples without any treatment exposure, was recorded at 3.35. Changes in the electrolyte leakage rate in cabbage were observed after treatments, with the values of 7.20, 7.41, 6.83 and 5.30 ELR, for the four different combinations applied (Table 4.5). The lowest value recorded amongst the treated samples was 5.30, which experienced the lowest ultrasound treatment exposure (10 min). This low ELR value (5.30) was the closest to the untreated control ELR of 3.35. It is, therefore, conceivable to suggest that cavitation induced stress and micro-streaming activity triggered structural changes in the cabbage leaf tissues. In a study by Alenyorege *et al*. (2019), where ultrasound was applied on Chinese cabbage, the results proposed that tissue damages had been initiated by a cell wall and protopectin collapse, increased ionic movement and ultimately a compromise in membrane strength. In comparison to other studies when increases in ELR were noted, it is plausible that the more substantial the texture or waxy cuticle of a vegetable leaf, the lower the rate of electrolyte leakage increase. Our study on cabbage had a range between 5.30 – 7.41 ELR, which is relatively low in comparison with other studies. Millan Sango *et al*. (2016) reported an ELR increase in the range of 12 – 21 on lettuce after applying an ultrasound-oregano combination, while Alenyorege *et al*. (2019) observed a range of 17 – 26 ELR on Chinese cabbage after applying an ultrasound-sodium hypochlorite combination. Additionally, it is possible to suggest that the mere presence of oregano essential oil or its increase in concentration, had an effect on ELR, as observed when 0.2 % v/v of oregano was used (Table 4.5). When oregano and thyme essential oils were added to lettuce leaves as individual treatments, their electrolyte rate increased notably in the range of 9 – 16 ELR (Millan-Sango *et al*., 2016). The increase in ELR in our study could, therefore, be due to the presence of the essential oil on the surface of the cabbage rather than the actual damage of leaf tissue cells.

Table 4. 5: Electrolyte leakage rate of cabbage samples after treatment with nisin, oregano and ultrasound at different levels and combinations.

Sample	Relative electrolyte leakage
Control	$3.35^{a} \pm 1.12$
400 IU/g, 0.125% v/v, 15 min	$7.20^{b} \pm 1.33$
600 IU/g, 0.2% v/v,15 min	$7.41^{b} \pm 1.28$
800 IU/g, 0.125% v/v, 15 min	$6.83^{b} \pm 1.01$
800 IU/g, 0.2% v/v, 10 min	$5.30^{ab} \pm 1.31$

Control was treated with distilled water. Values are represented as means ± SD (n=3). Means followed by different letters in a column are significantly different at $p<0.05$. The order of the treatment combinations being nisin (IU/g), oregano (% v/v) and ultrasound (min).

4.3.7 Sensory evaluation

The mean scores for consumer acceptability and sensory analysis are represented in Figure 4.4. To calculate the disparity between the samples, the nine-point hedonic scale was utilized, with an ascending scale in preference, where score 1 indicates ‘extremely dislike’ while score nine exhibits ‘extremely like’. The scores by the panellists noted a clear difference between the control and treated samples, in terms of aroma, flavour and overall liking. It would appear that the aromatic compounds inherent in oregano oil essential oil, at 0.125 and 0.2 percentiles, are too intense. Volatile compounds such as carvacrol, thymol and ϒ-terpinene have been identified as major components of oregano essential oil (Figiel *et al*., 2010). The panel reported adverse effects in terms of flavour, citing a mild bitter taste that was less favourable to their palate, thereby influencing the overall liking. A tangy taste was also picked by the panellists, which could have emanated from the slightly acidic nature of the nisin and oregano oil components of the treatment. However, in terms of the appearance and texture, scores were between 6-7, indicating noticeably acceptable scores, after the application of combined treatments.

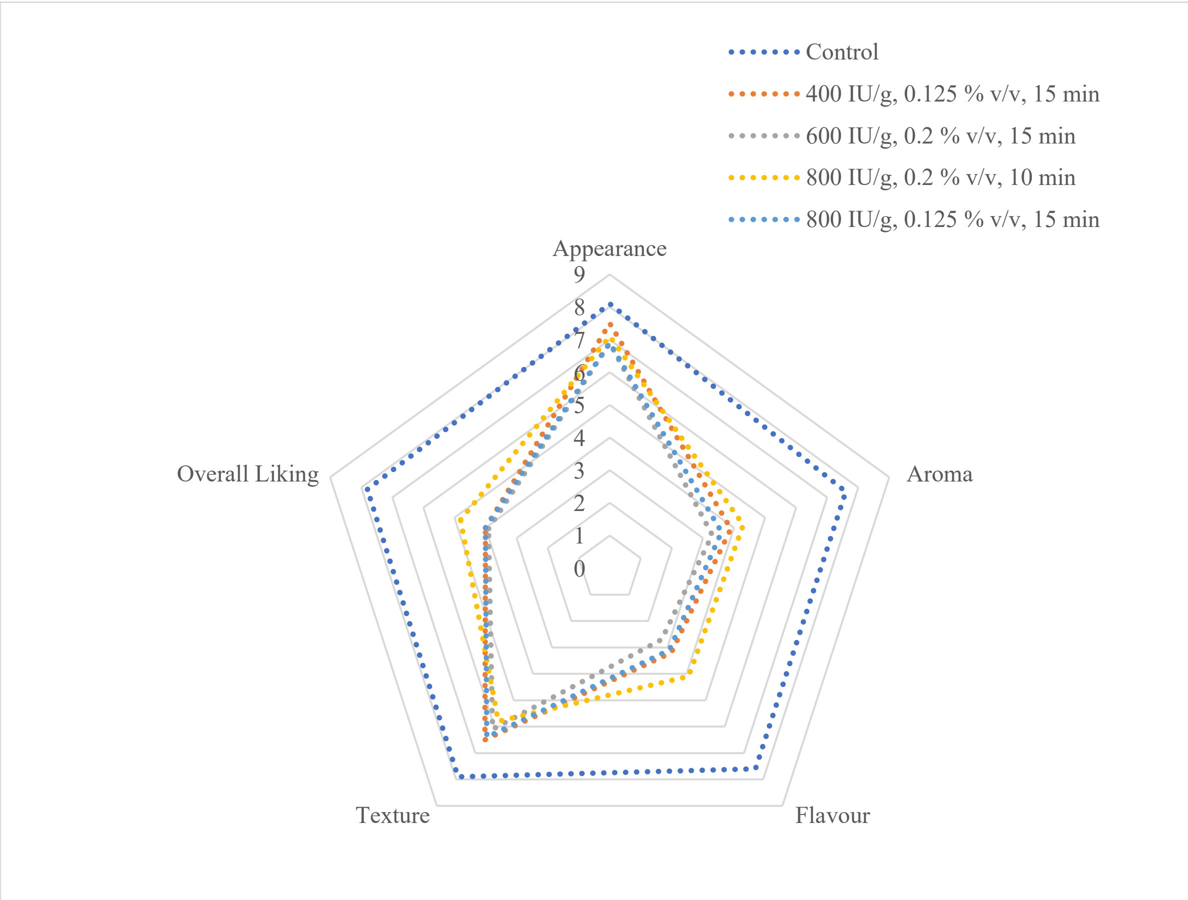

Figure 4. 4: Effect of combined treatments (nisin, oregano and ultrasound) on the sensory characteristics of cabbage. Control was treated with distilled water.

4.3.8 Scanning electron microscopy (SEM)

SEM was carried out to inspect the impact of the combined antimicrobial treatments on the structural properties of *E. coli* O157:H7 and *L. monocytogenes* before and after treatment. The microscopic images were able to show the ability of both bacteria to adhere to cabbage leaf surfaces effectively as shown in Figure 4.5x^1 and 4.5y^1. Also, in Figure 4.5x^1 and 4.5y^1, the SEM images of *E. coli* O157:H7 and *L. monocytogenes* cells observed in the control samples showed an even display of intact bacterial cells, with little to minimal visible damage.

The combined treatments were able to significantly reduce the microbial load of both pathogens as shown in Figure 4.5. These findings are in agreement with those of Srey *et al*. (2014) who was also able to show the bacterial elimination of *L. monocytogenes* biofilms on cabbage and lettuce after antimicrobial treatment. The effects of mechanical cell disruption and pore formation, all major components of our combination treatments, have been attributed in several studies as the causes of bacterial inactivation on inoculated cabbage (Duarte *et al*., 2018; Traore *et al*., 2020). Based on the SEM images obtained in this study, where the disruption of bacterial cells is apparent, it can therefore be concluded that the combined antimicrobial treatments are effective decontaminants in terms of the studied pathogens. It can also be noted through the SEM images, that no damages on the cabbage leaf surfaces were observed. A study by Millan-Sango *et al*. (2015) showed the effective use of ultrasound and essential oils without structural damage on the lettuce leaves, which also supports the findings of this study.

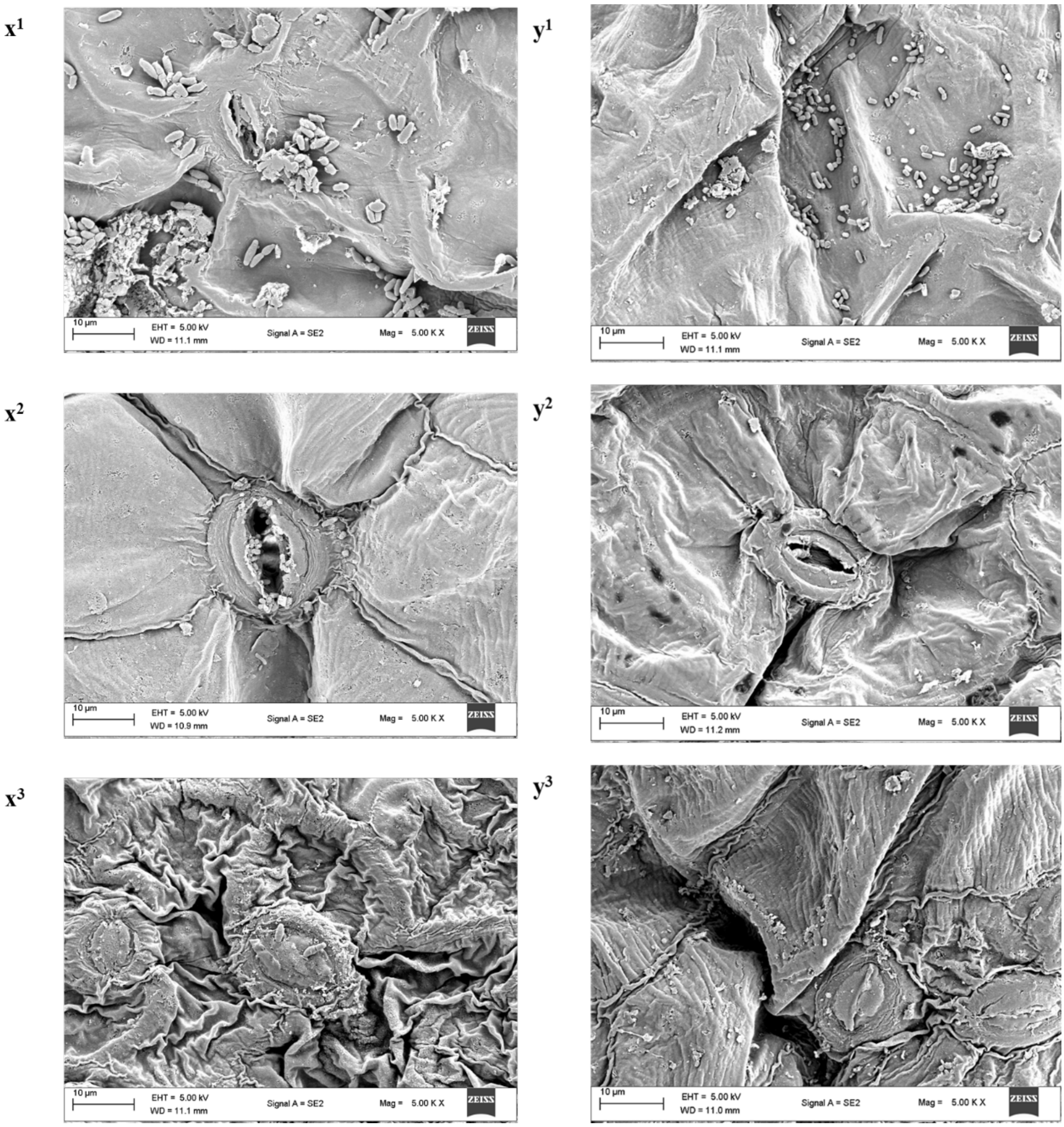

Figure 4. 5: SEM micrographs of inoculated cabbage leaf surfaces exposed to different antimicrobial combination treatments. Control was treated with distilled water. **X** represents *E. coli* O157:H7 inoculated samples while **Y** represents *L. monocytogenes* inoculated samples. **x^1**. Control; **x^2**. 607.85 IU/g, 0.2% v/v,14.98 min; **x^3**. 731.25 IU/g, 0.12% v/v, 13.21 min and **y^1**. Control; **y^2**. 607.85 IU/g, 0.2% v/v,14.98 min; **y^3**. 800 IU/g, 0.2% v/v, 10 min.

4.4 Conclusion

The use of combined treatment methods had a significant impact on the log reduction of *E. coli* O157:H7 and *L. monocytogenes* on cabbage. Response surface methodology was successfully used to develop an experimental system for potential use in investigating the effects of three variables, i.e., nisin, oregano and ultrasound on inactivation of *E. coli* O157:H7 and *L. monocytogenes* on cabbage. The use of this combined intervention method had a significant impact on the log reduction of *E. coli* O157:H7 and *L. monocytogenes*. The combination treatment had no significant differences in the colour and texture of cabbage, with only minimal effects on the electrolyte leakage, hence it could be utilized as an environmentally friendly alternative to other conventional methods. The results of this study are promising, but further research can be done to potentially explore other environmentally friendly antimicrobials, such as essential oil nano-emulsions. It could also be interesting to adopt the field of modern scientific exploits like artificial intelligence and machine learning, as tools for detailed predictive microbiology and development of robust antimicrobial treatments in fresh produce.

CHAPTER FIVE

5 General discussion, conclusion and recommendations

5.1 General discussion

In recent times, as demand for a healthier lifestyle increases through the consumption of fresh produce, the fresh produce industry has experienced notable and exponential growth. In contrast, this growth also conveys inherent safety challenges through pathogenic contamination causing disease outbreaks. *E. coli* O157:H7 and *L. monocytogenes* are two of the most implicated contaminants in the ready-to-eat vegetable industry. Concern regarding outbreaks of foodborne disease related to ready-to-eat vegetables has led to compulsory requirements for vegetable producers in relation to sanitation standards during the cultivation, harvesting and processing of their vegetables. An option that food processors could consider exploring, to improve the biological safety of vegetables such as cabbage and lettuce, is that of developing antimicrobial solutions that are environmentally friendly. This approach has stimulated research into the application of alternative decontamination techniques for ready-to-eat vegetables.

The aim of this research was to determine the impact of combining nisin, oregano and ultrasound treatments on microbial decontamination, physical properties, sensory properties and microstructure of lettuce and cabbage. The first part of this study investigated the antimicrobial efficacy of combined nisin, oregano and ultrasound treatments against pathogens such as *E. coli* O157:H7 and *L. monocytogenes*. The second part focused on the effects of the combined treatments on the physical and organoleptic properties of lettuce and cabbage.

Combination treatments of nisin (400 – 800 IU/g), oregano (0.05% - 0.2% v/v) and ultrasound (5 - 15 min) were formulated, using RSM. Microbial analysis results for lettuce and cabbage were expressed in the form of log reduction for both pathogens. The highest log reduction observed for *E. coli* O157:H7 was 3.43 CFU/ml and 3.66 CFU/ml for lettuce and cabbage, respectively. Inoculated lettuce and cabbage were also investigated for log reduction in terms of *L. monocytogenes*, showing values of 9.20 CFU/mL and 8.27 CFU/mL, respectively. The slight variations in the log reduction after treatments between lettuce and cabbage may emanate from the different morphological properties of these two vegetables. Cabbage has a waxy, smooth surface that detaches bacteria more readily than the more uneven and folded surface of lettuce which has a stronger attachment to bacterial cells (Srey *et al*., 2014). This phenomenon is especially evident through the representative 2D contour plots and 3D graphical plots, that showed more significant interaction of variables on cabbage as compared to lettuce (Fig 3.2, Fig 4.2). In the same vein, *L. monocytogenes* showed a significantly larger log reduction than *E. coli* O157:H7. This is possibly due to the differences in bacterial cell morphology, where one is either Gram-positive or Gram-negative. Combined antimicrobial treatments can target pathogens more effectively than the singular treatments (Traore *et al*., 2020). It was noticeable that ultrasound could be selective under different configurations, as it was more influential in *E. coli* O157:H7 disinfecting than the other factors. Several studies have reported similar observations on the evaluation of the ultrasonic effect on Gram-positive and Gram-negative bacteria (Li *et al*., 2016; Traore *et al*., 2020). It would also appear that an increase in concentration between the factors would result in increased log reductions even with the same bacterial strains (Table 3.2, Table 4.2). Nisin and oregano essential oil were very active in terms of disinfecting *L. monocytogenes* across both vegetables, while in terms of *E. coli* O157:H7, nisin was ineffective at any concentration. Sensitivity to various antimicrobial methods by different bacterial strains such as *E. coli* O157:H7 and *L. monocytogenes* have been documented in many studies (Forghani *et al*., 2013; Lee *et al*., 2014; Inatsu *et al*., 2017).

After application of treatments, the lettuce and cabbage were subjected to a series of physical and organoleptic tests which are indicators of the overall effects and consumer acceptability. It is more ideal to find results where there is minimal or no significant alterations in terms of colour, texture and electrolyte leakage. Both lettuce and cabbage had no significant differences in colour and texture. Similar findings in several studies, where no significant differences in colour and texture are well reported (Sagong *et al*., 2013; Srey *et al*., 2014; Millan-Sango *et al*., 2015). Differences in electrolyte leakage were noted when comparing lettuce and cabbage, where lettuce experienced a more prominent ELR value. This could be attributed to the stomatal and morphological structure of the leaves. Lettuce appears to facilitate osmotic processes more readily than the much more rigid cabbage leaves, resulting in a relatively higher ELR value. Similar studies when ready-to-eat vegetables were observed to experience increases in electrolyte leakage after application of essential oil-based treatments have been reported (Millan-Sango *et al*., 2016; Alenyorege *et al*., 2019).

5.2 Conclusion and future prospects

In general, the findings of this research provide an insight into the potential application of combined nisin, oregano and ultrasound treatments that are effective and safe to use in the fresh produce industry. The study presents evidence of the synergistic antimicrobial effects exhibited by the combination treatments, when applied to lettuce and cabbage, inoculated with *E. coli* O157:H7 and *L. monocytogenes*. A correlation between the increase in concentration between singular factors in combination form and a relative increase in log reduction was observed. Different degrees of influence in terms of the interaction between the treatments were shown, and depending on the type of vegetable, type of pathogen and level of concentration of the factors tested. There were no significant changes in terms of colour and texture indicating the potential of the hurdle treatments to achieve high levels of efficacy without negatively influencing its physical properties. There were slight differences observed in terms of electrolyte leakage rate, which can be expected as the antimicrobial solutions applied, nisin and oregano, have precedence of increasing ELR. However, when the treatments were applied to lettuce and cabbage, sensory evaluation tests highlighted the need to improve the influence of the treatments on the overall taste of the product. It would appear that the intensity of the oregano essential oil, is a bit too concentrated and masks the rest of the components with its strong aroma.

It is therefore evident that the sensorial influence of nisin and oregano essential oil requires further investigation to establish a favourable taste profile. Additionally, since the antimicrobials were expressed in liquid form in this investigation, aerosolization can be studied as an alternative. Production and commercialization of the combined treatment in the form of aerosols, serve to address the uneven topological and morphological structure of ready-to-eat vegetables through effective distribution and diffusion. Also, taking into consideration the advancement of other fields, like that of nanotechnology, incorporation of these highly effective antimicrobials to active biomaterials could present robust delivery systems. Furthermore, other researchers could delve into the application of combined nisin and oregano antimicrobials into packaging films and coatings, thereby ensuring sustained quality preservation in transport and storage facilities. The use of artificial intelligence in the fresh produce industry, in the form of predictive microbiology and mathematical modelling, presents several technological advantages enabling on-site risk assessment that is backed with strong data. Since the current study was focused on the present set of conditions, further studies can expand the scope in terms of different pathogens, different types of fresh produce and even various antimicrobials that present stronger efficacy.

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Appendix

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LWT

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Antimicrobial efficacy of nisin, oregano and ultrasound against *Escherichia coli* O157:H7 and *Listeria monocytogenes* on lettuce

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ABSTRACT

Ready-to-eat vegetables such as lettuce are prone to bacterial contamination, predominantly from *Escherichia coli* and *Listeria monocytogenes*, the leading causes of foodborne illnesses globally. Chlorine is widely used as a decontaminant in the fresh produce industry, however, potential carcinogenic risks limit its usage. As an alternative, green methods and hurdle technology are gaining interest. In this paper, the efficacy of a combination of nisin, oregano and ultrasound on the reduction of *E. coli* and *L. monocytogenes* on lettuce was studied using response surface methodology/Box-Behnken model design, and was found to be reliable ($p < 0.05$). A combination of 771.2 IU/g nisin, 0.185% v/v oregano and 14.65 min ultrasound was the most effective treatment on both pathogens, showing log reductions of 3.43 and 9.20 CFU/mL for *E. coli* and *L. monocytogenes*, respectively. The treated lettuce samples did not show any significant differences in textural properties; however, mild colour changes and a slight increase in the electrolyte leakage rate was observed, within permissible limits. Interestingly this is the first report on the combination of nisin, oregano and ultrasound, as a promising alternative to chemical treatments for the reduction of *E. coli* and *L. monocytogenes* on lettuce, without compromising its appearance and quality.