Application of Oregano oil Nano-emulsion to Control the Foodborne Pathogenic Bacteria *Salmonella* spp. in Alfalfa Seeds and Sprouts

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MASTER OF SCIENCE IN NUTRITION AND FOOD SCIENCE

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT

Sprouts are considered as highly nutritious food and in past few decades consumption of sprouts has increased among health-conscious consumers. On the flip side, along with plenty of health benefits, extent of foodborne outbreak from sprouts is higher due to ambient temperature and high moisture content. The purpose of this study was to test the efficacy of Oregano Oil Nanoemulsion in inhibiting the growth of *Salmonella* spp. on alfalfa seeds and sprouts. Strong antifungal, antibacterial and antiviral properties of essential oils make them possible food preservative. Areas investigated in this study are 1) antimicrobial susceptibility of Oregano Oil Nanoemulsion, 2) efficacy of nanoemulsion in inhibiting *Salmonella* on alfalfa sprouts 3) efficiency of antimicrobial nanoemulsion in reducing the growth of *Salmonella* while sprouting and post-harvest storage. 5% Oregano Oil nanoemulsion was made using sonication method, adding tween 80 as a food grade emulsifier. Different formulations of essential oil and emulsifier were used to identify the variation in stability of nanoemulsion with different proportion of surfactant. Dynamic light scattering was used to know the size particle of nanoemulsion. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of nanoemulsion was determined as 0.078%v/v. Treatment of 0.5% and 1% nanoemulsion on alfalfa seeds showed 1.47 log reduction and 3.25 log respectively in comparison with water control. Similar results were observed in the post-harvest treatment study. This data suggests that Oregano Oil Nanoemulsion can be used as effective, natural and safe potential antimicrobial agent on seeds and sprouts against *Salmonella.*

Keywords: Nanoemulsion, alfalfa seeds and sprouts, sonication, *Salmonella*.

1. INTRODUCTION

1.1 Introduction to Problem

In various developing nations, huge amount of food loss has been reported because of the microbial contamination (Bondi et al., 2017). Microbial contamination is one of the major reasons for various foodborne illnesses due to cross contamination at different levels of food production and processing. Over the past years there is an increase in consumption of seeds and sprouts such as mung beans and alfalfa sprout due to their high palatability and nutritional value. Sprouts are the immature growth produced by germination of seeds. These are rich source of fiber and nutrients. All kinds of bean sprouts (mung bean, alfalfa, broccoli, and radish) are classified as a "super-food" and mostly consumed by health-conscious consumers who prefers nutritionally rich diets.

Together with the proposed health benefits, there's also an inbred threat of foodborne illness. When sprouts are cooked, there's little threat of illness. Germination conditions like high humidity and ambient temperature make them high-risk food which are more likely to cause foodborne outbreaks. However, when consumed raw, the probability of foodborne illness is aggravated. The sprouting process provides right conditions microbial growth, which advancing to final aerobic counts of ≥ 9 log CFU/ g of sprouts. According to Centers for Disease Control and Preventions *Salmonella* causes about 1.3 million infections in the United States every year, recently in 2016 *Salmonella* outbreak associated with alfalfa seeds caused several illnesses in nine states. Majority of outbreaks attributed to alfalfa seeds, primarily caused by *Salmonella* which the most common pathogen to cause foodborne diseases followed by *Escherichia coli* and *Listeria monocytogenes.* The U.S department of health and human services has issued several reports highlighting the risk associated with the consumption of raw sprouts (Landry et al.,

2016). In various studies it has been founded that even low levels of initial contamination (0.1 log CFU/gm) can grow substantially during the sprouting because of favorable growth conditions (Fu et.al., 2008).

According to Centers for Disease Control and Prevention (CDC) 2016, multistate S*almonella* outbreak associated with alfalfa seeds caused 36 reported illnesses from nine different states out of which six people were hospitalized. Presently, used seeds disinfection techniques are ineffective in eliminating the pathogens from sprouts due to complex food matrix. As per U.S. Food and Drug Administration (FDA) regulation 20,000ppm calcium hypochlorite soak is used as disinfection technique prior to germination to reduce the risk of contamination (Thomas et.al 2003). However, industrial applications of this method are limited due to the safety concern of works and adverse effect on environment. Therefore, there is need to find a suitable safe and effective alternative for the seeds and sprouts decontamination which will possess lower effect on the environment.

Alternative approach to eliminate calcium hypochlorite is the application of plant based essential oils. Essential oils are naturally occurring secondary metabolites of plants which generally produced by several aromatic plants which have shown the strong antioxidant, antimicrobial and antiradical properties (Brut, 2004). Essential oils can be obtained from leaves, barks, flowers, bud and fruits of numerous aromatic plants. Plant based essential oils are generally recognized as safe (GRAS) for their use as flavoring agents in the consumption of humans. Hydrodistillation is considered as the most effective and easy method for extraction essential oils from plant parts (Baldim et al., 2019). These days application of essential oils gained popularity because of increase in awareness for addition of synthetic food additives

including antimicrobial agents, essential oils are natural antimicrobial agents (Chang et al., 2011).

Until now, plenty of research demonstrated the antimicrobial efficacy of essential oils, however practical applications in the industry are limited due to several factors. Researchesrs Harmankaya and Vatansever (2017) conducted a study that suggested the inhibitory effect of R. officinalis (Rosemary) and S. aromaticum (Cloves) essential oils against numerous gram positive and gram negative pathogenic bacterias including *Salmonella enteritidies* in chicken meat, application of essential oil can expand the shelf life of meat for 24-48 hrs. Also in another study conducted by de Sá Silva et al. (2019) suggested that effectiveness of tea tree essential oil against Listeria monocytogenes at minimal concentration in grounded beef, minimum inhivitory concentration (MIC) and minimum bactericidal concentration (MIC) were 0.10 and 0.15 µl/ml, respectively.

Essential oils of aromatic plants like thyme, nutmeg, clove, cinnamon, oregano and black pepper showed multiple inhibitory effects against twenty-five bacterial strains including pathogens from plant and animal origin (Dorman and Deans, 2000). Among all the listed oils thyme oil exhibit the widest range of antibacterial activity followed by oregano, cinnamon and clove essential oil. Due to the presence of outermost membrane in gram-negative bacteria, they are highly resistant to essential oils in comparison with gram positive bacteria, this membrane acts as a barrier against essential oils which makes the Eos less effective against gram-negative pathogenic bacteria. In some studies, it has been showed that essential oil emulsion containing surfactant could be a solution to this problem. Nanoemulsion based delivery system enhances the efficiency of essential oils by stabilizing the antimicrobial compounds in these and releasing

them in the food without interference with organic compounds from fresh produce. In this study, we have used the ultra-sonication for the preparation of 5% Oregano Oil Nanoemulsion.

1.2 Hypothesis and Objectives of the Study

1.2.1 Hypothesis

Oregano Oil Nanoemulsion (OONE) exhibit antimicrobial properties and will inhibit the growth of *Salmonella* Spp. on alfalfa seeds and sprouts during germination and storage as well.

1.2.2 Objectives and Specific Aims

Primary goal of this research is to develop a natural antimicrobial nanoemulsion in order to improve the microbial safety of seeds and sprouts by reducing the *Salmonella* contamination on seeds during storage and sprouting. Specific aims to attain this objective are as follow:

1. Preparation and characterization of Oregano Oil Nanoemulsion (OONE) along with determining the stability of emulsion.

2. To evaluate the efficacy of antibacterial nanoemulsion for the reduction of *Salmonella* on alfalfa seeds

3. To determine the effect of essential oil nanoemulsion for reducing *Salmonella* on sprouts during sprouting and post-harvest storage

2. REVIEW OF LITERATURE

Due to several factor food safety has always been on pinpoint, continuous increases in foodborne illness outbreaks caused by pathogenic bacteria and viruses make it more versatile. As per Centers for Disease Control and Prevention (CDC 2013) datasheet, approximately 48 million illnesses occur in United States every year, causing 3000 deaths and 128,000 hospitalizations which cause high economic burden because of health loss (Scharff 2012). Average annual cost for medication of major foodborne diseases were estimated in between $2.3 to $ 4.3 billion (Buzby et al., 1996). Instead of modernization and evolution in food safety strategies by novel technologies, the occurrence of foodborne outbreaks is not reducing in recent decades. For that reason, research is still required to find an effective strategy to eliminate threats to food safety at global food supply.

Numerous conventional methods for food preservation including heat and cold treatments, controlled packaging, synthetic antimicrobials are still commercially used to ensure food safety. In last three decades, implication of hurdle technology gained lots of popularity, utilizing several preservation methods combined (heat, controlled pH and packaging conditions) are widely applicable in industry all around the globe (Leistner, 2000). Out of all other preservation methods, food antimicrobials have notable roles in inhibiting pathogenic and spoilage causing microorganisms in food (Davidson et al., 2005).

Application of synthetic antimicrobials in food have been approved by many regulatory bodies, organic acids and esters including acetic acid, benzoates, lactic acid and propionates. In comparison with synthetic preservatives, naturally driven food grade antimicrobial agents are more preferred by customers which enhanced the demand for plant based natural antimicrobials at commercial level. Development of essential oils-based delivery system can the best approach

to address the issue of food safety and satisfying the customer need. Seeds and sprouts are highly prone to occurrence of foodborne outbreak due to favorable sprouting conditions for microbial growth.

2.1 Alfalfa seeds

Alfalfa seeds scientifically called as ***Medicago sativa,*** these comes from a perennial flowering crop, perennial crops are those which grows for many years after planting. In many parts of the world, it is cultivated as forage crop, usually planted in the spring or fall season. Size of the seeds is tiny around 1-2 millimeters, because of smaller size need to be planted very close to the surface of soil. These are called alfalfa basically in North America, while in New-Zealand, Australia, South Africa and United Kingdom commonly known as lucerne. Plant resembles the closer family member Clover, especially when it is immature, later while matured leaflets get more elongated. Cultivation of small number of seeds can grow out in huge quantity, 15-25 pounds of seeds can be planted per acre of land, there are around 200,000 alfalfa seeds in one pound. At immature stage, plant is fragile and must be protected from weed. Once, from a crown (complex network of leaflets) seeds become vigorous and can re0grow many times after harvesting (around three to eleven times a year).

Roots can grow deeper than 15 feet. Warmer temperature conditions are required for the growth of plant, which is why alfalfa is native to warmer climate zones. Remains of alfalfa seeds older than 6000 years found in Iran and the mentioned in a writing from Turkey, dating 1300 B.C. So, from this evidence it can be assumed that seeds were probably cultivated first around Iran, Turkey, Turkmenistan and other regions in Asia. During the wars alfalfa seeds were of great importance to Greeks, Persians and Romans, they used alfalfa for feeding horses. The name alfalfa came from Arabic and Persian words, meaning best horse fodder. Presently many

varieties of alfalfa seeds are available to growers in United States and Canada, it is widely grown from coast to coast and is the nation's 4th largest cultivated crop. These are 3rd in value after Corn and Soyabean in United States, holding the national value of more than $ 8 billion yearly. One of the major reasons for high value of crop is ability to work as nitrogen fixative, unlike other staple crops nitrogen fertilizers are not required for the growth of seeds.

Table 2.1 Nutritional value of alfalfa sprouts (Akinori Yanaka 2017)

Major Nutrients	Unit	Amount
Energy	kcal	12
Water	g	96
Protein	g	1.6
Lipid	g	0.1
Carbohydrates	g	2
Minerals	g	0.3
Sodium	mg	7
Potassium	mg	43
Calcium	mg	14
Magnesium	mg	13
Phosphorus	mg	37
Iron	mg	0.5
Vitamins		
E	mg	1.9
B1	mg	0.07
B2	mg	0.09
B6	mg	0.01
C	mg	5
Dietary Fiber		
Water Soluble	g	0.1
Insoluble	g	1.3
Total	g	1.4

2.2 Alfalfa Sprouts (Production and Consumption)

Sprouts can be elucidated as a young plant that has emerged from the seed. Further classification of sprouts is based on stem (thin hypocotyl) and leaves (little immature cotyledons) which pop up in pairs. Harvesting of sprouts is basically dependent on the variety of sprouts, typically it is within 1 to 8 days of planting, factors like maturity and required length of sprout also considerable (DeEll, 2014). Most widely consumed varieties of sprouts are alfalfa, clover, broccoli, wheatgrass and mung bean (Oregon Public Health Division et al., 2015). These are generally consumed as raw or slightly cooked.

As per United States Department of Agriculture (USDA) summary of vegetables (2015), fresh produce production of the year was over 400 million hundred by weight, which was harvested from 1.55 million acres. Value of the harvested produce was estimated to be approximately 11.9 billion dollars. According to USDA 2012's Vegetable and Pulses Yearbook, sprouts were aligned under the category of special vegetables. Approximately 80 million pounds of alfalfa seeds are produced annually in the US (Mueller, 2008). However, a very small part of these seeds is used to grow alfalfa, while the majority are produced for alfalfa hay sprouts (Mueller, 2008). In the range of 15 to 20 million pounds, alfalfa and mung beans produced in the United States annually and most of these beans are sprouts are eaten (Oregon Public Health Division et al., 2015). Moreover, about 75% of beans consumed worldwide are sourced from China and Japan (Oregon Public Health Division and others, 2015). The International Sprout Growers Association estimates that 10% of Americans are farmers routinely consumes sprouts (Sikin et al., 2013). According to Oregon Public Health Division et al. (2015) and Matos et al. (2002), alfalfa sprouts are the most popular sprout in the country. The mature alfalfa plant is a legume that is frequently fed to cattle. Alfalfa sprouts typically take 3 to 7 days to develop and

reach harvest size. They are harvested when they reach a height of around 3.8 cm (1.5 inches), and they can be recognized by their slim white stems and short, dark green leaves.

Alfalfa sprouts are frequently used to lend a crunchy texture to sandwiches and salads and have a mild nutty flavor (Oregon Public Health Division et al., 2015).

2.3 Microbiology of Sprouts

All the optimal conditions for pathogen growth, including nutrients, pH, time, temperature, oxygen, and moisture, are present throughout the sprouting phase. In 2-3 days during the sprouting process, seeds can reach a bacterial concentration of 10^9 CFU/g (Liao, 2008). For the purpose of assessing the efficiency of antimicrobial treatments (thermal processing, post-harvest washing, etc.) on food, a 5-log reduction in pathogens is the industry standard. Most of the time, a 5-log reduction in pathogens is sufficient to dramatically lower the likelihood of a foodborne disease if the food is consumed. However, the risk for foodborne illness increases when produce has pathogen contamination more than 5 logs and a post-harvest treatment is performed that is unable to remove the to acceptable levels.

For instance, according to research by Fett (2002), unwashed alfalfa sprouts had about $\log_{10}$ CFU/g of mesophilic aerobes, 7 $\log_{10}$ CFU/g of coliforms, and 3 to 4 $\log_{10}$ CFU/g of yeast and mold. These sprouts can still contain enough microorganisms, even after a postharvest treatment, which results in a 5-log reduction in the number of microbes. If consumed, these microorganisms can make someone sick. Because sprouts are known to contain a high level of microorganisms, the sprout industry relies on washing the seeds before sprouting to minimize contamination.

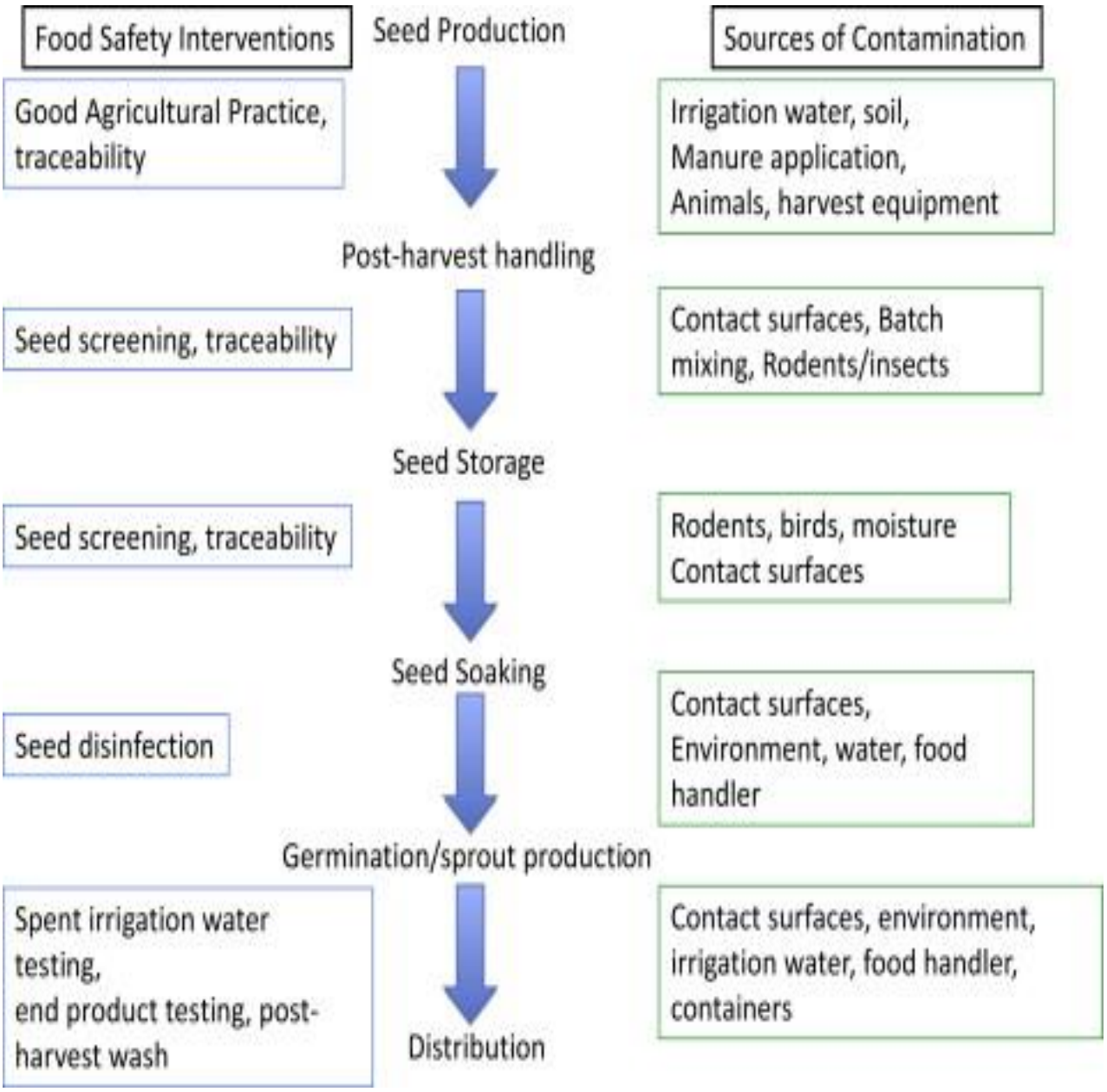

Figure 2.1 Process of alfalfa seeds from production to distribution

The current guideline is to rinse the seeds in a 20,000-ppm calcium hypochlorite solution for 15 minutes (Fett, 2001) in order to eliminate these microorganisms. Although this method effectively lowers contamination, it does not completely eradicate all germs (FDA, 1999b). According to Sikin et al. (2013), calcium hypochlorite treatment of seeds reduces contamination

by 2.5 log CFU/g on average, however results may vary depending on the type of seed used. Any pathogen that is already on the seed when sprouting starts or is added during sprouting can easily develop and multiply if the growing conditions are favorable for pathogen growth. According to Fett's study from 2002, untreated alfalfa sprouts had 8 log10 CFU/g of mesophilic aerobes, 7 log10 CFU/g of coliforms, and 3 to 4 log10 CFU/g of yeasts and molds when they were harvested. In a study by Kim et al. (2009b), it was discovered that 45 samples of sprouts from retail markets had more than 7 log CFU/g of total aerobic bacteria, as well as yeasts and molds. Gram-negative bacteria from the Enterobacteriaceae family, *Klebsiella* and *Enterobacter*, have been reported to test positively for coliforms and fecal coliforms (Beuchant, 1998). Because of this, positive coliform tests may not actually indicate the presence of fecal coliforms.

In the first 48 to 72 hours after sprouting, S*almonella* has been reported to multiply rapidly (Liao, 2008). According to Liao's study from 2008, there was a direct correlation between the total amount of *Salmonella* on alfalfa sprouts and the amount of *Salmonella* that was initially present on the seeds prior to sprouting. *Listeria monocytogenes* and *Escherichia coli* O157:H7 both attained their optimum growth within 48 to 72 hours of the sprouting process, according to additional research by Palmai et al. (2002) and Stewart et al. (2001).

2.4 *Salmonella*

A facultative anaerobe with a Gram-negative status, *Salmonella enterica* has about 2500 serovars and six subgroups. *Salmonella* is a genus of Gram-negative, rod-shaped (bacillus) bacteria belonging to the Enterobacteriaceae family. *Salmonella enterica* and *Salmonella bongori* are the two types of *Salmonella. S. enterica* is the most common foodborne pathogen, suspected of being responsible for roughly 1.3 billion cases of foodborne illness each year, with symptoms ranging from minor intestinal discomfort to bacteremia and death. With cell lengths

ranging from 2 to 5 m, peritrichous flagella, and a non-spore-forming life cycle, *Salmonella* species are largely motile enterobacteria. They are chemotrophs, which means that they need organic substances to fuel oxidation and reduction reactions. Additionally, they are facultative anaerobes, able to produce ATP either anaerobically (without oxygen) or aerobically (with oxygen) depending on the available electron acceptors.

Salmonella species are intracellular pathogens, and some serotypes of these organisms can cause disease. The majority of diseases are brought on by eating food that has been contaminated by either human or animal excrement, such as by a food service employee at a restaurant. There are two primary categories of *Salmonella* serotypes: typhoidal and nontyphoidal. Nontyphoidal serotypes are zoonotic and can spread from person to person as well as from animal to human. They often only affect the digestive system and cause salmonellosis, whose symptoms can be treated without the use of antibiotics. Nontyphoidal *Salmonella*, on the other hand, can be invasive in sub-Saharan Africa and result in paratyphoid fever, which necessitates prompt antibiotic therapy. Typhoid and paratyphoid fevers are all brought on by typhoidal serotypes, which can only be passed from person to person.

Salmonellosis is a serious bacterial intestinal ailment that affects both humans and animals and has a range of negative health effects. *Salmonella* species have been found to be able to grow in a wide variety of temperatures, from 8 to 45 degrees Celsius, and pH levels of 4 to 9.5 (Chlebicz, Liewska, & Health, 2018). As a result, they have become a big worry for the food sector. Due to the ingestion of contaminated food, there are over 1.4 million cases of salmonellosis per year in the United States. From these 35,000 instances, public health laboratories have determined the *Salmonella* serotype, and they have electronically forwarded the results to the Centers for Disease Control and Prevention (Control & Prevention, 2008).

Table 2.2: Recent Outbreaks related to *Salmonella* (CDC, 2022)

Year	Stereotype	Illness	Hospitalizations	Death	Food
2021	*Salmonella* Typhimurium	31	4	0	Pre-packed Salad
2018	*Salmonella* Montevideo	10	0	0	Raw Sprouts
2016	*Salmonella Reading, Salmonella Abony*	36	7	0	Alfalfa Sprouts
2016	Salmonella *Muenchen,* Salmonella *Kentucky*	26	8	0	Alfalfa Sprouts
2014	*Salmonella Enteritidis*	115	29	0	Bean Sprouts
2011	*Salmonella Enteritidis*	25	7	0	Alfalfa and spicy sprouts
2010	*Salmonella Typhimurium*	138	33	0	Alfalfa Sprouts

2.5 Oregano Oil

The herb *Origanum vulgare* produces Essential Oregano Oil (EOO), a volatile essential oil. One of the most popular essential oils (EOs) used all over the world is oregano. It is made primarily of carvacrol and thymol and is derived from Origanum vulgare L. (Teixeira et al., 2013). Carvacrol and thymol are both monoterpenes made comprised of a single phenolic ring created by the union of two isoprene molecules with three functional group substituents (Memar et al., 2017). They give EOO its antibacterial and antioxidant characteristics in addition to its

anticancer and anti-inflammatory functions because of their chemical composition (Sakkas and Papadopoulou, 2017; Sharifi-Rad et al., 2021). EOO and its components have gained attention as a result of these efforts and are currently the subject of extensive research for use as a food preservative, for active packaging, and in the treatment of various illnesses such infections (Bhalla et al., 2013). Terpenoid and phenolic compounds are prominent in it. In addition to rosmarinic acid, which is a potent antioxidant, the presence of carvacrol and thymol is credited with much of EOO's antioxidant activity. These substances predominantly capture free radicals to produce their antioxidant properties. According to reports, thymol and carvacrol have antibacterial actions on the fungi's and bacteria's outer membranes, inactivating them. The main terpenes found in the many oregano species are carvacrol, thymol, p-cymene, and -terpinene; other compounds include terpinen-4-ol, linalool, -myrcene, trans-sabinene hydrate, and -caryophyllene. Chemotypes within the same species are determined by the ratio of these and other EOO components. The primary element of the EO, such as carvacrol, thymol, -citronellol, 1,8-cineole, etc., is typically the term given to the chemotype.

The EOs from herbs and spices have been the subject of substantial research because of their anti-pathogenic qualities. To assess the potential antibacterial, antiviral, and antifungal properties of EOs, several in vitro and in vivo studies have been carried out (Rodriguez-Garcia I. et al., 2016 & Adame-Gallegos J.R et al., 2016). Due to the growth of antibiotic-resistant strains, the rise in the population with lower immunity, and the rise in drug-resistant biofilm linked infections, studies of this nature are crucial. This research has concentrated on both individual EOs and combinations of EOs, or by extracting the EOs from various herbs and spices using a variety of methodologies.

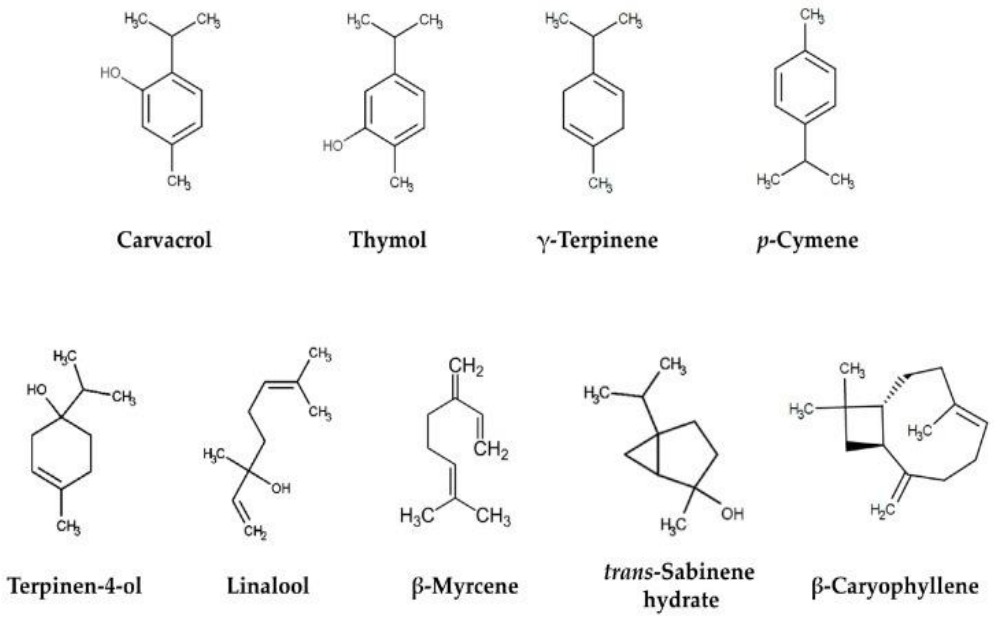

Figure 2.2 Main constitutes of Essential Oregano Oil

2.6 Mode of Action of Essential Oils

Even though numerous modes of action have been hypothesized and researched, the precise mechanisms of EOs' antibacterial activity remain unclear (Lambert et al., 2001; Sikkema et al., 1994). The hydrophobic properties of EO components, which allow them to enter the cell membrane and disrupt its structure and increase its permeability and cause the leakage of cell contents including ions, ATP, nucleic acids, and amino acids, are related to a generalized model (Burt, 2004). Gram-positive and Gram-negative bacteria are two examples of distinct microorganisms that may respond differently to various EOs or EO components. The interaction between the hydroxyl group on the phenolic rings and the cell membrane determines a major portion of the mode of action for phenolic drugs (Thormar, 2011).

The main phenolic ingredient in oregano and thyme oil- carvacrol has the ability to rupture Gram-negative bacteria's outer membrane, releasing lipopolysaccharides and enhancing membrane permeability (Burt, 2004).

2.7 Nanoemulsion Technology

Emulsions are a diverse range of EO delivery systems that are created using biopolymers that are amphiphilic, like as protein, or synthetic surfactants like polysorbates. Microemulsions, nanoemulsions, and macroemulsions are the three subcategories that they belong within. Oil droplets are often contained in small, thermodynamically stable surfactant micelles with a radius of less than 25 nm in microemulsions, which can develop spontaneously (Rao and McClements, 2011b). Due to the higher solubility of EO components in the aqueous phase, carvacrol and eugenol included in nonionic surfactant micelles efficiently prevent the development of Escherichia coli O157:H7 and Listeria monocytogenes (Gaysinsky et al., 2005).

There are many benefits to creating nanoemulsions with essential oils (NEO), including protection from oxidation of volatile EO compounds, higher solubility in the aqueous phase, controlled release of bioactive chemicals, and increased bioactivity due to the emulsion's increased surface area. With the aim of enhancing the microbial inhibitory activities, antioxidant capabilities, and utilization in real food systems, nanoencapsulation has become a significant approach to entrap EOs and bioactive compounds (Dwivedy et al., 2018; Chaudhari et al., 2021a). The term "nanoencapsulation" refers to the process of encapsulating natural substances or compounds in a suitable polymeric matrix having at least one dimension below 100 nanometers (Prakash et al., 2018). Short-term losses in free EO availability at applied food surfaces limit their potential in the food system. Therefore, controlled release may hold great promise for extending the shelf life of food goods. To preserve meat, a controlled release of EOs loaded on zein nanoparticles has been proposed (Xavier et al., 2021).

Energy is needed for the formation of nanoemulsions and can be found in mechanical devices or chemical energy that has been stored in the system (Gutiérrez et al., 2008; Maali and

Mosavian, 2013). The preparation of nanoemulsions can be categorized as either high energy emulsification or low energy emulsification. Surfactants and co-surfactants' physicochemical properties are crucial for low-energy emulsification (Anton et al., 2008). As the system compositions or ambient circumstances are changed, nanoemulsions may spontaneously develop. The two most popular techniques are spontaneous emulsification and phase inversion temperature (PIT) (McClements and Rao, 2011). Spontaneous emulsification is a pretty easy process. A pure aqueous phase and an organic phase made up of an oil, a surfactant, and a water-miscible solvent are combined at a specific temperature to create nanoemulsions (Anton and Vandamme, 2009). High pressure homogenizers, ultrasonic homogenizers, and microfluidizers are a few examples of mechanical tools that can produce strong disruptive forces to reduce droplet size during high energy emulsification (McClements, 2004a). Great pressure homogenizers are the most used emulsifying device to create nanoemulsions in the food sector because of their many benefits, such as easy scaling up, the lack of organic solvents, and high efficiency (Maali and Mosavian, 2013). (McClements, 2004a). According to tradition, high-shear mixer-produced coarse emulsions are pumped into a chamber inside the homogenizer and then forced through a small valve at high pressure (50–200 MPa) (Maali and Mosavian, 2013). This results in strong disruptive forces like turbulence, hydraulic shear, and cavitation that can break up large droplets into smaller ones (Lovelyn and Attama, 2011). Numerous bioactive components, including pharmaceuticals (drugs), nutraceuticals (food components with health benefits), flavor enhancers, and antioxidant properties in foods, can be encapsulated, protected, and delivered using nanoemulsions. It is difficult to effectively use essential oils as an antibacterial agent in food products. Nanoemulsions with EO show improved antibacterial characteristics, a higher surface-to-volume ratio, and easier control over distribution.

3. METHODOLOGY

3.1 Materials

All the physio-chemical tests were conducted in the lab of the Department of Human Environmental Science. The microbiological tests were carried out under the Biosafety Level 2 (BSL2) hood in the microbiology lab at the Department of Biology, STEM building at University of Central Oklahoma, Edmond, OK. In the food science lab of the Department of Human Environmental Sciences, OONE's antioxidant capacity and stability were assessed. Dynamic light scattering method was used to measure the particle size Of Oregano Oil Nanoemulsion using DynaPro Plate Reader II, Wyatt Technology provided by Stanton Young Biomedical Research Center at University of Oklahoma Health Sciences Center, Oklahoma City, OK. Alfalfa seeds and Oregano essential oil was ordered from Amazon online market.

Figure 3.1 Alfalfa seeds

The materials used to perform experiments were Xylose Lysine Deoxycholate (XLD) Agar (Fisher Chemical, Inc.) which is dehydrated culture media, TWEEN®-80 (SigmaAldrich, Inc.) as a surfactant, ultra sonicator (QSONICA, Q700, Hudson Fusion LLC.) to prepare nanoemulsion, magnet stirrer (MS-H280-Pro, Scilogex, LLC.), PBS, distilled water (double

distilled), Themo Scientific alamarBlue TM Dye (Fisher Chemical, Inc.), 96-well plate, Absolute Alcohol 200 (Fisher Chemical Inc.), glutaraldehyde 25% (Fisher Chemical Inc.), Phosphate Buffer Saline Solution (Fisher Chemical Inc), polypropylene bacterial spreader. Pure bacterial cultures of Salmonella enterica (strain- 4293), *Salmonella enteric* subspecies enterica serovar Newport (strain- 2725), *Salmonella enteric* (strain-1708), *Salmonella enteric* (strain-1975), *Salmonella enteric* (strain0172), *Salmonella enteric* (strain-20740) were provided by Dr. Hari Kotturi, Department of Biology, the University of Central Oklahoma, Edmond, OK.

3.2 Bacterial Strains Used and their Source

In this research six strains of *Salmonella* were used from different sources to determine the efficacy of Oregano Oil Nanoemulsion against bacteria from varied sources.

Table 3.1 *Salmonella* Strains Used in this study

Bacterial Strain	Letter Designation	Source	Origin
Salmonella enterica subsp. enterica	Sal_NR_4293	2004 Pennsylvania Tomato Outbreak, Serovar Anatum, Isolate 3	Found in domestic and wild animals
Salmonella enterica subsp. enterica	Sal_NR_172	Larry R. Beuchat, Center for Food Safety, University of Georgia,	Isolated in 1993 from a patient with salmonellosis associated with tomatoes.
Salmonella enterica subsp. enterica	Sal_NR_20740	Tennessee, strain IN01	Isolated in December 2006 from the stool of a 52-year-old patient

Salmonella enterica subsp. enterica	Sal_1708	E2002001708 (Serovar Newport)	Isolated from patient
Salmonella enterica	Sal_1975	Strain 11975 (Serovar Newport	Isolated in 2006 from cattle feces in Washington,
Salmonella enterica subsp. enterica	Sal_2725	SL254 (E20002725) (Serovar Newport)	Isolated in 2000 from a human stool in Minnesota

3.3 Preparation of Nanoemulsion

Oregano Oil Nanoemulsion, 5% v/v oil-in-water was formed using high energy ultrasonication approach. To prepare 50ml of nanoemulsion, 46.25ml of distilled water was measured using a pipette, added in 2.5 ml of oregano essential oil and 1.25 ml of Tween 80 (emulsifier). Initially, Oregano Oil was crudely mixed with Tween-80 (which is a food-grade surfactant) and distilled water using a magnetic stir plate for 30 minutes at a constant speed of 700rpm.

Ultra sonicator (QSONICA, Q700, Hudson Fusion LLC.) was used for the preparation of nanoemulsion through sonication. Throughout the preparation time probe depth was maintained at 3/4th of an inch, diameter of the probe is 0.5 inch. Standardized process was followed for making the nanoemulsion, for which amplitude was set to 60. Frequently, depth of the probe was checked and adjusted accordingly. Range of watts used were between 50-70. Pulse on time was set to 5 seconds and pulse off time was 3 seconds. To counter the heat produced during sonication, the experiment was carried out in a water bath containing ice. Total time for sonication was set to 20 minutes. In addition, a control batch was prepared without Oregano Oil

using only distilled water and Tween 80. Concentrations of Tween 80 and water were changed correspondingly.

Figure 3.2 Ultra sonicator (QSONICA, Q700, Hfffudson Fusion LLC.)

3.4 Formulations of Nanoemulsion

To find out the most stable nanoemulsion composition, several Oregano oil and Tween 80 ratios were tested. To evaluate the stability of the emulsion, Oregano Oil and Tween 80 were mixed in the following ratios: 2:1, 1:1, 1:2, and 1:3. The samples were kept for 3 months in order to test the stability of the Oregano Oil Nanoemulsion. The ideal emulsion for the research was one with the highest stability. The samples were run at 3300 rpm for 30 minutes in a centrifuge machine to examine the stability of the Oregano Oil Nanoemulsion.

3.5 Emulsion Characterization

By employing the technique of dynamic light scattering, the particle size of emulsions was determined (DynaPro Plate Reader II, Wyatt Technology), the formulation with the smallest particle size was chosen for this research.

3.6 Stability of Nanoemulsion

By centrifuging the samples at 3300 rpm for 30 minutes, the stability of the Oregano Oil Nanoemulsion was examined. The stability of the Oregano Oil Nanoemulsion was examined over period of six-month period by centrifuging the sample every 30 days.

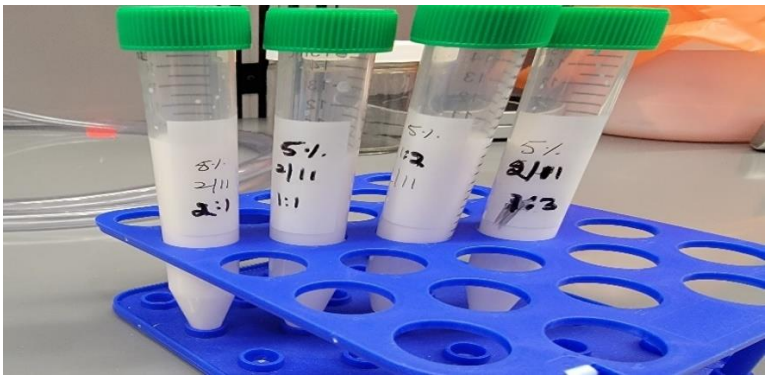

Figure 3.3 Stability of Oregano Oil Nanoemulsion

3.7 Antimicrobial Susceptibility Testing

3.7.1 Broth Micro-Dilution Method

According to recommendations from the National Committee for Clinical Laboratory Standards (NCCLS) for *Salmonella* spp, the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) values were determined using the broth micro-dilution method. To obtain final concentrations of 2.5%, 1.25%, 0.625%, 0.6325, 0.15625%, 0.078125%, 0.039062x5%, 0.01953125%, 0.009765625%, 0.004882813%, 0.002441406%, and 0.001220703% v/v, prepared stock solutions of oregano essential oil (50µl/L) were serially diluted in 96-well plates along with fifty microliters of the overnight prepared inoculum (0.5 McFarland) added to each well. For the purpose of identifying any cross-contamination from one well to another during the handling of plates, negative controls without the corresponding organism and the tested oils were added. After incubation of 24hrs, dye-almar blue was added

15µl/L was added to each well to differentiate the viability of cell, then after 4 hours of incubation with dye, plate was observed through absorbent wavelength 570 and 600 nm with linear shake of 5 seconds at 23°C. A loop of aliquots from wells of MIC and next three diluted concentration of MIC where no visible growth was observed were plated on XLD Agar and incubated at 37°C for 24 hours. Testing was carried out in triplicates.

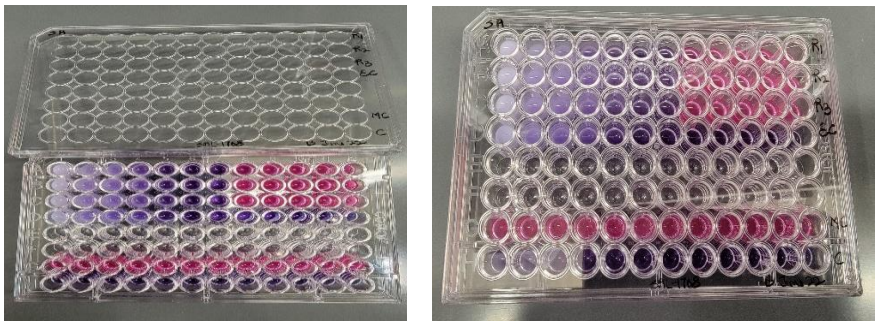

Figure 3.4 MIC on 96-well plate

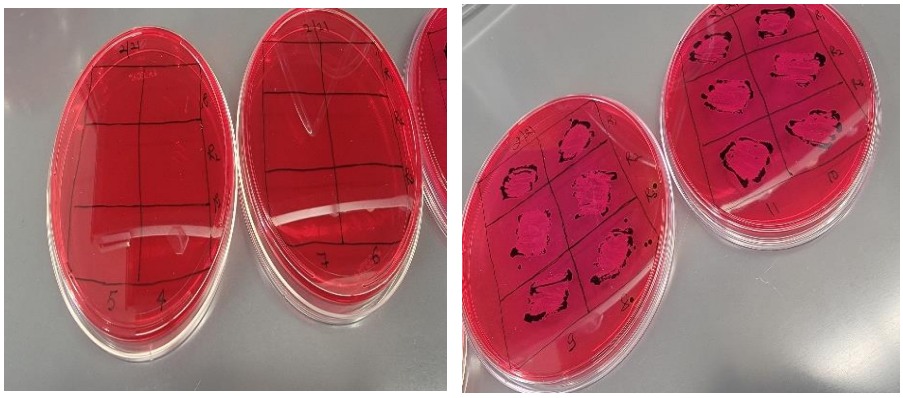

Figure 3.5 MBC confirming the results of MIC

3.8 Kinetic Time-Kill Assay for Nanoemulsions against *Salmonella* spp.

Three strains of *Salmonella* sal_1708, sal_NR_172 and sal_1975 were incubated at 37°C to achieve 0.5 McFarland turbidity of bacterial suspension. Bacterial cultures (2ml) were added to 2ml of 0.078% (MIC value) and 0.15% (2xMIC value) dilutions of 5% OONE. For viability

testing, aliquots of the samples were taken and diluted appropriately in saline solution at room temperature at 0minutes, 5 min, 15min, 30 minutes and 60 minutes.

XLD Agar was spread with measured volumes (100µl) using a disposable spreader, and colonies developed overnight at 37°C in the incubator. The average numbers of colonies were counted from the set of triplicates.

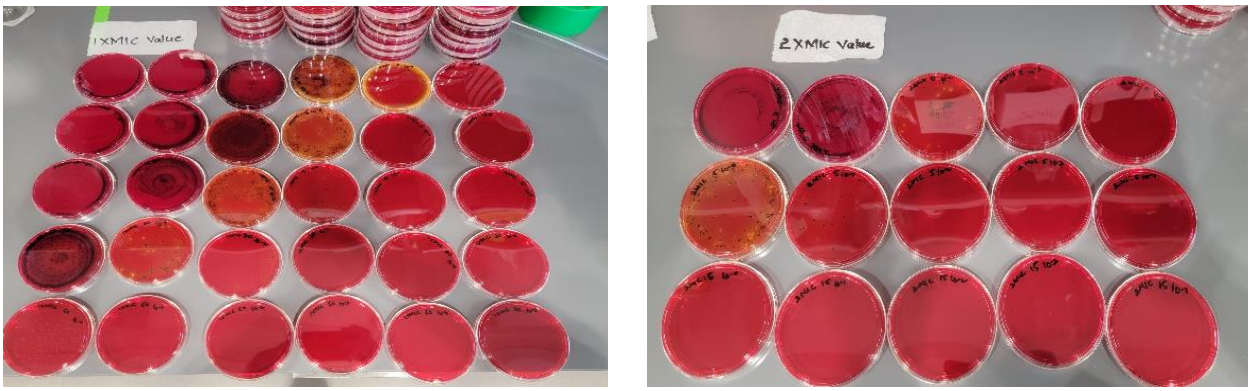

Figure 3.6 Time kill assay (1XMIC value left and 2XMIC value right)

3.9 Preparation of different Oregano Oil Concentrations

The formulation with the smallest particle size was then diluted to obtain two different concentrations of Oregano Oil, 0.5% and 1.0%. using sterile DI water.

3.10 Preparation of Inoculum

For this study, a bacterial cocktail formed of the serotypes NR-2725, NR-4293, and NR-1975 of S. enterica was used. Two of these strains, NR-2725 and NR-2787, were found in hospitals, whilst NR-4336 was found in farms. The different bacterial strains were successively loop-transferred in 15 ml of Nutrient Broth (NB) which was incubated at 37 °C for 24 hours, to produce all of these bacterial strains. All the bacterial strains were mixed and adjusted to 0.5 McFarland using nutrient broth for dilution.

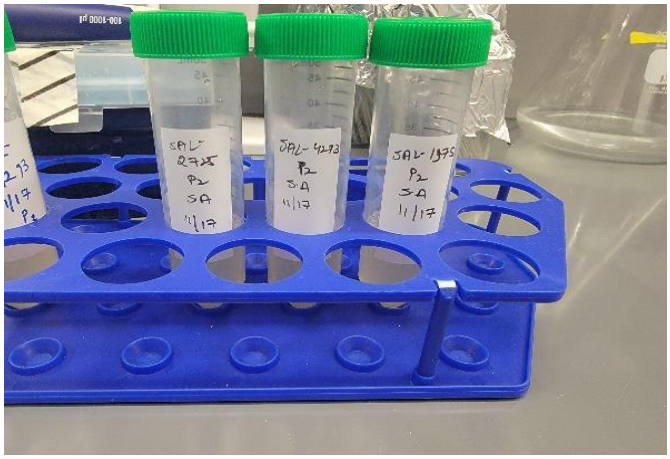

Figure 3.7 *Salmonella* strains

3.11 Practical Application of Oregano Oil Nanoemulsions on Alfalfa seeds and sprouts

3.11.1 Effect of nanoemulsion on germination of alfalfa seeds

Two batches of 100 alfalfa seeds were treated with 1% and 0.5% concentration of Oregano Oil Nanoemulsion. One batch with solution of tween 80 in distilled water was served as control (C1) and another with distilled water only was considered as C2 (Control). Emulsion control for 0.5% and 1% two was also performed. Moistened Whatman No. 1 filter paper with 4ml of nanoemulsion (1% and 0.5%) was placed in petri dishes. Hundred seeds of alfalfa were placed in the petri dishes and 4 ml of water was added. The petri dishes were kept at 25 ± 2 °C for 24 hours in a growth/germination chamber. Seeds that had a 1 mm protruding radicle were considered germinated. To observe the germination of seeds after 24 and 72 hours, regular monitoring was conducted. The number of both germination-proficient and non-proficient seeds was determined after 7 days.

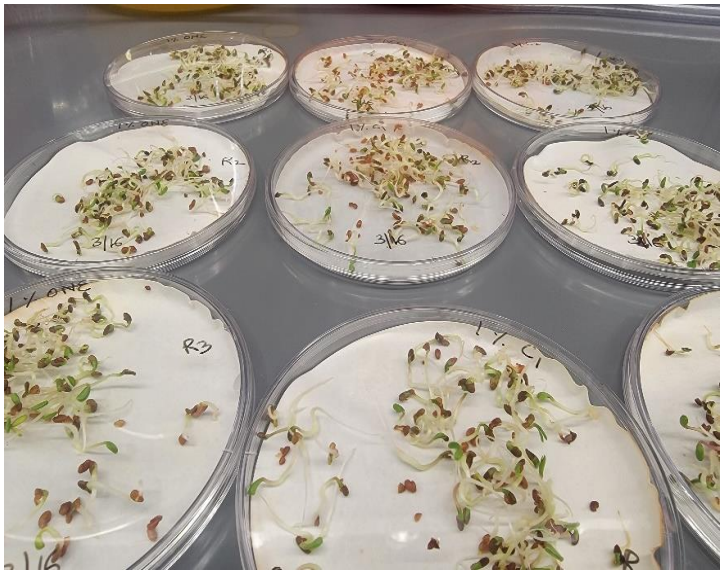

Figure 3.8 Germination of alfalfa seeds

3.11.2 Antimicrobial efficacy of Nano emulsions on alfalfa seeds: Pre-harvest study

To remove any initial contamination, surface of alfalfa seeds was sanitized using Calcium Hypochlorite (20,000 ppm). Alfalfa seeds were coated with *Salmonella* by dipping in the bacterial cocktail suspension, gently agitated and soaked for 5 minutes at room temperature. Suspension was drained and seeds allowed to dry in biosafety cabinet for 24 hours. A total of 7 groups of 1gm dried seeds were divided. After 24hrs of drying, control sample was taken to obtain the initial bacterial load on seeds.

Group 1 seeds underwent a 30-minute treatment/wash in sterile water. For 30 minutes, seeds in groups 2, 3, and 4 were immersed in 0.5% and 1.0% of an emulsion control, respectively. The seeds from the remaining groups were gently stirred into Oregano oil Nano emulsions with concentrations of 0.5% and 1.0% for 30 minutes. Every sampling period (day 0, 3, and 5) involved immersing alfalfa seeds in 50 ml of 1% Phosphate Buffer solution and vortex it several times. For the enumeration of *Salmonella*, diluted samples from each treatment were

individually plated on XLD agar. To determine the antibacterial effectiveness of the nanoemulsion in preventing *Salmonella* contamination of alfalfa seeds, number of colonies formed by surviving pathogens were counted.

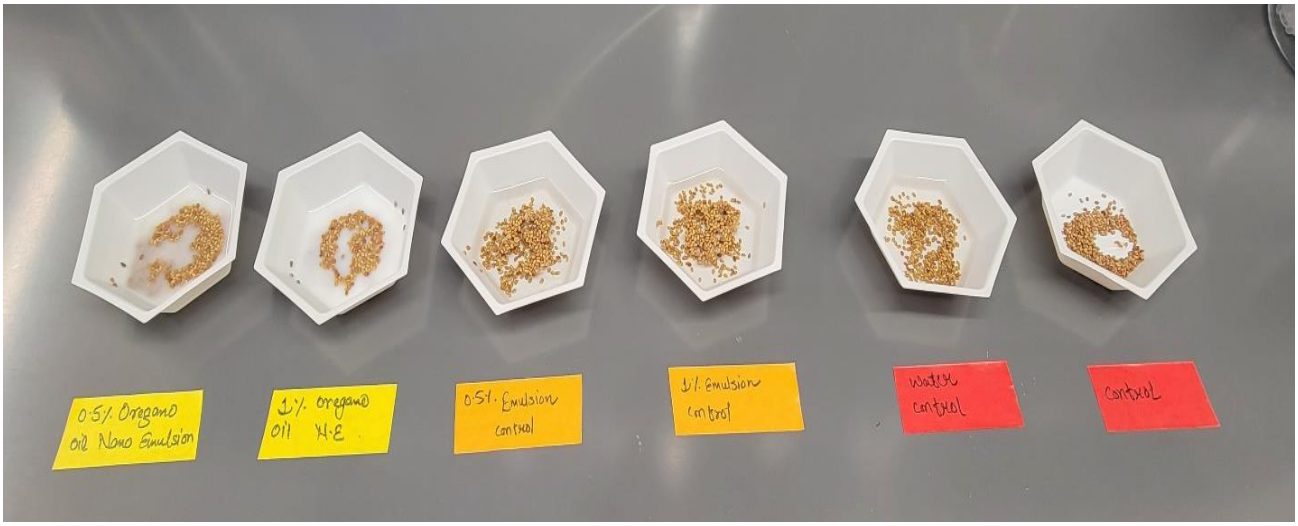

Figure 3.9 Different treatment groups for pre-harvest study

3.11.3 Antimicrobial efficacy of Nano emulsions on alfalfa Sprouts: Post-harvest study

Alfalfa seeds were taken in petri dishes and each dish was filled with 4ml of water. For 24–48 hours, the petri dishes were kept in a growth/germination room at 25 2 °C. Seeds that had a 1 mm protruding radicle were considered germinated. After surface sterilization of alfala sprouts with Calcium Hypochlorite (20,000), sprouts were added to a suspension of *Salmonella*, gently stirred, and then allowed to soak for three minutes at room temperature. Sprouts were rinsed and dried for 24 hours in a biosafety cabinet. A total of 7 groups of 25 dried sprouts were divided. After drying, the control sample was obtained to identify the initial extent of bacterial inoculation. For 30 minutes, sprouts in group 1 were treated/washed in sterile water.

Figure 3.10 Washing of alfalfa sprouts with OONE and EC

Sprouts in groups 2, 3, and 4 were immersed in 0.5% and 1.0% emulsion controls for 30 minutes, respectively. Sprouts from the remaining groups were gently stirred for 30 minutes while submerged in Oregano Oil Nanoemulsions with concentrations of 0.5% and 1.0%, respectively. Every sampling period (day 0, 3, and 5) involved dipping alfalfa sprouts in 50 ml of 1% Phosphate Buffer solution and repeatedly vortexed. Samples from each treatment were diluted and plated on XLD agar for the corresponding identification of the *Salmonella* species. To determine the antibacterial effectiveness of the nanoemulsion in reducing the contamination of alfalfa sprouts with *Salmonella*, the number of pathogens that survived was counted.

3.12 Microbial counts

After culture inoculation at 0day, 3day and 5 days of storage, the bacterial counts were processed in triplicate. Sterilized conditions were used for every treatment. For each treatment, 1 gram of alfalfa sprouts and seeds were used. Following that, samples were diluted and plated onto XLD agars for the identification of *Salmonella* species. The experiments were all carried out in triplicate.

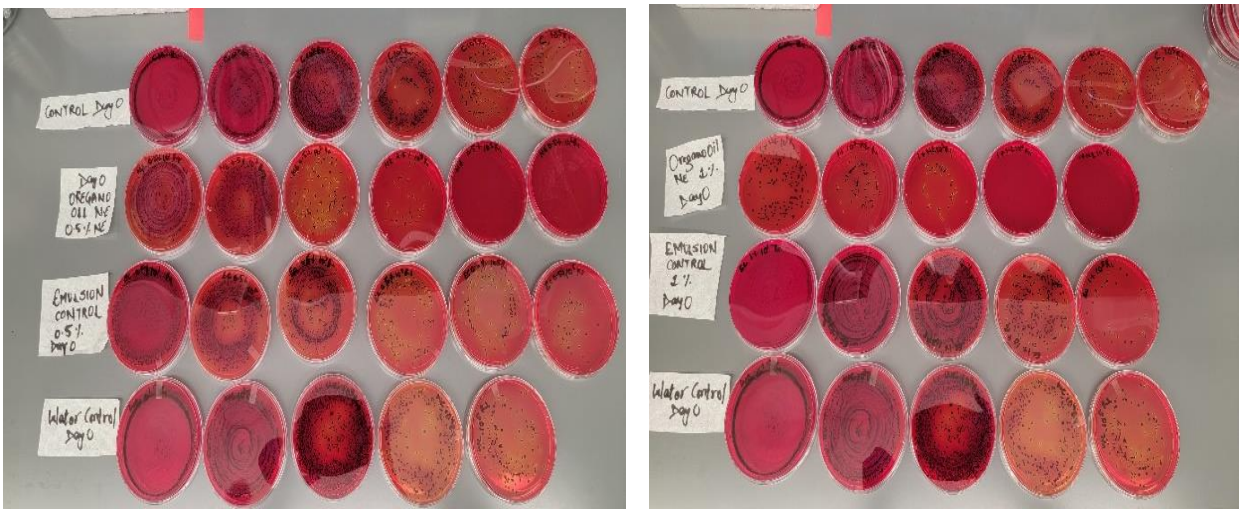

Figure 3.11 Plate count in triplicates

3.13 Statistical Analysis

All the experiments were performed in triplicates. *Salmonella* population reduction was identified, and the effectiveness of various time treatments was examined. Using a standard Microsoft Office Excel program, simple statistics analysis was carried out to assess the significant difference between all types of treatments and control samples.

4. RESULTS & DISCUSSION

4.1 Nanoemulsion formulation and Characteristics

Table 4.1 Characterization of different formulations of nanoemulsion

Formulation	Radius (nm)	Polydispersity (nm)
Control-2.5%T, 10 min sonication	2157.18±184.42	1231.31±36.01
NE-2.5%T, 10 min sonication	139.85±0.41	74.74±0.89
Control-2.5%T, 20 min sonication	3571.23±1.26	2038.12±7.26

NE- 2.5%T, 20 min sonication	149.83±4.89	50.61±0.77
Control-5%T, 10 min sonication	5582.61±354.91	3186.53±24.8
NE-5%T, 10 min sonication	101.08±2.52	34.08±0.77
Control-5%T, 20 min sonication	5606.78±6.39	3201.98±3.37
NE- 5%T, 20 min sonication	88.18±17.05	32.53±3.98

Legend: T= tween 80 (food grade surfactant), S= ultrasonication, nm= nanometer

All samples of emulsion contain 5% of Essential Oregano Oil. The creation of Oregano Oil Nanoemulsion involved the application of high energy ultrasonic emulsification. This is an effective approach to deliver plant based essential oils into food matrix. Emulsion used in this research has longer stability and smaller particle size. Important factors like particle size and polydispersity were determined using the approach of dynamic light scattering. The size of particle affects polydispersity. Particle size of an emulsion needs to be smaller in order to get more homogeneous nanoemulsion. The compressive and tensile stresses produced by these ultrasonic waves ultimately lead to a reduction in the mean droplet diameter size (MDDS). All of the various oregano oil formulations' droplet sizes and polydispersity were measured using the dynamic light scattering (DLS) method. It operates by shining a laser beam through the sample and using a quick photon detector to track variations in scattered light at a predetermined angle (Ren et al., 2019). The formulation 5% Nanoemulsion with 20-minute sonication has radius of 88.18±17.05 and polydispersity of 32.53±3.98.

Particle and polymer size and mass distribution are topics covered by polydispersity. The particle size distribution is more homogenous the lower the polydispersity index is. In my

investigation, a formulation made with 2.5% tween and 5% oregano oil was employed with an ultrasonication time of 20 minutes. According to data gathered using the dynamic light scattering method, the average particle has a radius of 149.83 nm, is 50.61 nm polydisperse, and has a polydispersity index of 0.33. The computed standard deviation for the chosen formulation was 4.98, 0.77, and 0.0, respectively.

The homogeneity in the distribution of molecules in a given mixture is often represented by the polydispersity index (PI), also known as the heterogeneity ratio. The nanoemulsion formulation's lower PDI values shows that the droplet size distribution is homogeneous and supports the homogeneity of the droplets as previously reported. The optimal formulation ratio was chosen for the creation of the nanoemulsion based on the optimization analysis. It serves as a gauge for the molecular weight distributions in the emulsion solution that has been created. The degree of consistency and uniformity of the particles in the solution is also estimated using PDI values (Gulotta, Saberi, Nicoli, McClements, & Chemistry, 2014). The distribution of droplet sizes is diverse when the PDI value is close to 1 (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, Martn Belloso, & Technology, 2013). The average polydispersity index For Oregano Oil Nanoemulsion was determined to be 0.39, which, accordingly, ensures acceptable homogeneity and consistency between the distributions of size droplets. The average droplet size and polydispersity index reported in a study by Bhargava et al., 2015 on the use of Oregano Oil Nanoemulsion to suppress foodborne pathogen on fresh lettuce, agree with my findings for particle size analysis. Particle size, PDI, and zeta potential of cinnamaldehyde emulsions made with various formulations were measured in another study by Asmawati et al. (2015). According to the study, the average particle size was 105.53 nm, and the polydispersity index was 0.28. All these findings matched the outcomes I found for oregano oil.

4.2 Nanoemulsion Stability

To create a stable Oregano Oil Nanoemulsion, various nanoemulsion formulations were created in order to identify the ideal ratio of tween 80 and ultrasonication time. The OONE made with 2.5% tween and an ultrasonication time of 20 minutes was determined to be as stable as 5% tween 80 nanoemulsion formulation. Nnaoemulsions were prepared as 1:1 ratio of Oregano Oil and Tween 80 and 2:1 ratio or EOO and Tween 80. Oregano Oil Nanoemulsion compositions were centrifuged at 3300 rpm for approximately 25 minutes to test their stability (Moghimi et al., 2016). Both the emulsions were stable for 3 months without showing any separation of oil and water or layer formation on the surface of nanoemulsion.

4.3 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration

Table 4.2 MIC and MBC value of OONE

Bacterial Strain	MIC	MBC
Sal_1708	0.078%	0.078%
Sal_NR_172	0.078%	0.078%
Sal_1975	0.078%	0.078%
Sal_20740	0.078%	0.078%
Sal_2725	0.078%	0.078%
Sal_NR_4293	0.078%	0.078%

The average Minimum Inhibitory concentration for *Salmonella* strains and the average Minimum Bactericidal Concentration of Oregano Oil Nanoemulsion for *Salmonella* were both 0.078% v/v. Volatile components of oregano oil also showed inhibitory properties against

Salmonella, in MIC volatiles components from Oregano Oil Nanoemulsion were interfering the growth of bacteria in control. In a related work, Bhargava et al. (2015) discovered that the Oregano Oil Nanoemulsion had a higher MIC value for *L. monocytogenes, S. Typhimurium*, and *E. coli* at 0.0625% than what we found for cinnamon oil nanoemulsion for *Salmonella*. In a similar vein, Bhargava et al. (2015) reported MBC values for *S. Typhimurium* at 0.625%, which is greater than what we have for *Salmonella*, but reported a lower MBC of 0.125% for E. Coli, which is considerably lower than ours. Tween 80 had no impact on bacterial growth, according to studies as well.

According to reports, essential oils often exhibit more antibacterial action against Gram-positive bacteria than Gram-negative bacteria (Semeniuc et al., 2017). The nature of Gram-positive bacteria allows the hydrophobic molecules of oils to easily pass-through cell walls and hence can affect both the cell membrane and the cytoplasm, which accounts for the increased antibacterial action of EO (Bakkali, et al., 2008). The bioactive components of oregano oil are able to penetrate through a thin outer barrier that is present outside of a thin peptidoglycan layer when a nanoemulsion is created by sonication, which enhances the antibacterial activity of oregano oil on Gram-negative bacteria as well (Landry et al., 2016).

The MIC of nanoemulsified thymol/eugenol against *L. monocytogenes* Scott A in TSB was greater than that of the free thymol/eugenol in earlier investigations where thymol (Shah et al., 2013a) and eugenol (Shah et al., 2013b) nanoemulsions were produced with WPI-MD conjugate utilizing the emulsion. After being prepared as nanoemulsions with smaller droplets, eugenol and carvacrol similarly displayed decreased antibacterial activity (Terjung et al., 2012). This indicated a stronger degree of interaction with the emulsifier Tween 80 and correlated with a lower concentration in the continuous aqueous phase. The identical MICs of the nanoemulsion

generated with PG and free thymol, which are lower than those of the emulsion without PG, may be physically related to the weaker binding of thymol with WPI-MD conjugate by PG due to a modest change in polarity and perhaps due to the synergistic antibacterial activity of thymol and PG. The obtained MICs for *Cladosporium sp*. exceeded those discovered by Zabka et al. for *Cladosporium cladosporoides* (0.028-0.066 g/ml). Daferera et al. and Stevi et al. discovered MICs of oregano essential oil for *Fusarium sp*. that were 150 g/ml and 70-1160 g/ml, respectively—values that were significantly higher than those in this investigation.

4.4 Kinetic Time killing

Salmonella enterica (strain-1708), *Salmonella enterica* (strain-1975),and *Salmonella enterica* (strain-0172), were tested for the kinetics of Oregano Oil Nanoemulsion's antimicrobial activity. Dilution ranges were 0.5% and 1.0%. The graph below displays the study's overall presentative data.

All the *Salmonella* strains had bacterial colony counts were counted for 1XMIC value and 2XMIC value of Oregano Oil Nanoemulsion. All bacterial strains had decreased by about 2 logs by the end of 30 minutes in both the concentration treatments. In data is shown that all the bacterial cells were dead after 60 minutes of treatment with 1xMIC value Oregano Oil Nanoemulsion. However, 2xMIC value of nanoemulsion showed more effective and rapid results against *Salmonella enterica* strain 1708, 1975 and NR-172.

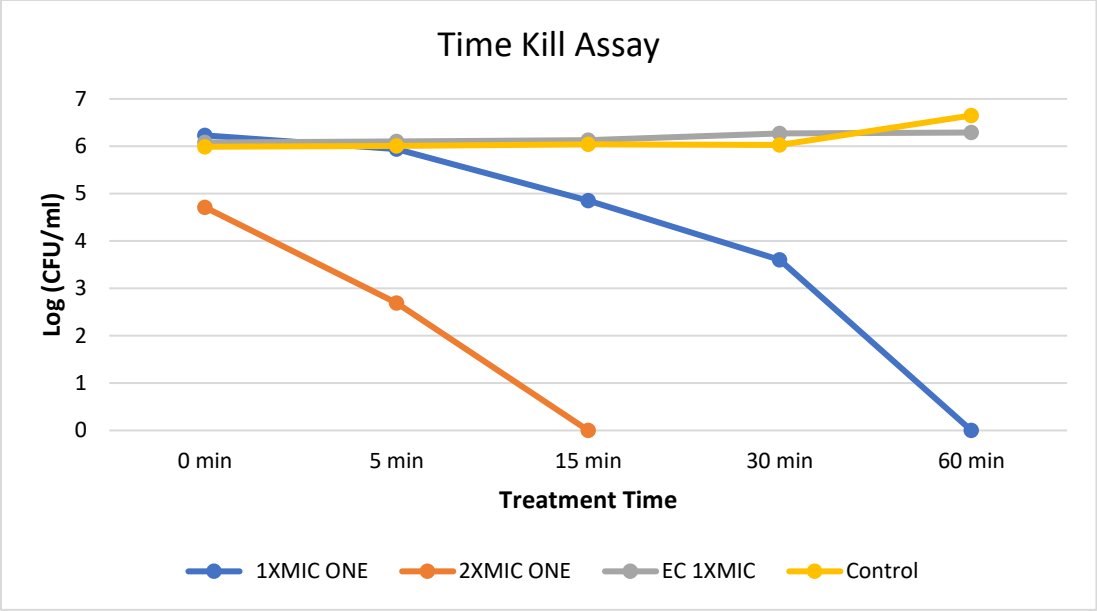

Figure 4.1 Kinetic time killing with nanoemulsion

4.5 Effect of Oregano Oil Nanoemulsion on germination of Alfalfa seeds

Outcomes showed nanoemulsion does not have any inhibitory effect on the growth of sprouts in alfalfa seeds with the treatment group of 1% ONE and 0.5% ONE. Our findings indicated a negligible lesser germination rate in the treatment groups. With both water and emulsion control, alfalfa seeds germinated at nearly the same rate. That is supported by the statistics and charts.

ONE: Oregano Oil Nano Emulsion

GP= Germination Percentage

$$GP = \frac{n}{N} x100$$

n = Number of newly germinating seeds.

N = Total number of seeds.

GS= Germination Speed

GS = $R_s = \sum_{i=1}^{n} Si/Di$

R_s = germination speed (number of seeds per day).

Si = Number of germinated seeds.

Di = Number of days.

Germination speed and germination percentage over the period of 48hrs is calculated by above mentioned formulas.

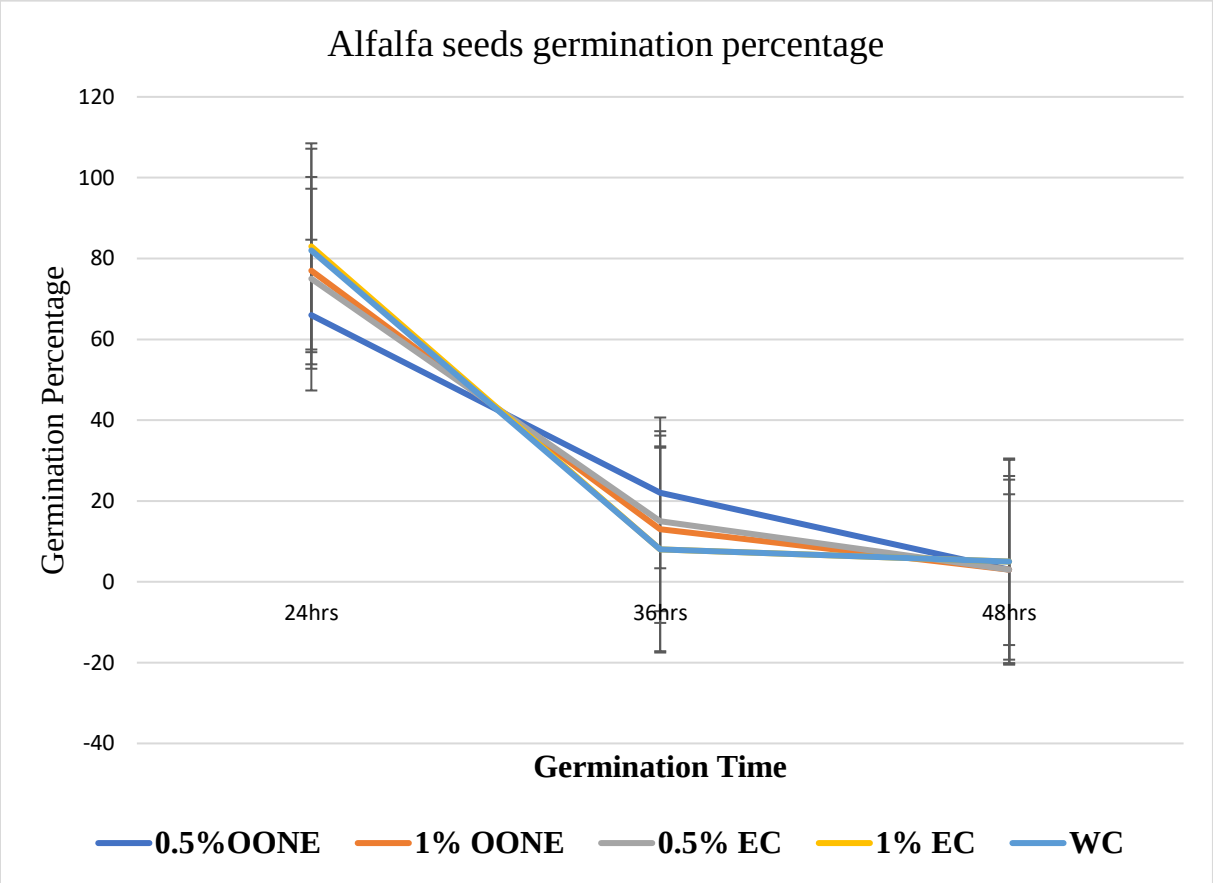

Figure 4.2 Germination percentage of alfalfa seeds

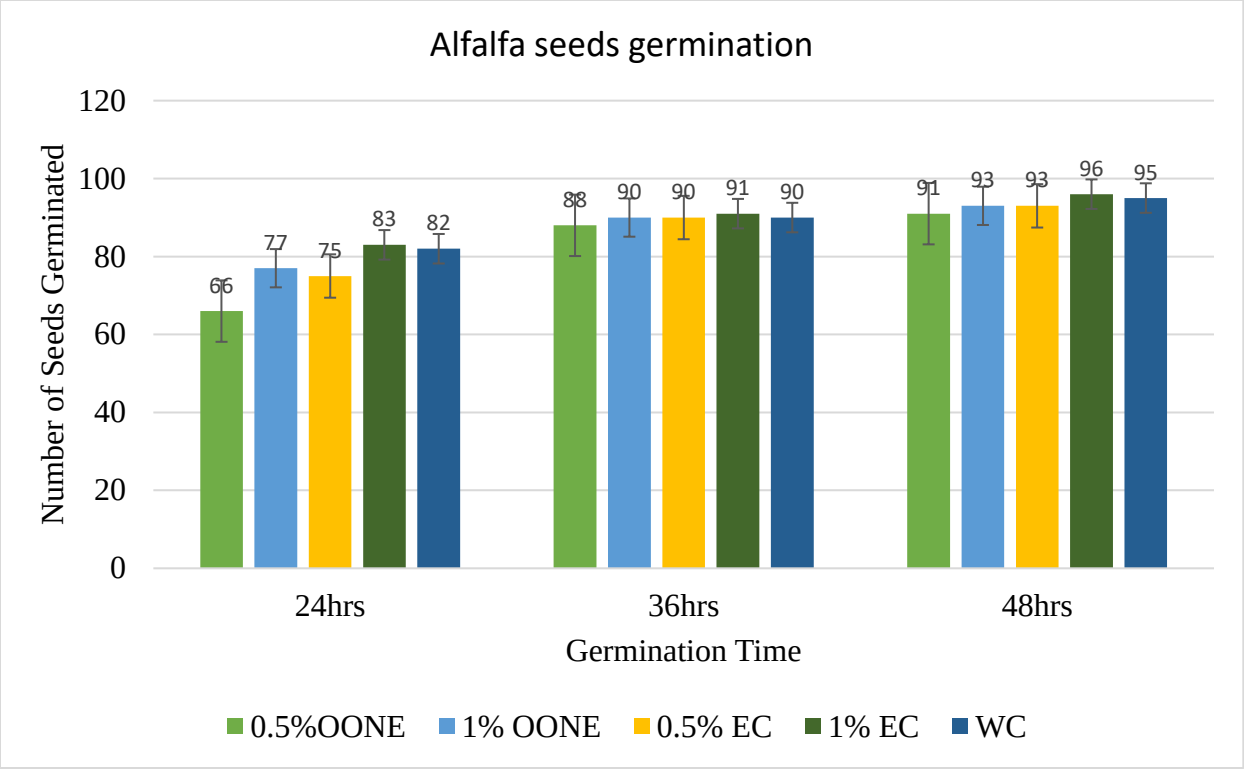

Figure 4.3 Number of seeds germinated in 48 hrs

4.6 Efficacy of Oregano Oil Nanoemulsion to control *Salmonella* in alfalfa seeds: Pre harvest

For alfalfa seeds, the initial *Salmonella* concentration was 6.96(CFU/gm) on day 0 before treatment with different concentrations of nanoemulsion. Microbial populations were measured following treatment at three separate time points (0 hours, 72 hours, and 120 hours) (Figure). Oregano Oil Nanoemulsion's ability to prevent microbial growth was effective at both concentrations 1% and 0.5%. *Salmonella* levels significantly differed between the treatments of 0.5% and 1.0% at 0 day and 3 day ($p<0.05$) compared to the control, which eliminates the null hypothesis of no difference in the control and treatment groups. On day 5, there was also a discernible difference between the treatments and the controls ($p<0.05$)

Table 4.3 Bacterial count during storage of seeds after treatment

Treatment Group & Storage time	Day 0 Log (CFU/gm)	Day 3 Log (CFU/gm)	Day 5 Log (CFU/gm)
Control	6.96	7.78	8.69
Water Control	6.08	7.76	8.63
0.5% OONE	4.61	5.61	6.10
1% OONE	2.83	4.93	5.67
0.5% EC	5.94	6.79	9.02
1% EC	6.05	6.97	8.88

One gram of alfalfa seeds was inoculated with 0.5 McFarland culture of *Salmonella* cocktail suspension, after drying for 24 hrs, samples for control were taken to know the initial bacterial load, remaining groups of 1gm seeds were treated with Nanoemulsions and Emulsion controls. Initial population on the seeds found to be 6.96 log (CFU/gm). On day 0, treatment with 0.5% OONE showed 1.47 log reduction as compared to water control (6.08), however, 1% OONE treatment showed better results with reduction of 3.25 log in comparison with water control.

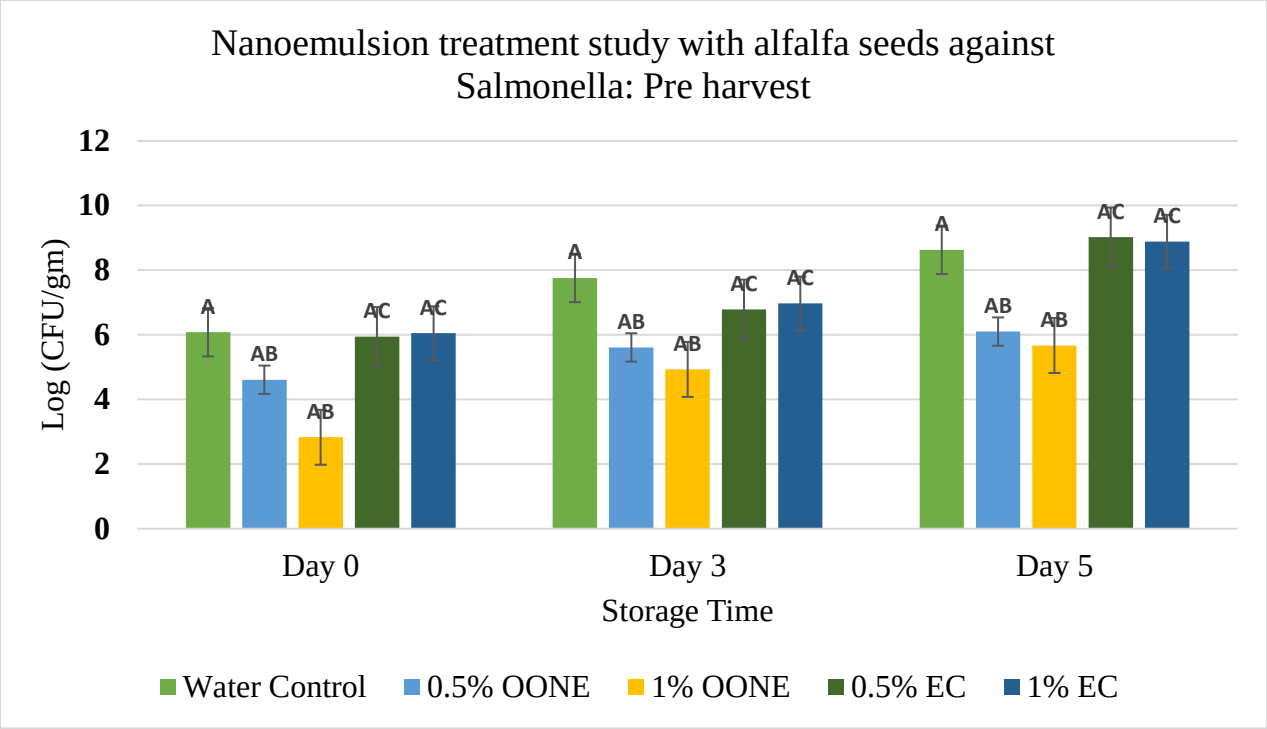

Figure 4.4 Nanoemulsion treatment study with alfalfa seeds against *Salmonella*: Pre-harvest

Emulsion Control= the mixture of Tween 80 and water only with ultrasonication; 1%= 1% of Oregano Oil Nanoemulsion; 0.5%= 0.5% of Oregano Oil Nanoemulsion. Error bars represent the standard deviation from mean value among sampling intervals (0day, 3day and 5day) for each treatment.

A = Water Control, AB= Where p value or significance is lesser than 0.05 and AC where p value is higher than 0.05.

During the storage time of day 3 and 5, there was a significant difference between the microbial load on oregano oil treated seeds and water control. However, emulsion control had no impact on the bacterial count in the seeds which means $p>0.05$. Both the concentrations of Oregano Oil Nanoemulsion showed inhibitory effect against the *Salmonella* in pre-harvest treatment study.

When Oregano Oil Nanoemulsion was applied to fresh lettuce in a study conducted by Bhargava et al. (2015), the results showed a log reduction of 2.18 log at 0.1% versus 2.31 log reduction at 0.05% at 24 hours for *Salmonella*. My findings agree with the log reductions reported by Bhargava et al. (2015). *Salmonella* has a lower log decline than other foodborne infections because it has an extra layer of protective polysaccharides outside the cell. This layer guards the Oregano Oil Nanoemulsion's active ingredients from damaging the cell cytoplasmic membrane and altering the structure of the membrane.

4.7 Efficacy of Oregano Oil Nanoemulsion to control *Salmonella* in alfalfa sprouts: Post harvest

The initial bacterial count on sprouts was higher than seeds, it was 9.38 log (CFU/gm), after washing with water, bacterial load reduced by 1.37log and evaluated as water control to determine the efficacy of nanoemulsion in comparison with water washed sprouts. 0.5% OONE treatment showed 1.83 log reduction at 0 day of treatment and 1% OONE treatment inhibit the *Salmonella* growth by 2.7 log.

Table 4.4 Bacterial count during storage of sprouts after treatment

Treatment Group & Storage time	Day 0 Log (CFU/gm)	Day 3 Log (CFU/gm)	Day 5 Log (CFU/gm)
Control	9.38	11.22	14.18
Water Control	8.01	11.03	13.06
0.5% OONE	6.18	8.06	8.94
1% OONE	5.31	7.15	7.97
0.5% EC	8.10	11.10	12.96
1% EC	7.96	11.01	13.04

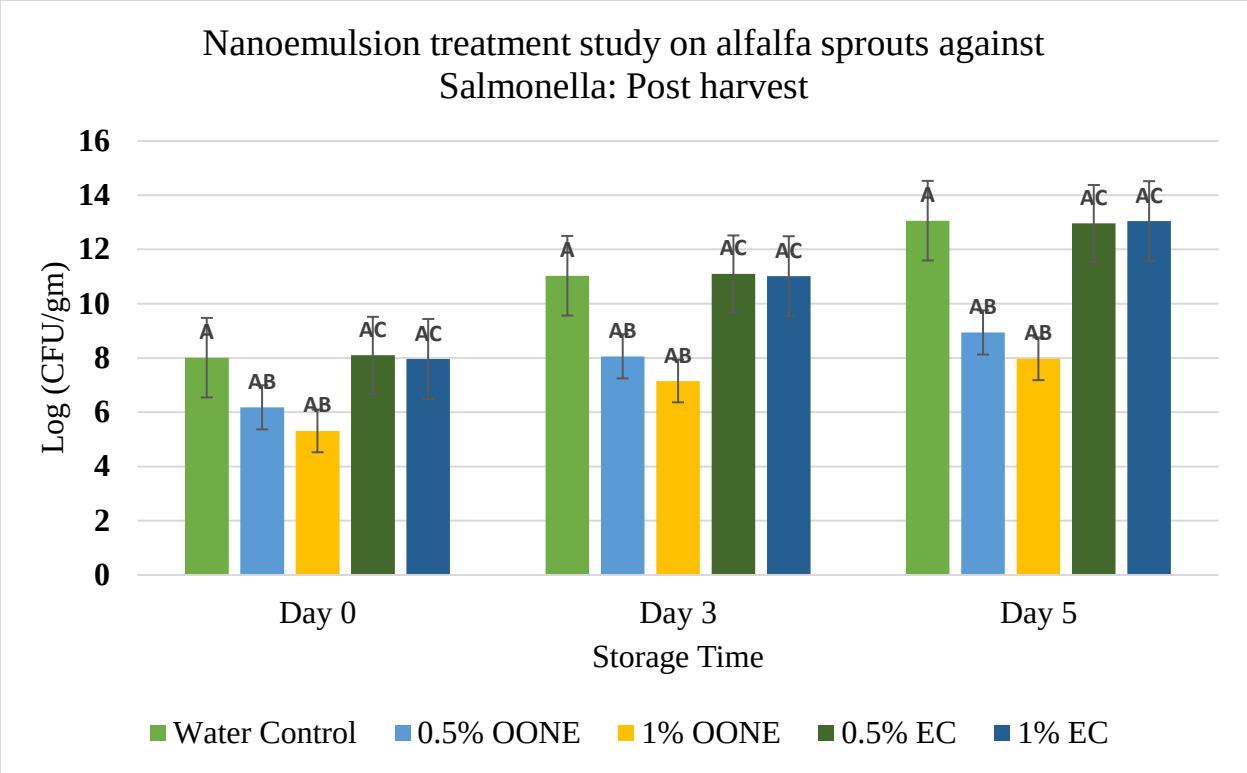

Figure 4.5 Nanoemulsion treatment study on alfalfa sprouts against *Salmonella*: Post harvest

Emulsion Control= the mixture of Tween 80 and water only with ultrasonication; 1%= 1% of oregano oil nanoemulsion; 0.5%= 0.5% of oregano oil nanoemulsion. Error bars represent the standard deviation from mean value among sampling intervals (0day, 3day and 5day) for each treatment.

A = Water Control, AB= Where p value or significance is lesser than 0.05 and AC where p value is higher than 0.05.

During the storage time of day 3 and day 5, increase in bacterial count in OONE treated samples was lesser than water and emulsion controls. In the first 3 days of storage, water control had around 70% more bacterial growth than OONE treatments and by 5^{th} day it was almost doubled the nanoemulsion treatment groups. At 0day, 3 day and 5-day storage, there was a significant difference in the control and treatment groups ($p<0.05$).

Fresh vegetables can become contaminated by foodborne pathogens at any stage along the line from farm to fork. However, fieldwork, initial processing, and home preparation of fresh vegetables are all potential points of contamination (Jacobsen & Bech, 2012). Finding a treatment with potent antibacterial effects while preserving the sensory qualities of food products is therefore crucial. Thyme, cinnamon, oregano, and clove are just a few examples of the bioactive chemicals found in plant essential oils that have been shown to have somewhat greater antibacterial action (Sipailiene et. al., 2006).

This study showed the antimicrobial efficiency of Oregano Oil Nanoemulsion against *Salmonella* in seeds and sprouts by significant reduction in bacterial plate count. However, the growth of *Salmonella* continued during storage, but it was slower than control groups, this is due to the volatile antimicrobial compounds of oregano oil were not intact on seeds longer so during the storage period efficacy of nanoemulsion reduced. The results of a study conducted by Bhargava et al. (2015) on the use of Oregano Oil Nanoemulsion to reduce foodborne bacteria on lettuce showed 3.26 log reductions, which is a little greater than what we discovered during our studies. This might be because alfalfa seeds and sprouts had a considerably greater starting concentration of *Salmonella* (6.96 log and 9.38 log respectively). Additionally, compared to Gram-positive bacteria, Gram-negative bacteria are said to be more resistant to essential oils. *Salmonella* is more resistant to treatment with essential oils due to the existence of an extra lipopolysaccharide layer that blocks the entry of any hydrophobic substances into the bacterial cell.

5. CONCLUSION & FUTURE DIRECTIONS

In this research Oregano Oil Nanoemulsion was formulated using a high energy sonication technique by applying different time intervals and energy in order to obtain different characteristics of nanoemulsion. Also, the emulsion was prepared in various concentrations. The basis of the selection of nanoemulsion for research application is radius size and polydispersity index. Nanoemulsion formulation with the smallest radius size and minimum polydispersity index was considered as the most optimized formulation and used that standardized process to make Oregano Oil Nanoemulsions used throughout the research project.

The radius of the nanoemulsion formulation with 2.5% tween-80 sonicated for 20 minutes was found to be 149.83±4.89 nm, and the polydispersity index (PI) was found to be 0.33 0.0% using the dynamic light scattering (DLS) method. The antimicrobial efficacy of OONE was evaluated against six different strains of *Salmonella enterica* obtained from different sources. Results of antimicrobial susceptibility tests (Minimum Inhibitory Concentration and Minimum Bactericidal Concentration) were 0.0781%v/v for *Salmonella*, MIC and MBC were determined by using the broth dilution method. This result indicates the lowest concentration of Oregano Oil Nanoemulsion which can inhibit the growth of pathogenic bacteria *Salmonella.*

Numerous formulations of Oregano Oil Nanoemulsion were utilized on the alfalfa seeds and sprouts against *Salmonella*, from 5% original OONE two other formulations of 0.5% and 1%v/v were prepared by dilution and used in pre-harvest and post-harvest study treatments. Kinetic time killing was evaluated to determine the time minimum concentration of OONE takes to inhibit the bacteria, it was observed 60 minutes for 1XMIC value and 10 minutes for 2XMIC

value of OONE. We concluded the results that 2XMIC value has a higher efficiency in killing the bacteria and reduced the bacterial load to half within 5 minutes.

Furthermore, 1% Oregano Oil Nanoemulsion showed higher log reduction in both seeds and sprouts against *Salmonella*, which is 3.25 log in seeds and 2.7 log in post-harvest treatment (sprouts). However, 0.5% OONE reduced the bacterial count by 1.47 log and 1.83 log in seeds and sprouts respectively. Hence, both the concentrations of Oregano Oil Nanoemulsion are effective in inhibitive the growth of foodborne pathogenic bacteria *Salmonella* in fresh produce. The log reduction was compared with water controls which means the *Salmonella* coated seeds were washed with water once to obtain the bacterial count in water control for identifying the effect of washing on *Salmonella*. In order to know the initial bacterial population one group of seeds was considered as control. All the experiments were conducted in triplicates and emulsion control (mixture of tween 80 and water) was kept as a group to check whether the presence of tween 80 (surfactant) has any inhibiting effect on *Salmonella.*

With the help of the germination investigation, we concluded that nanoemulsion is not inhibiting the alfalfa seed germination. Overall, various Oregano Oil Nanoemulsion compositions were effectively created and optimized. The concentration of oregano oil utilized, and the length of storage time affect the overall efficacy of Oregano Oil Nanoemulsion treatments. In alfalfa seeds and sprouts, both concentrations of Oregano Oil Nanoemulsion (0.5%, 1%) were discovered to be efficient in preventing the growth of various *Salmonella* strains in both pre-harvest and post-harvest treatments. This recommends that Oregano Oil Nanoemulsion be used as a safe and sustainable food preservation technique for fresh produce.

Future studies about the process of active components of oregano oil like carvacrol which has antimicrobial properties and inhibit bacterial growth can be done to figure out the mechanism

of action of these active compounds. Moreover, during antimicrobial susceptibility testing it was found that volatile compound of oregano oil also has inhibitory properties, OONE volatiles interfere with the control groups in adjacent wells of control samples and hindered the growth of *Salmonella.* So, application of oregano oil in vapor form in food matrix can be considered as future research scope. Moreover, studies on safety of Oregano Oil Nanoemulsion treated food can be done to assure there is no harm in consuming food treated with essential oils.

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