10-25-2017

Isolation, Detection, and Prevention of Foodborne Bacteria in Irrigation Water and Dairy Food

Mohammed Saleemallah Alhejaili
Louisiana State University and Agricultural and Mechanical College

ISOLATION, DETECTION, AND PREVENTION OF FOODBORNE BACTERIA IN IRRIGATION WATER AND DAIRY FOOD

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

School of Nutrition and Food Sciences

by
Mohammed Saleemallah W Alhejaili
BSc, Taibah University, 2008
MSc, Heriot Watt University, 2011
December 2017

To my family and beloved city of Medina, Saudi Arabia

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my major advisor, Dr. Marlene E. Janes for guidance and support. Also, I would like to thank my research committee members, Dr. Kayanush J. Aryana and Dr. Achyut Adhikari for their guidance, assistance, support, and encouragement during my research and for allowing me to complete my projects in their laboratories.

I would like to express my deepest gratitude to Dr. Douglas Olson and Dr. Charles Boneke for helping me make yogurt in the dairy science plant. I would like to acknowledge Dr. M. Antonieta Guerrero-Plata for accepting to serve as the Dean's representative.

I also would like to thank my lab mates, Dr. Naim Montaziri, Dr. Evylen Watts, Dorra Djebbi-Simmons, and Carmen Velázquez for their assistance and participation.

My sincere appreciations to Mr. Anthony Bridges for allowing me to collect water samples from the Ben Hur Farm and also for Stephen Peterson R&D Ph.D. from Zeotech Corporation for providing me with the zeolites.

This work is supported by the USDA National Institute of Food and Agriculture, Hatch project accession number 1008762. Also, I would like to thank the farmer at Ben Hur farm for their help in collecting water samples.

Last but not least, my love goes to my family in Saudi Arabia and to my beloved city of Medina.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Foodborne microorganisms can be beneficial to humans or cause disease. *Salmonella* is one of the most causative foodborne disease outbreaks in the U.S.A. *Salmonella* has been detected in surface water used in irrigation, and can survive for weeks to years in water. The purpose of the study was to detect low levels of *Salmonella* spp. by comparing surfactant modified zeolite (SMZ) filtration and Activated Carbon (AC) filtration with the Environmental Protection Agency (EPA) method 1200. Also, to determine the generic *E.coli* counts and if there was a direct correlation of *Salmonella* to generic *E. coli* in irrigation water. The EPA method detected *Salmonella* at lower levels compared to the SMZ and AC methods. Generic *E.coli* counts for surface water had no significant difference between EPA 1603 and Quanti-Tray methods (p value > 0.05). Whereas, Generic *E.coli* counts for surface and sediments using Quanti-Tray method had significant difference (p value < 0.05). Base on the Food Safety Modernization Act for agricultural water's rule, the generic *E.coli* were higher than 126 > CFU/100 ml which shows correlation to *Salmonella* in irrigation water. On the other hand, probiotics are good bacteria to consume for the digestive system. Yogurts contain the probiotic bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. In order to protect yogurt from contamination and spoilage, an antimicrobial called Myrrh was added to the yogurt and studied for 5 weeks. Myrrh is a natural flavoring substance approved by FDA as a food flavor. The objective was to determine the effect of myrrh on *S. thermophilus* and *L. bulgaricus* counts in peptone water and in the yogurt matrix. The pH, titratable acidity, viscosity,

and color of yogurts measured for 5 weeks. Myrrh dispersion was prepared and incorporated into yogurt at a 1% v/v level. A control with no myrrh was also prepared. There was significant difference between *S.thermophilus* counts and *L. bulgaricus* counts in yogurt myrrh and control yogurt. Only minimum changes during storage were observed for color. With little to no change in yogurt pH and titratable acidity, and viscosity yogurt culture bacteria can survive in the presence of myrrh within yogurt.

CHAPTER 1. LITERATURE REVIEW

1.1 Microbial indicators in water

The indicator bacteria is used to estimate and detect fecal contamination in water and used to indicate health concerns. There are seven criteria for a good microbial indicator of fecal contamination in water. Microbial indicators should have the following criteria: (I) The bacteria should present in higher counts in human intestine tract and feces (II) The bacteria should be non-pathogenic (III) The bacteria should be cheap, reliable, and easily be detected in the environmental waters. (IV) The bacteria counts should be stable and do not multiply outside of the enteric environment. (V) Most importantly, in environmental waters, the bacteria may be exist in high counts than other pathogenic bacteria; (VI) The indicator bacteria and pathogenic bacteria should have similar death phase (VII) If human fecal contamination can be separated from the animal source, the indicator bacteria should be easily distinguish from the intestine of domestic and farm animals *(5)*.

1.2 Escherichia .coli

1.2.1 *Escherichia .coli* morphology

The first identification was by the German pediatrician Theodor Escherichia during his researches on infants intestinal flora in 1885 *(11)*. *E.coli* is Gram-negative bacteria non-spore forming, facultative anaerobic of the enterobacteriaceae family *(1)*. *E. coli* is rod shape cells 2.0-6.0 um length and 1.1-1.5 um in width that are motile by flagella. They produce gas to ferment carbohydrates *(11)*.

1.2.2 Survival in the environment

E. coli can be detected as a part of microbiota in the gastro intestinal tract of mammals. *E. coli* is well known that it can survive in the environment but cannot reproduce*(11)*.

E.coli is generally harmless, however, it can be an opportunistic pathogen that may cause infection *(1)*. *E.coli's* routes of transmission and exposure in humans are fecal-oral involving contaminated water, food, and person to person. Water contaminated with sewage is considered one of the major causes for water outbreaks *(11)*.

The U.S. Environmental Protection Agency recommends the use of *E.coli*, as an indicator organism for freshwater*(2)*. The logical reason behind the use of *E. coli* as the indicator organism for environmental fecal contamination is because that the *E.coli* does not live in nonhost environments and it is presence in the environment results from excretion of waste by warm blooded hosts *(32)*.

The survival of fecal indicator bacteria in environmental waters depends on biological, chemical ,and physical factors*(2)*. Also, The survival conditions of *E. coli* in intestinal system is much higher favorable than in water, soil, and manure *(29)*. In water, *E.coli* is so sensitive to chlorine and other disinfectants *(11)*. Several studies showed that *E. coli* grows and divides 1 day in water, 1.5 days in sediment, and 3 days in soil (32). Therefore, *E. coli* does not live in nonhost environments and always are shedding from human and animal sources sustain a constant population outside warm blood hosts *(32)*. Environmental conditions have important role in the growth and death rates

such as the availability of nutrients and energy sources*(29)* therefore, *E. coli* populations decline rapidly in freshwater *(32)*.

There is a new evidence shows that the *E.coli* in the tropical environment can survive and multiply (11). Subsequently, the *E.coli* indicates fecal pollution but not in the tropical environment this not the case and further studies and investigation is required *(11)*. Nonetheless, Soil factors such as surface area, porosity, macropore structure, and bulk density play big roles in the leaching of invading bacteria in water by their influence in gravitational movement and adsorption *(29)*.

1.2.3 Quanti-Tray MPN

Several studies have done in the United States and the United Kingdom showed that The Quanti-Tray method was statistically as good as, the reference Swedish multiple-tube fermentation and membrane filtration methods for qunification of *E. coli* cells in drinking water *(10)*. The Quanti-tray MPN method is based on ß -d-galactosidase activity, an essential enzyme for lactose fermentation, possessed by both coliforms and *E. coli*. B-d-galactosidase can hydrolyse ortho-nitrophenyl-ß-D-galactopyranoside (ONPG), releasing the yellow-coloured product o-nitrophenol. Also, b-glucuronidase, expressed by the majority of *E. coli*, can hydrolyse MUG, forming the fluorescent product 4-methylumbelliferone *(8)*.

1.2.4 Membrane vacuum filtration

The membrane filtration method is used to detect low level of microorganisms in large volume of water. The membrane filtration method can enumerate viable cells in environmental samples including drinking and recreational water. This method filters a known volume of liquids that range from 100 to 1000 ml through a sterile membrane filter that has 0.22 or 0.45 um pore size. The microorganisms remain on the membrane during the liquid filtration. After filtration, the membrane is placed on to an agar medium plate. The plates are incubated and the colonies are counted as the number of colony forming units (CFU). The original number of microorganisms in the sample have the units of CFU/ml. This is calculated by taking the total colony counts taken from the incubated membrane filter plates and combined with the respective membrane filtered sample volume. Normal counting ranges are between 20 to 80 CFU *(13)*.

1.3 Salmonella

1.3.1 *Salmonella* morphology

Salmonella spp. are facultative anaerobic gram-negative rod-shaped bacteria''. *Salmonella* belong to the Enterobacteraese, some are motile bacteria and have flagella. However, some strains are non-flagellated such as *S***.** entrica. servors Gallinarum and Pullorum (22). Furthermore, some *Salmonella* are non-motile strains resulting from dysfunctional flagella. *Salmonella* is a chemoorganotrophic that has the ability to metabolize a wide range of the organic substrates. Nonetheless, *Salmonella* has the ability to metabolize nutrients by

respiratory and fermentative pathways as well. The optimum growth temperature for *Salmonella* is 37°C (22).

1.3.2 *Salmonella* isolation

Salmonella has been detected in irrigation, recreational and drinking water *(19)*. *Salmonella* can survive for weeks to years in water and soil *(13)* . Irrigation water is a common route for contamination of produce with *Salmonella (19). Salmonella* can enter the aquatic environment through two routes, direct route by feces of infected human or animals or indirect route through the agricultural land run off or sewages discharge . This pathogen has been detected in different water sources, i.e. lakes, rivers, coastal waters and contaminated ground water *(19)*. The growth of *Salmonella* in water supplies is possible *(19)*. Mainly because it has the ability to colonize on the surface of distribution system pipes and form biofilms. Generally, water is suggested to be a significant source for the transmission of *Salmonella* for the typhoid and non-typhoid serovars *(19)*. Any food contaminated by fecal matter, such as vegetables and fresh fruits can be serve as a source of *Salmonella* infection *(13)*.

1.3.3 *Salmonella* symptoms

One of the most usual foodborne diseases is caused by non-typhoid *Salmonella* , which has the ability to invade the small intestine. *Salmonella* cause gastroenteritis in humans that can last between 1 to 7 days with other symptoms that include abdominal pains, vomiting, diarrhea, chills and nausea that can lead to dehydration and headaches. Susceptible individuals such as infants and elderly people and immunocompromised people could develop septicemia or chronic conditions such as

reactive arthritis. Although *Salmonella* has a low death rate < 1%, this figure can be higher amongst some groups especially elderly people *(17)*.

The infective does can vary widely but generally high number between 10^5 - 10^6 cells of *Salmonella* is required to be consumed to cause the illness. Furthermore, the infective does depends on several factors, more importantly consuming amount, and possible serotype involved. The incubation time is between 6 to 48 hours and most commonly between 12 – 36 hours. Individuals recovering from *Salmonella* infection still can shed *Salmonella* in their stools for some time *(17)*.

1.3.4 *Salmonella* outbreaks

In the United States of America, from 1971 to 2000 the CDC collaborated with the EPA and state health epidemiologists, started a surveillance program to collect and report water-borne outbreaks. The CDC reported that non-typhoid zoonotic *Salmonella* were the causes of 15 drinking water-borne outbreaks. Most of the outbreaks were associated with the ground water and public water systems for human consumption. Furthermore, the outbreaks were caused by using inadequate treated of ground water and distribution systems i.e. from the source to consumers in irrigation facilities or water agents *(19)*. Recently, in Tennessee, two outbreaks occurred due to inadequate water treatment. The source of infection for the first report linked to untreated water supply. During the investigation, it was discovered that the water had been collected from a spring that had been stored in a small-unprotected reservoir subject to contamination from wildlife and runoff. This affected 5 people in the rural

community *(19)*.The second report occurred in 2008 in Alamosa, Colorado. The public water system that supplies drinking water to the community had become contaminated with *Salmonella*. This outbreak caused one death and 442 reported illnesses. The source of infection was linked to water wells that had not been chlorinated. It was assumed that animal feces contaminate the ground level water storage reservoir *(19)*.

In 2002 tomatoes grown in Virginia, caused a *Salmonella* Newport outbreak that resulted in 510 illnesses in 26 states with no deaths. In 2005 the same strain was isolated and FDA in conjunction with states and local authorities conducted an investigation to determine the source of tomatoes eaten by the cases, inspected tomato farms, and tested irrigation ponds for *Salmonella*. They found that at least one irrigation pond was contaminated with *Salmonella* that was used to dilute pesticides for spraying the tomatoes fields *(14)*. In 1999 in the United States, a mango outbreak had occurred, 78 patients from 13 different states were infected with the outbreak strains. Two died, 15 were hospitalized. 50 % of infected people had consumed mango 5 days earlier before the illness occurred. It was linked to a Brazilian farm that was washing the mangos with contaminated water *(27)*. In 2012, in the United States an outbreak strain of *Salmonella* typhimurium and *Salmonella* newport were reported from 24 states. The total infected were 261 people with outbreak strains of *Salmonella* Typhimurium (228 persons) and *Salmonella* newport (33 persons). Three deaths and 94 ill persons were hospitalized. Epidemilogic, laboratory and traceback investigations conducted by officials in state, local, agriculture and regulatory agencies linked this outbreak to cantaloupe originating from chamberlain farms produce in Owensville, Indiana. Eighty-

one (65%) of 123 ill persons interviewed reported consuming cantaloupe in the week before their illness began *(6)*.

In developed countries such the United Kingdom, 71% of irrigation water is draw from surface water that may be contaminated with treated sewage effluent. In contrast, in developing countries sewage contaminated water is commonly used to irrigate crops. Therefore, the risk of microbial contamination is high *(19)*. A study on seed sprouts in Norway showed that *Salmonella* was detected in the spent irrigation water. The contamination of spent irrigation water with *Salmonella* was probably due to the use of contaminated seeds. Seeds are the main source of contamination and diseases in sprouts. However, the contamination might be low but the conditions and process may amplify the number of *Salmonella*. Furthermore, the use of fecal manure as a fertilizer can contaminate irrigation water. Animals such as deer, dogs and wolfs could contaminate water, seeds and crops *(24)*.

1.4 Zeolite

1.4.1 Zeolite structure

Natural zeolites can be used for water treatment because they have the ability to remove pathogenic bacteria and viruses from water *(31)*. Natural zeolite has a large surface area and cation exchange capacities and capable to form stable aggregates that can be ground and sieved to any desired permeability *(25)*. Zeolite have unique channels with three dimensional cage like structures *(20)*. Zeolite are hydrated aluminosilicate mineral with cage like framework structures, that contains $SiO4^{4-}$ and AlO_4^{5-} tetrahedral units, these are linked and brought together by the sharing of oxygen

atoms. The substitution and replacement of the Al^{3+} to Si^{4+} in the tetrahedra framework will lead to a negative charge which can be equalized and balanced by the presence and existence of alkali metal or alkali earth metal cations such as Ca2+ , K+, and Na+ that are found in the zeolite cavities. Those cations are mobile and can be changed by other cations. Since zeolite mineral possess a high cation exchange capacity they are not appropriate for anion contaminates removal in water *(30)*. They have excess negative change on the surface. The natural zeolite belongs to the cationic exchangers group. However, the zeolite could be modified chemically *(20)*. Zeolite are aluminosilicates substance that are naturally occurring and can be characterized by outside parts and surface areas and high cation exchange capacities *(20)*.

1.4.2 The modification of the zeolite

The modification of negatively charged zeolite surface can be conducted by forming layers of adsorbed cationic surfactants on the zeolite surface *(30)*. Charge balancing cations (typically K^+, Na^+, Ca^+ and Mg^+) are normally presented on the surfaces of natural zeolites and can be substituted by high molecular weight quaternary amines such as hexadecyltrimethylammonium HDTMA *(4)*. HDTMA is a format production surfactant that is used in mouthwash and hair conditioners. HDTMA surfactant has the ability to exchange the charge balancing on the outside of zeolite surface and forms an unchangeable surfactant bilayer. The forming of bilayer creates a hydrophobic environment and changes the zeolite surface from negative to positive *(25)*. HDTMA is a heavy organic cation that is to huge to enter inside the channels of natural zeolites. HDTMA- Br is a sorption cationic surfactant. HDTMA-Br has two

stages. The first stage when HDTMA is below critical micelle concentration (cmc) in this stage it adsorbed as a monolayer by electrostatic effect. The second stage when the surfactant concentration increased which leads to exceed the micelle concentration (cmc) in the solution. This leads to the increase in the surfactant sorption that causes a hydrophobic effect that will form a bilayer of surfactant on the zeolite surface *(30)*.

The negative charge on the surface of the zeolite can be reversed by treatment with long chain cationic surfactants such as HDTMA-Cl *(25)*. This modification will result in the creation of Surfactant Modified Zeolite (SMZ) which can remove about 100% of *E.coli* presented in water *(25)*. SMZ can be used as a sorbent for removing inorganic anions, inorganic cations, and neutral organics in water treatment *(4)*. Also, SMZ has the ability to remove high concentrations of bacteria and viruses from water *(25)*. Furthermore, SMZ are suitable for big volume treatments such as wastewater and subsurface permeable barriers for ground water pollution control *(4)*. The amount zeolite relies on the water treatment method and particles, the size distribution, temperature, pressure and the concentration of contaminants *(20)*.

1.4.3 Zeolite advantages

There are many advantages of using natural zeolites in water treatment. The natural zeolites have the ability to go through ion exchange and adsorption. In term of water contamination, they can be modified and treated *(16)*. Furthermore, they are high porous materials, a unique structure. The natural zeolite can be used as a bio filter for removing bacteria. Natural zeolite is cheap and economical and it does not require high

technology system *(16)*. Studies are required and needed to test the feasibility of using SMZ as a filter pack for long periods *(25)*.

1.5 Activated carbon

Activated carbon, otherwise known as activated charcoal, is a generic term for a family of highly porous carbonaceous materials, none of which can be characterized by a structural formula or by chemical analysis *(21)* . It is unique and it has an extended surface area and has been used throughout the ages (Figure 1) *(7)*. Activated carbon has been used in many applications due to it is capacity for adsorption from the gas and liquid phases. Activated carbon has been used widely in drinking water purifications and wastewater treatments. Activated carbon has several advantages due to its high degree of porosity, thermal stability, and because adsorb materials rapidly *(15)*.

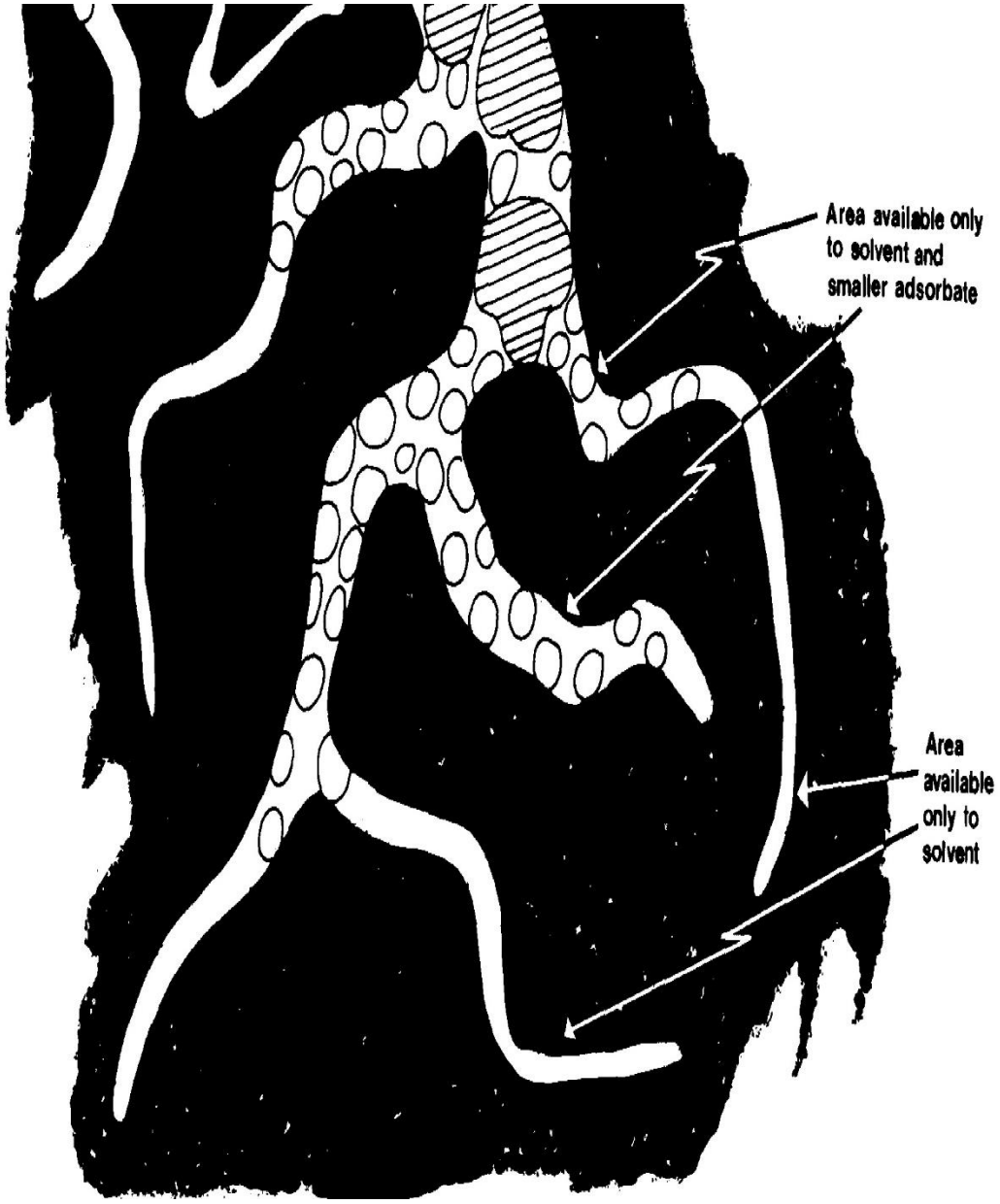

Figure 1. Structure of Activated carbon *(21)*.

The rate of water flow controls the length of contact time and activated carbon materials. This has a significant effect on the adsorption of the contaminants. The more contact time between the activated carbon and water, the greater adsorption *(18)*.

Activated carbon has been used in many important applications that are related to potable water. Such as the removal of color, taste, odor, and organic materials for the treatments of industrial and domestic waste water *(21)*. Charcoal has also been used in food poisoning cases, due to its ability to adsorb toxins emitted by bacteria *(7)*.

Activated carbon has two types of adsorption process: chemical and physical adsorption. Chemical adsorption manipulates the homopolar forces: covalent and ionic bonds, which are irreversible. Physical adsorption manipulates the diplo-diplo interactions and hydrogen bonding, which breaks weak Van der Waals forces, which is reversible. Therefore, the adsorption classified as being physical in nature *(21)*. Several factors influence the adsorption behaviors in aqueous solution, such as temperature and pH. The increasing temperatures decrease the adsorption process. The pH value of the solution effects the ionicity *(21)*.

1.6 Detection methods

1.6.1 Serology

Agglutination is a term used to express the aggregation of particular antigens test. The bacterial cells contain specific antigen that can bind specific antibody that produced in response to that antigen when it introduced to the host. The quality of the test results depends on several factors: (1) The incubation time of the antibody; (2) the amount of the antigen conjugated to the carrier and; (3) the environmental condition of the test such as pH and protein concentration *(28)*.

Serological tests for *Salmonella* is widely used especially in surveillance. The serology test for *Salmonella* depends on determining the O-antigen and the

lipopolysaccharide (LPS) and, the flagellar protein (H-antigens) components. The O-antigen is variable, and has unique structure on each serotype. The LPS of *Salmonella* is well known for binding to specific antigen. The amount of agglutination of *Salmonella* is based on the antigen-antibody reaction among *Salmonella* serotypes *(3)*.

1.6.2 DNA extraction

A simple method to extract *Salmonella* DNA is by taking a single colony into 500-µl dH_2O in a 1ml micro-centrifuge tube. The tube is placed into a water bath and heated for 5 minutes at 95 °C. After heating, the tube is immediately placed into an ice bucket to lyse bacterial cells. This method is used for the detection and identification of pure colonies with the use of RT-PCR *(12)*.

1.6.3 Real-Time PCR

Real-time polymerase chain reaction is also called quantitative real-time PCR or qPCR or Q-PCR. The real-time PCR is a technique developed in recent years and used to amplify a specific DNA fragment to a billion folds in an hour*(9)*. It can be used to determine the presence and absence of a particular DNA sample *(9)*. The technique is based on exponential amplification of DNA by the Taq polymerase that is produced by the thermostable bacteria *Thermous aquaticus (23)*. The fundamental goal of the real time PCR is to measure and distinguish a sample even at low levels *(26)*. It requires a reaction mixture that contain DNA molecule to be amplified, nucleotide triphosphates (dNTPs), thermostable polymerase, and the two primers to synthesis DNA, specific buffer and Mg^{2+} *(9)*.

The polymerase chain reaction (PCR) includes three main steps: denaturation, annealing, and extension. During the denaturation, the reaction mixture is heated to 95°C in order to break hydrogen bonds and separate the strands of the DNA. Temperature is then lowered to 55°C during the annealing step. The forward and reverse primers bind to the DNA strands that are temperature dependent. Finally, the temperature is increased to 72°C, which is necessary for the thermostable polymerase to work in the extension step. The nucleotides are added to enlarge the DNA strand in the 5'-to-3' direction. After 20 to 25 cycles, millions of DNA molecules are formed from a single segment of double stranded DNA *(26)*. The real-time PCR systems use a fluorescent reporter to bind the DNA in the minor groove *(9)*.The real time PCR is used in food microbiology for several reasons. It is rapid and accurate for detecting pathogenic bacteria and for tracing outbreaks of pathogenic bacteria in the food supply *(23)*. PCR is not time consuming, requires less labor, and can quantify nucleic acid at wide ranges that reach at least 5 log units *(26)*.

1.7 Justifications

This study clearly shows the need for future investigations identifying more suitable methods for detecting *Salmonella* spp. in surface water, ground water, and irrigation waters. The overall goal of the project is to detect low levels of *Salmonella* spp. in irrigation water by using SMZ or activated carbon.

1.8 References

1. Adams, M. R., and M. Moss. 2007. Food microbiology. Royal society of chemistry.P 216-217.

2. Anderson, K. L., J. E. Whitlock, and V. J. Harwood. 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Applied and environmental microbiology*. 71:3041-3048
.
3. Aribam, S. D., M. Elsheimer-Matulova, H. Matsui, J. Hirota, K. Shiraiwa, Y. Ogawa, H. Hikono, Y. Shimoji, and M. Eguchi. 2015. Variation in antigen-antibody affinity among serotypes of *Salmonella* O4 serogroup, determined using specific antisera. *FEMS microbiology letters*. 362.

4. Bowman, R. S., E. J. Sullivan, and Z. Li. 2000. Uptake of cations, anions, and nonpolar organic molecules by surfactant-modified clinoptilolite-rich tuff. *Natural zeolites for the third millennium*:287-297.

5. Cabral, J. P. 2010. Water microbiology. Bacterial pathogens and water. *International journal of environmental research and public health*. 7:3657-3703.

6. CDC. 2012. Multistate Outbreak of *Salmonella* Typhimurium and *Salmonella* Newport Infections Linked to Cantaloupe. Access link: https://www.cdc.gov/salmonella/typhimurium-cantaloupe-08-12/

7. Çeçen, F., and Ö. Aktaş. 2011. Water and wastewater treatment: historical perspective of activated carbon adsorption and its integration with biological processes. *Activated Carbon for Water and Wastewater Treatment: Integration of Adsorption and Biological Treatment*:1-11.

8. Chao, W. 2006. Evaluation of Colilert-18 for the detection of coliforms and *Escherichia coli* in tropical fresh water. *Letters in applied microbiology*. 42:115-120.

9. chaudhuri, k. 2014. Microbial Genetics. *The Energy and Resource Institute*.

10. Eckner, K. F. 1998. Comparison of membrane filtration and multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and *enterococci* used in drinking and bathing water quality monitoring in southern Sweden. *Applied and Environmental Microbiology*. 64:3079-3083.

11. Embrey, M., P. Hunter, J. Sellwood, P. Wyn-Jones, S. L. Percival, and R. Chalmers. 2004. Microbiology of waterborne diseases: Microbiological aspects and risks. Academic Press.

12. Goktepe, I., V. K. Juneja, and M. Ahmedna. 2006. Probiotics in food safety and human health. CRC Press.

13. Goldman, E., and L. H. Green. 2009. Practical handbook of microbiology. CRC Press.

14. Greene, S., E. Daly, E. Talbot, L. Demma, S. Holzbauer, N. Patel, T. Hill, M. Walderhaug, R. Hoekstra, and M. Lynch. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. *Epidemiology & Infection*. 136:157-165.

15. Hesas, R. H., A. Arami-Niya, W. M. A. W. Daud, and J. Sahu. 2013. Preparation and characterization of activated carbon from apple waste by microwave-assisted phosphoric acid activation: application in methylene blue adsorption. *BioResources*. 8:2950-2966.

16. Kalló, D. 2001. Applications of natural zeolites in water and wastewater treatment. *Reviews in mineralogy and geochemistry*. 45:519-550.

17. Lawley, R., L. Curtis, and J. Davis. 2015. The food safety hazard guidebook. Royal Society of Chemistry.

18. Lemley, A., L. Wagenet, and B. Kneen. 1995. Activated Carbon Treatment of Drinking Water. Cornell Cooperative Extension, New York State College of Human Ecology.

19. Levantesi, C., L. Bonadonna, R. Briancesco, E. Grohmann, S. Toze, and V. Tandoi. 2012. *Salmonella* in surface and drinking water: occurrence and water-mediated transmission. *Food Research International*. 45:587-602.

20. Margeta, K., N. a. Z. Logar, M. Šiljeg, and A. Farkas. 2013. Natural zeolites in water treatment–how effective is their use. *In*, Water treatment InTech.

21. McDougall, G. 1991. The physical nature and manufacture of activated carbon. *Journal of the Southern African Institute of Mining and Metallurgy*. 91:109-120.

22. Montville, T. J., and K. R. Matthews. 2012. Food microbiology: an introduction. ASM Press.

23. Overbergh, L., A. Giulietti, D. Valckx, and C. Mathieu. 2010. Real time polymerase chain reaction. *Molecular diagnostics*:87-105.

24. Robertson, L. J., G. S. Johannessen, B. K. Gjerde, and S. Loncarevic. 2002. Microbiological analysis of seed sprouts in Norway. *International Journal of Food Microbiology*. 75:119-126.

25. Schulze-Makuch, D., S. D. Pillai, H. Guan, R. Bowman, E. Couroux, F. Hielscher, J. Totten, I. Y. Espinosa, and T. Kretzschmar. 2002. Surfactant-modified zeolite can protect drinking water wells from viruses and bacteria. *EOS, Transactions American Geophysical Union*. 83:193-201.

26. Seifi, M., A. Ghasemi, S. Heidarzadeh, M. Khosravi, A. Namipashaki, V. M. Soofiany, A. A. Khosroshahi, and N. Danaei. 2012. Overview of Real-Time PCR Principles. *In*, Polymerase Chain Reaction InTech.

27. Sivapalasingam, S., E. Barrett, A. Kimura, S. Van Duyne, W. De Witt, M. Ying, A. Frisch, Q. Phan, E. Gould, and P. Shillam. 2003. A multistate outbreak of *Salmonella* enterica Serotype Newport infection linked to mango consumption: impact of water-dip disinfestation technology. *Clinical Infectious Diseases*. 37:1585-1590.

28. Turgeon, M. L. 1990. Immunology & Serology in Laboratory Medicine-E-Book. Elsevier Health Sciences.

29. Van Elsas, J. D., A. V. Semenov, R. Costa, and J. T. Trevors. 2011. Survival of Escherichia coli in the environment: fundamental and public health aspects. *The ISME journal*. 5:173.

30. Wibowo, W., T. Utari, and R. T. Yunarti. 2011. Anion exchange capacity of chromate on modified zeolite clinoptilolite with HDTMA-Br and its regeneration. *Makara Journal of Science*.

31. Widiastuti, N., H. Wu, M. Ang, and D.-k. Zhang. 2008. The potential application of natural zeolite for greywater treatment. *Desalination*. 218:271-280.

32. Winfield, M. D., and E. A. Groisman. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli. Applied and environmental microbiology*. 69:3687-3694.

CHAPTER 2. CORRELATION OF *SALMONELLA* SPP. TO GENERIC *ESCHERICHIA.COLI* IN IRRIGATION WATER

2.1 Introduction

The Centers for Disease Control (CDC) collaboration with the Environmental Protection Agency (EPA) and state health epidemiologists started surveillance program focus on water-born outbreak. The survey was conducted from 1971 to 2000. Non-typhoid zoonotic *Salmonella* has been reported as the cause of 15 outbreaks in ground water and drinking water over a 29 year period. The program found that the cause of the outbreaks was the result of using ground water and distribution systems that were not properly treated for bacteria *(19)*

Salmonella Belongs to the Enterobacterae family, anaerobic, gram negative, rod shaped, motile, and has a flagella *(23) Salmonella* has the potential to survive in water and soil for years, and it can be found in recreational, irrigation, and drinking water, which can become an issue (19). *Salmonella* enters the water system in one of two ways: directly via excrement of infected animals and humans or indirectly via sewage discharge or agricultral water runoff (19). *Salmonella* bacteria have been found in lakes, rivers, coastal waters, and contaminated ground water*(19) Salmonella* is one of the most common foodborne diseases that causes gastroenteritis in humans which can last between 1 and 7 days. Gastroenteritis symptoms include abdominal pain, vomiting, diarrhea, chills, and nausea, which lead to dehydration and headaches *(17)*

Natural zeolites are good substances for water treatment because they have the ability to remove pathogenic bacteria and viruses from water via water filtration *(27)*.

The properties of natural zeolites are a negative charge and large surface area with cation exchange capacities *(25)*.

The negative charge on the zeolite can be altered by insulating its surface with cationic surfactants *(26)* Hexadecyltrimethylammonium (HDTMA) is a high molecular quaternary amines that can substitute the charge balancing cations to form an unchangeable surfactant bilayer. The charge balancing cations are usually K^+, Na^+, Ca^+ and Mg^+, which are on the surface of natural zeolites *(3)*. The bilayer formed from the HDTMA and the zeolite changes the surface from negative to positive and creates a hydrophobic environment *(25)* The surface change in the zeolite results in the Surfactant Modified Zeolite (SMZ) which can remove up to 100% of *E.coli* presented in water *(24)*

The advantages of using natural zeolites are numerous: zeolite is cost effective, they can be modified to prevent water contamination. Natural zeolite does not require a high technology system, and they can go through ion exchange and adsorption *(16)*.

The activated carbon otherwise known as activated charcoal, is a generic term for a family of highly porous carbonaceous materials, none of which can be characterized by a structural formula or by chemical analysis *(22)* Activated has been used throughout the ages due to its unique structure and extended surface area *(5)* Activated carbon has many applications due to its capacity for adsorption from gas and liquid phases. The applications include, but are not limited to: purification of drinking of drinking water and waste water. The advantages of activated carbon include thermal stability, quick material adsorptions, and a high degree of porosity *(14)*.

In 2014, University of Florida conducted a study to isolate and detect *Salmonella* from irrigation water by using the cross-streaking method. The developed method did not show promising results and not recommended to use *(20)*. In 2011, in Taiwan another study was conducted to detect *Salmonella* in environmental waters by using three analysis processes. The first analysis process detected zero positive out of 116 samples. The second analysis process detected 10 positive out of 116 results. The third analysis process detected 7 out of 116 samples *(20)*.

These studies illuminate the need of a more reliable and accurate way to detect *Salmonella* in irrigation systems. The objective of the research is to detect low levels of *Salmonella* in irrigation water by using Activated Carbon and Surfactant Modified Zeolite which will be compared to the Environmental Protection Agency (EPA) method 1200.

2.2 Materials and methods

2.2.1 *Salmonella* culture condition

The *Salmonella* typhimurium ATCC 14028 strain was cultured in 10 ml tryptic soy broth (Acumedia, Lansing, MI., U.S.A.) statically at 37 °C for 24 h before being used. This was followed by centrifugation (5000 rpm, 5 min, 20 °C; Thermo Fisher Scientific model multifuge X1R, Langenselbold, Germany). The supernatant was discarded and replaced with 80% of TSB and 20 % of sterilized glycerol solution and mixed. Next, 1 ml of the mixed solution was transferred into the cryogenic vial and stored at -80 °C. This served as a positive control until further use.

2.2.2 Optimization of surfactant modified zeolite

Granular zeolites (Zeolites® Xtreme) (provided by Mr. Stephen Peterson from Zeotech Co., Fortworth, TX) was used in this study. The zeolite granules have a size range of 14 × 13 mesh (0.3-1.4 mm dia). Zeobrite®Xtreme has been modified by its manufacture through a chemically bonded carbon chain that will make the zeolite ‘’dual-charged’’. The modified zeolite has 10 times more surface area then sand; resulting in attracting negatively charged particles onto its surface and positive particles in to its interior.

The zeolite was treated to change the charge on the surface. The zeolite was soaked in water at 1:3 ratio for 24h. After that, the zeolite was placed in an oven to dry at 350 °F for 30 minutes. This was followed by treatment with two different surfactants. The zeolite was treated with different concentrations of HDTMA–Br (Sigma Aldrich CO, St. Louis, MO) (5 %, 10 %, 20, and 30 %) by weight and HDTMA–Cl (Sigma Aldrich CO, St. Louis, MO) (5 %, 10 %, 20 %, and 25 %) by volume to make the surfactant modified zeolite. Zeolite (7.5g) was added into a 50 ml tube that contained 30 ml of final volume of different concentrations of dissolved HDTMA–Br and HDTMA-Cl. Each zeolite treated sample was placed onto a shaker (Red Rotor, Hoefer Scientific Instruments, San Francisco, CA) and agitated at 6 rpm for 8h. After mixing, each zeolite sample was placed at room temperature for 48 h. Next, the zeolite treated tube was washed 2 times manually and 2 times by centrifuge (5000 rpm, 5 min, 20°C; Thermo Fisher Scientific model multifuge X1R, Langenselbold, Germany) and then was poured into aluminum dishes (Mini loaf pans, 3.25 W x 2” D x 5.75’ L, Ar, U.S.A.) and placed

in an oven at 255°F for 60 min. Finally, the Surfactant Modified Zeolite (SMZ) was stored at room temperature until used for water filtration *(25, 26)*.

2.2.3 Surfactants modified zeolite and activated carbon filtration for detection of *Salmonella*

An in-vitr study was done with different dilution *Salmonella* of that were filtered through 2 grams of SMZ in vitro with different concentration of CTAC to assess the best CTAC concentration to be used in the study. Next, the SMZ and activated carbon filtration methods were conducted by using vacuumed filter flasks and filtrated 300 ml of irrigation water samples through 3 grams of SMZ or 3 grams of activated carbon. After filtration, the 3 grams of the best SMZ attachment percentage and activated carbon were transferred into 10 ml of *Buffered Peptone Water* (BPW) (Difco, Becton Dickinson, Sparks, MD) and then placed into a shaker incubator at 37 °C for 24h. After that, an ml of each BPW tube was transferred into 10 ml of Tetrathionate Broth (TT Broth) (Acumedia, Lansing, MI) and incubated at 37 °C for 24h. After that, a loopful of TT Broth was streaked into XLD agar and incubated 37 °C for 24h in duplicates. Followed by re-streaking black colonies onto XLD Agar and incubated at 37 °C for 24h in duplicates.

2.2.4 Sampling sites

Irrigation water samples were collected every month for two years from an LSU agricultural research station called Ben Hur located on Nicolson drive Baton Rouge, LA. The water samples were collected from ponds that are close to a cattle farm. The water samples were collected by using sterilized water sampling dipper for surface water and sediment. After that, the water samples placed in ice chest and transferred to the Food

Microbiology Laboratory and immediately tested for Generic *E.coli* and *Salmonella*. Rainfall fluctuated in Baton Rouge during this study's period: Flooding occurred in August 2016, causing the farm to be submerged for 3 days. On the other hand, no rain was recorded in the months of November and December resulting in the farm drying up and no samples collection. Water accumulation in the pond resumed in January 2017.

2.2.5 Chlorine concentration and pH analyses

All irrigation water samples were tested for chlorine concentration using (Chlorine Test Papers, Code-4250BJ, Maryland, and U.S.A.). The temperature of the pond water was measured for each visit. The pH of the irrigation water was measured (Thermo Scientific™ Orion™ 2-Star Benchtop pH meter, Center Beverly, MA, U.S.A.). The electrode was calibrated with pH 7.00 and 4.00 and 10.00 buffer solutions (VWR International, West Chester, PA, U.S.A.) prior to use.

2.2.6 EPA 1603 membrane filtration method

The EPA 1603 modified membrane – thermotolerant *Eschericia coli* agar modified mTEC (Difco, Becton Dickinson, Sparks, MD) method was used to determine the bacterial counts of generic *E. coli* in irrigation water(EPA 1603 date) . The filtration method was used to enumerate viable cell of 100 ml of irrigation water sample. The vacuum (Chemical Duty Pump, Model WP6111560, 115 V/60 Hz, Millipore, Billerica, Massachusetts, U.S.A.) was used to accelerate filtering process. After filtration the membrane filter (Millipore, MF membrane filters 0.45 µm, Billerica, Massachusetts, U.S.A.) was placed onto mTEC agar The mTEC agar plates were incubated at 42 °C for 2h then followed by a second incubated at 37°C for 22h. The plates were in duplicates

and a count method was used to count red magenta colonies under microbial counter. A Quebec Darkfield Colony Counter (Leica Inc., Buffalo, NY, U.S.A.) was used to assist in enumerating the colonies.

2.2.7 Quanti-Tray most probable number

A Quanti-Tray method was used to quantitate generic *E.coli* counts of 100 ml of irrigation water samples. A Colilert reagent (Colilert, IDEXX, Maine, U.S.A.) were added and mixed with the 100 mL irrigation water before pouring into the Quanti-Tray. The Quanti-Tray (Quanti-Tray 2000, IDEXX, Maine, U.S.A.) was sealed by the Qunti-Tray sealer (Qunti-Tray Sealer, Mode 2X idexx, Westbrook, Maine, U.S.A.) and incubated at 35°C for 24h. Next, All trays were counted under the U.V. light (U.V. SPECTROLINE Model CM-10A, Fluorescence Analysis Cabinet, Westbury, U.S.A.) to determine the positive wells based on the fluorescences. The MPN results were determined using the Quanti-tray Table.

2.2.8 The EPA 1200 method

The EPA method 1200 Analytical Protocol for Non-Typhoidal *Salmonella* in Drinking Water and Surface Water was used. The EPA 1200 method was used to estimate low levels of *Salmonella* in undiluted environmental water samples (EPA 2012). Five 50 ml tubes containing 20 ml of 2X *tryptic soy broth (*TSB) (Sigma Aldrich CO, St. Louis, MO) and 20 ml of the undiluted irrigation water sample were incubated at 37 °C for 24 h along with a positive and a negative control (EPA 2012). An ml of each tube transferred into TT Broth and incubated at 37 °C for 24h. After that, a loopful of TT Broth was streaked into XLD agar and incubated 37 °C for 24h in duplicates. Followed

by re-streaking black colonies onto XLD Agar and incubated at 37 °C for 24h in duplicates.

2.2.9 Validation of *Salmonella*

The verification of *salmonella* colonies was done for Surfactants Modified Zeolite Filtration Method, the Activated Carbone filtration method, and The EPA 1200 method. All black colonies from XLD plates were isolated and streaked onto tryptic soy agar (TSA) (Acumedia, Lansing, Mich., U.S.A.). All TSA plates were incubated at 37°C for 24h. Serological studies were done by using *Salmonella* latex agglutination tests (Microgen Bioproduct, Camberley, U.K.). This was followed by observation of the agglutination reaction, which indicates a positive result. All serological positive colonies were confirmed by Real Time PCR (RT-PCR).

The DNA was extract from a colony that tested positive for the serological analysis by using thermos osmatic lysis. Each colony was added to 500 µl of Milli Q water in Eppendorf tube. Then, the tubes was heated in a water bath at 95 °C for 10 minutes, after heating samples were placed into shaved ice followed centrifuged (13.5 rpm, 8 min, 4°C; Eppendorf model 5415 D, Brinkmann Instruments Inc., Westbury, NY). The supernatant that contained the extracted DNA was collected (12)**.** The real time PCR method was adopted from De Paola et Al, (2010) (8). The primers and probe sequences used for *invA* gene detection are given in Table 1. The master mix (50 µl) for the RT-PCR reaction contained the following: one bead of OminiMix H5 (Cephied, Sunnyvale, CA) 200 nM each forward and reverse *invA* and 200 nM probe for *invA* gene. The final volume for the RT-PCR reaction tube was equal to 25 µl. Cepheid

SmartCycler® II system (Sunnyvale, Calif.,U.S.A.) was used and the cycling parameters were set at 95°C for 2 min followed by 45 cycles of 94°C for 10 s, 63°C for 15 s, and 72°C for 15 s. The manual threshold fluorescence units setting was adjusted to 15 *(8)*.

Table 1. Primers and probe for detection of *Salmonella* spp.

	Salmonella spp. (*invA*) gene
Forward	CAACGTTTCCTGCGGTACTGT
Reverse	CCCGAACGTGGCGATAATT
Probe	TxRed-CTCTTTCGTCTGGCATTATCGATCAGTACCA-BHQ2

2.3 Statistical analysis

The results were analyzed with JMP Software Pro 13 one-way analysis of variance (ANOVA), followed by compare means, all pairs Tukey-Kramer, a P value < 0.05 was considered significant.

2.4 Results

2.4.1 *Salmonella* SMZ optimization

Salmonella attachment percentages using CTAC/SMZ and CTA/SMZ are represented in Table2.

Table 2. Attachment percentage of *Salmonella* using different concentrations of Hexadecyltrimethylammonium-Cl/SMZ (CTAC/SMZ) and Hexadecyltrimethylammonium - Br/SMZ (CTAB/SMZ).

Surfactant Concentration (wt/vol %)	Attachment Percentage (%)	
	CTAC/SMZ	CTAB/SMZ
Zeolite [a]	2.60 ± 0.88	2.60 ± 0.90
10%	6.55 ± 1.13	2.61 ± 0.19
20%	42.29 ± 9.87	29.28 ± 28.27
25%	31.68 ± 9.7	nd [b]
30%	nd [b]	18.33 ± 5.34

[a] Zeolite not treated with surfactants. [b] nd: Not Done.

Table 3. Comparison of all pair means for the CTAC and CTAB.

Surfactant Concentration	CTAC Mean	CTAB Mean
Zeolite	2.605000 [a]	2.605000 [a]
10%	6.690000 [ab]	2.610000 [a]
20%	42.295000 [c]	29.285000 [a]
25%	31.685000 [bc]	nd [d]
30%	nd [d]	18.335000 [a]

[abc] Levels that are not connected with the same letter within the table are significantly different based on Tukey-Kramer HSD one-way ANOVA (P value < 0.05). [d] nd: Not Done.

The attachment percentage of *Salmonella* using different concentrations of Hexadecyltrimethylammonium-Cl/SMZ (CTAC/SMZ) and Hexadecyltrimethylammonium-Br/ SMZ (CTAB/SMZ) are shown in Table 2. The zeolite attachment of *Salmonella* was 2.60 ± 0.88. The attachment percentage decreased at 10% but increased significantly at 20% where it reached the highest observed value of 42.29 ± 9.87 (P value < 0.05). Whereas in the case of CTAB/SMZ surfactant, statistical analysis showed no significant difference between the different surfactant concentrations (P value > 0.05) as shown in Table 3.

Surfactant Modified Zeolite (SMZ) has been used to remove *E.coli* and viruses from water with 100 % efficiency *(25)*. Also, zeolite was used as a broiler feed additive at different concentrations and was significantly effective in reducing *Salmonella* at the broiler farm *(1)*. Moreover, SMZ could be used in several water treatment processes as a sorbent/ion exchanger *(3)*. As an example, the long chain cationic surfactants

HDTMA-Cl can be used to reverse the negative charge on the surface of the zeolite *(25)*. Another example of a sorption cationic surfactant is the HDTMA- Br, that has two stages: The first one exists when HDTMA is below critical micelle concentration and the surfactant is adsorbed as a monolayer due to electrostatic effect. In the second stage, the surfactant concentration increases leading to an excess in the micelle concentration in the solution. Consequently, the surfactant sorption increases causing a hydrophobic effect resulting in the formation of a bilayer of surfactant on the zeolite surface *(26)*.

2.4.2 Irrigation Water physicochemical properties

Table 4. Irrigation water physicochemical properties.

Month	Temperature (°C)	Chlorine (ppm)	pH
May	27	<10	7.5
June	28	<10	7.6
July	29	<10	7.7
August	30	<10	7.2
September	27	<10	7.6
October	24	<10	7.7
January	17	<10	7.5
February	19	<10	7.3
March	21	<10	7.9
April	23	<10	8.0
May	23	<10	7.1
June	26	<10	7.6

All values are the mean of duplicate.

The pH, chlorine concentration, and temperature measurements are shown in Table 4. All the measurements were in the *Salmonella* survivability range (Table 4). Chlorine residue of at least 0.2mg/l are sufficient to eliminate *Salmonella* from the distribution drinking water system *(10)*. In this study, the chlorine concentration was

measured with a test paper with 10 ppm sensitivity. The strip color indicated a chlorine concentration below sensitivity limit (Table 4) which may be at the lower end and does not affect *Salmonella* growth. *Salmonella* has the ability to grow in a pH ranging from 4.5 to 9.5 with the optimum being 6.5 to 7.5 *(23)*. *Salmonella* is able to grow at temperature ranging from 10-43 °C *(10)*. Furthermore, *Salmonella* can grow at higher temperature of 54 °C and lower of 2-4 °C *(23)*.

2.4.3 Quantification of Generic *E.coli* in irrigation water

Table 5. Quantification of Generic *E.coli* (log_{10} CFU/100 ml) in irrigation water using two methods.

Month	EPA 1603	Quanti-Tray
May	2.28	2.24
June	2.75	3.03
July	2.47	3.58
August	1.99	2.22
September	1.89	2.27
October	2.72	4.15
January	3.82	3.92
February	3.92	3.91
March	3.05	3.07
April	2.69	2.66
May	3.37	3.55
June	1.95	2.31

All values are average of duplicates.

Table 6. Comparison of all pair means for the EPA 1603 and Quanti-Tray methods

EPA 1603	-0.07437
Quanti-Tray	-0.40662

Positive values show pairs of means that are significantly different based on Tukey-Kramer HSD one-way ANOVA

For the Quantification of Generic *E.coli* in irrigation water, two methods were used: The vacuum filtration method (EPA 1603) and Quanti-Tray method that are shown in Table 5. The vacuum filtration method has an accurate generic *E.coli* counts whereas the Quanti-Tray is an estimation method for the generic *E.coli* counts. Although the Quanti-Tray method showed higher log counts than the vacuum filtration method, there was no significant difference in the data shown in Table 6.

The results were compared with the FDA Food Safety Modernization Act (FSMA)'s produce safety agricultural water microbial quality criteria. The total number of water samples was 12 (n=12) and 10 samples from this 12 months study showed *E.coli* levels above the regulatory threshold of > 126 CFU/100 ml. The irrigation water volume had been fluctuating over the last 2 years which resulted in changing the pond's water content and the osmotic balance. According to Winfield and Groisman (2003) the *E.coli* counts decreases significantly in the presence of osmotic stress which leads to the death of the *E.coli.* Whereas, *Salmonella* can survive for longer time in the presence of osmotic stress and other stresses associated with environmental fluctuating in irrigation water *(28)*. Furthermore, they reported that although *E. coli* does not live in nonhost environments, the continuous bulk transfer from animals and human sources sustains a

steady *E. coli* population outside animal hosts. Furthermore, the lack of nutrients might be a significant factor for non-survival of generic *E.coli* in water *(28)*.

Furthermore, during collecting the irrigation water samples throughout all the 12 months, a wide range of animals have been observed such as cows, snakes, turtles, raccoon, and birds, all of which are natural for *Salmonella*. This can explain the fluctuation in the numbers of generic *E.coli* counts as well as *Salmonella* presence.

According to Eckner (1998) the Quanti-Tray method could present a viable alternative method for testing water quality for *E. coli* in both drinking water and freshwater. It has the same sensitivity as Swedish standard methods for detecting *E. coli* in bathing water samples. In comparison with the reference Swedish multiple-tube fermentation and membrane filtration methods, the Quanti-Tray method showed statistically higher *E. coli* counts in drinking water. This finding was similar to results published by many studies performed in the United States and the United Kingdom (9).

2.4.4 Quantification of Generic *E.coli* using the Quanti-Tray for surface and sediments in irrigation water

Table 7. Generic *E.coli* counts (log_{10} CFU/100 ml) in irrigation water using the Quanti-Tray method.

Month	Quanti-Tray Surface	Quanti-Tray Sediments
May	2.24	3.20
June	3.03	3.66
July	3.58	4.09
August	2.22	3.59
September	2.27	3.66
October	4.15	5.38
January	3.92	4.23
February	3.91	3.76
March	3.07	3.79
April	2.66	3.10
May	3.55	4.46
June	2.31	3.97

All values are average of duplicates.

Table 8. Comparison of all pair means for the Quanti-Trays surface water and sediment.

Quanti-Tray Surface	0.46376
Quanti-Tray Sediments	-0.38170

Positive values show pairs of means that are significantly different based on Tukey-Kramer HSD one-way ANOVA

The quantification of Generic *E.* coli using QuantiTray method for surface and sediments is illustrated in Table 7. The results found that *E. coli* counts in surface samples for all months were less than the sediments except for the month of February.

Statistical analysis is shown in Table 8 and there was significant difference between the counts obtained through the two methods ($P < 0.05$).

Several studies have found higher levels of bacteria in sediment more than in surface water due to higher survivability of bacteria in sediments which is in accord with this study. For instance, Craig et al. (2002) reported higher fecal coliform concentrations in sediment than in surface water *(7)* .Benjamin et al. (2013) reported that the generic *E. coli* counts in sediments were generally higher than in surface water source. Favorable conditions such as high microbial concentration in the surface water and slow water flow rate can cause accumulation of bacteria in sediments *(13)*. Furthermore, higher microbial counts in water may cause safety risk and contamination to fresh crops in the case of flooding *(2)*.

The longer survival of *E. coli* in the sediment could be attributed to the greater content of organic matter present in the sediment *(11)*. Chandran, et al. (2011) indicated that sediments present an adherence microbial substratum and provide bacteria with protection against bacteriophage attacks and protozoan consumption of bacteria as well as nutrition. Also, bacterial survival in sediments was shown to be influenced by sediments composition and characteristics such as sand, silt, clay and organic carbon content *(6)*.

2.4.5 The detection of *Salmonella* in irrigation water using three methods.

Table 9. The detection of *Salmonella* in irrigation water using three methods.

Month	Environmental Protection Agency 1200	Surfactant Modified Zeolite	Activated Carbon
May	+	-	**nd***
June	-	-	**nd**
July	-	-	**nd**
August	+	+	**nd**
September	+	-	**nd**
October	+	-	**nd**
January	+	+	+
February	+	-	+
March	+	-	-
April	+	-	-
May	+	-	+
June	+	-	-

Water was sampled once every month. +/-: Presence or Absences of *Salmonella* .
*nd: Not Done

The environmental Protection Agency 1200 method detected *Salmonella* in irrigation water 10 months from May 2016 to May 2017 (Table 9). All monthly samples were positive except June and July 2016 and during these months, the cattle were not on the farm.

The Surfactant Modified Zeolite was the least sensitive for detecting *Salmonella* in irrigation water. *Salmonella* was detected in only two months, August 2016 and January 2017. In August 2016, Louisiana State flooded and the farm was submerged for 3 days. This could have caused the number of the *Salmonella* to increase. In November and December 2016, the farm ultimately dried up and no samples were collected. In January 2017, the water began accumulating in the pond and this possibly

caused *Salmonella* to be recovered in the farm water. In this study apparently, qualitative SMZ method can only detect *Salmonella* in higher counts. Whereas, the activated Carbon method showed better results compared to the Surfactant Modified Zeolite. However, these two methods were less efficient compared to the EPA 1200 method. For instance, a study conducted in Tanzania reported a high efficiency for the EPA 1200 method which has been used for detection of *Salmonella* from lakes and ponds and all samples were positive *(21)*.

Activated carbon could attach nutrients which would allow already attached bacteria to grow in cracks and crevices forming therefore an extracellular slime layer. Moreover, *Salmonella* Typhimurium mixed with heterotrophic plate count microorganisms also showed attachment to the activated carbon *(4)*.

Schulze-Makuch, et al. (2002) reported that, SMZ removed 100 % of *E.coli* and 99% of viruses from water *(25)*. Schulze-Makuch, et al. (2003) stated that the use of SMZ in a filter pack can remove 100% of *E.coli* for a year and a half *(24)*. Furthermore, the sole use of a lower concentration of surfactant such as HDTMA can kill bacteria. However, once bound to the zeolite, HDTMA surfactant becomes less toxic *(25)*.

Contrarily to our findings, Jones et al. (2015) reported that in irrigation water, the presence of *Salmonella* was not strongly correlated to the presence of the *E.coli* (15). Similarly, Martha Embrey (2004) stated that the absence of *E.coli* from treated drinking water is not an adequate assurance that *Salmonella* will be absent *(10)*.

2.5 Conclusions

The correlation between *Salmonella* and Generic *E.coli* in irrigation water was studied for 12 months. The majority of generic *E.coli* counts were higher than 126 CFU/ 100 ml. Based on the microbial quality criteria of the Food Safety Modernization Act for agricultural water, there was a correlation between generic *E.coli* and *Salmonella* in irrigation water. Statistical comparison of Generic *E.coli* counts obtained by EPA 1603 and the Quanti-Tray method showed no significant difference while Generic *E.coli* counts for surface and sediments waters using Quanti-Tray method were statistically significant. The Surfactant Modified Zeolite treatment was used with HDTMA for chloride and bromide separately at different concentration to attach *Salmonella*. Based on statistical analysis CTACT 20% concentration was selected for this study. EPA 1200 method showed the best results for attaching *Salmonella* followed by activated carbon then SMZ.

Further investigation is required to understand why the surface water sample could have lower E.*coli* level during flooding in comparison to high levels of *Salmonella*. This information would be very valuable to grower using surface water in the produce production environment.

2.6 References

1. Al-Nasser, A. Y., S. F. Al-Zenki, A. E. Al-Saffar, F. K. Abdullah, M. E. Al-Bahouh, and M. Mashaly. 2011. Zeolite as a feed additive to reduce *Salmonella* and improve production performance in broilers. *International Journal of Poultry Science*. 10:448-454.

2. Benjamin, L., E. R. Atwill, M. Jay-Russell, M. Cooley, D. Carychao, L. Gorski, and R. E. Mandrell. 2013. Occurrence of generic *Escherichia coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. *International Journal of Food Microbiology*. 165:65-76.

3. Bowman, R. S., E. J. Sullivan, and Z. Li. 2000. Uptake of cations, anions, and nonpolar organic molecules by surfactant-modified clinoptilolite-rich tuff. *Natural zeolites for the third millennium*:287-297.

4. Camper, A. K., M. W. LeChevallier, S. C. Broadaway, and G. A. McFETERS. 1985. Growth and persistence of pathogens on granular activated carbon filters. *Applied and environmental microbiology*. 50:1378-1382.

5. Çeçen, F., and Ö. Aktaş. 2011. Water and wastewater treatment: historical perspective of activated carbon adsorption and its integration with biological processes. *Activated Carbon for Water and Wastewater Treatment: Integration of Adsorption and Biological Treatment*:1-11.

6. Chandran, A., S. Varghese, E. Kandeler, A. Thomas, M. Hatha, and A. Mazumder. 2011. An assessment of potential public health risk associated with the extended survival of indicator and pathogenic bacteria in freshwater lake sediments. *International journal of hygiene and environmental health*. 214:258-264.

7. Craig, D., H. J. Fallowfield, and N. Cromar. 2002. Enumeration of faecal coliforms from recreational coastal sites: evaluation of techniques for the separation of bacteria from sediments. *Journal of Applied Microbiology*. 93:557-565.

8. DePaola, A., J. L. Jones, J. Woods, W. Burkhardt, K. R. Calci, J. A. Krantz, J. C. Bowers, K. Kasturi, R. H. Byars, and E. Jacobs. 2010. Bacterial and viral pathogens in live oysters: 2007 United States market survey. *Applied and Environmental Microbiology*. 76:2754-2768.

9. Eckner, K. F. 1998. Comparison of membrane filtration and multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and *enterococci* used in drinking and bathing water quality monitoring in southern Sweden. *Applied and Environmental Microbiology*. 64:3079-3083.

10. Embrey, M., P. Hunter, J. Sellwood, P. Wyn-Jones, S. L. Percival, and R. Chalmers. 2004. Microbiology of waterborne diseases: Microbiological aspects and risks. Academic Press.

11. Gerba, C. P., and J. S. McLEOD. 1976. Effect of sediments on the survival of Escherichia coli in marine waters. *Applied and environmental microbiology*. 32:114-120.

12. Goktepe, I., V. K. Juneja, and M. Ahmedna. 2006. Probiotics in food safety and human health. CRC Press.

13. Goyal, S. M., C. P. Gerba, and J. L. Melnick. 1977. Occurrence and distribution of bacterial indicators and pathogens in canal communities along the Texas coast. *Applied and environmental microbiology*. 34:139-149.

14. Hesas, R. H., A. Arami-Niya, W. M. A. W. Daud, and J. Sahu. 2013. Preparation and characterization of activated carbon from apple waste by microwave-assisted phosphoric acid activation: application in methylene blue adsorption. *BioResources*. 8:2950-2966.

15. Jones, L. A., R. W. Worobo, and C. D. Smart. 2014. Plant-pathogenic oomycetes, Escherichia coli strains, and *Salmonella* spp. frequently found in surface water used for irrigation of fruit and vegetable crops in New York State. *Applied and environmental microbiology*. 80:4814-4820.

16. Kalló, D. 2001. Applications of natural zeolites in water and wastewater treatment. *Reviews in mineralogy and geochemistry*. 45:519-550.

17. Lawley, R., L. Curtis, and J. Davis. 2015. The food safety hazard guidebook. Royal Society of Chemistry.

18. LeChevallier, M. W., T. S. Hassenauer, A. K. Camper, and G. A. McFETERS. 1984. Disinfection of bacteria attached to granular activated carbon. *Applied and Environmental Microbiology*. 48:918-923.

19. Levantesi, C., L. Bonadonna, R. Briancesco, E. Grohmann, S. Toze, and V. Tandoi. 2012. *Salmonella* in surface and drinking water: occurrence and water-mediated transmission. *Food Research International*. 45:587-602.

20. Luo, Z., G. Gu, M. C. Giurcanu, P. Adams, G. Vellidis, A. H. van Bruggen, and A. C. Wright. 2014. Development of a novel cross-streaking method for isolation, confirmation, and enumeration of Salmonella from irrigation ponds. *Journal of microbiological methods*. 101:86-92.

21. Lyimo, B., J. Buza, W. Smith, M. Subbiah, and D. R. Call. 2016. Surface waters in northern Tanzania harbor fecal coliform and antibiotic resistant Salmonella spp. capable of horizontal gene transfer. *African Journal of Microbiology Research*. 10:348-356.

22. McDougall, G. 1991. The physical nature and manufacture of activated carbon. *Journal of the Southern African Institute of Mining and Metallurgy*. 91:109-120.

23. Montville, T. J., and K. R. Matthews. 2012. Food microbiology: an introduction. ASM Press.

24. Schulze-Makuch, D., R. S. Bowman, S. D. Pillai, and H. Guan. 2003. Field Evaluation of the Effectiveness of Surfactant Modified Zeolite and Iron-Oxide-Coated Sand for Removing Viruses and Bacteria from Ground Water. *Groundwater Monitoring & Remediation*. 23:68-74.

25. Schulze-Makuch, D., S. D. Pillai, H. Guan, R. Bowman, E. Couroux, F. Hielscher, J. Totten, I. Y. Espinosa, and T. Kretzschmar. 2002. Surfactant-modified zeolite can protect drinking water wells from viruses and bacteria*. EOS, Transactions American Geophysical Union*. 83:193-201.

26. Wibowo, W., T. Utari, and R. T. Yunarti. 2011. Anion exchange capacity of chromate on modified zeolite clinoptilolite with HDTMA-Br and its regeneration*. Makara Journal of Science*.

27. Widiastuti, N., H. Wu, M. Ang, and D.-k. Zhang. 2008. The potential application of natural zeolite for greywater treatment*. Desalination*. 218:271-280.

28. Winfield, M. D., and E. A. Groisman. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli. Applied and environmental microbiology*. 69:3687-3694.

CHAPTER 3. INFLUENCE OF THE ANTIMICROBIAL MYRRH ON YOGURT CULTURE BACTERIA OVER YOGURT SHELF LIFE

3.1 Introduction

Myrrh is extracted from *Commiphora* trees of the *Burseraceae* family (*Commiphora* species are small trees or shrubs with short, thorny branches). *Commiphora myrrha*, a variable species found in southern Arabia and northeast Africa as far south as northeast Kenya. Genus *Commiphora* comprises more than 200 species *(4)* Myrrh is composed of essential oil (2-10%), ethanol soluble resin (25-40%), and water soluble gum (30-60%) *(4)*.

Myrrh has considerable antimicrobial activity and is used to cure variety of diseases. It has antibacterial and anti-fungal activity against standard pathogenic strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans (6)*. Furthermore, it shows strong inhibitory activity for the *Salmonella* Typhimirium*, E.coli* O157:H7, and *Listeria* monocytogenes on lettuce *(5)*. Myrrh has antibacterial and antifungal because of Sesquiterpenes furanodiene-6-one and methoxyfuranoguaia-9-ene-8-one. The antibacterial and antifungal properties due to Five furanosesquiterpenoids; 3-methoxy-4-fruanogermacrea-10(15)-dien-6-one, 2methoxy-4-furanogermacra-1(10)-en-6-one,furanogermacra-1(10)-4-dien-6-one and curzerenone (6,7-dihydro-5-isopropenyl-3, 6-dimethyl-6-vinyl benzofuran-4-one were isolated from myrrh gum *(1)*.

Myrrh is a safe, natural flavoring substance approved by the US Food and Drug Administration *(6)*. Extracts from many types of plants have been used as flavoring and seasoning agents in foods and beverages and also as folk medicines and food

preservatives, since ancient time. It adds characteristic flavor and prolongs the shelf life of foods by their antioxidant, bacteriostatic, and bactericidal activity*(5)*.

The aroma of myrrh is described as 'warm-balsamic, sweet, and somewhat spicy-aromatic, sharp and pungent when fresh'. In taste, myrrh possesses an acrid-aromatic bite-burning taste. Therefore, it has been used in mouthwash and toothpaste *(12)*. Myrrh alongside with honey and bee propolis can help to cure chronic non-healing wounds such as diabetic foot disease *(6)*.

Yogurt is manufactured with two cultures of bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus (11)*. *Streptococcus thermophilus* bacterium is gram positive, facultative anaerobic, non motile, nonsporeperforming and homofermentative. The optimum growth of the bacterium is at 40-45 ° C, the minimum growth is at 20-25 °C and the maximum growth is at 47-50 ° C and it can resist heat at 60 ° C up to 30 minutes. Therefore, it has been used in yogurt manufacturing *(2)*. The *Streptococcus thermophilus* bacterium is thermotolerant and it requires incubation temperature between 35-43 °C *(11)*. *Lactobacillus bulgaricus* bacterium is gram positive, facultatively anaerobic,non motile, nonsporeforming and obligatory homofermentative, and it resist acidity *(2)*.

Yogurt contains an abundant sources of nutrients of milk, carbohydrate, proteins and minerals such as phosphorous and calcium, and vitamins such as thiamin and riboflavin, niacin, folate and cobalamin. The availability of milk proteins provides most essential amino acids that are important to maintain good health. The consumption of yogurt has been increased in the dairy market in particularly in standard yogurt and yogurt drinks *(10)*.

Several studies showed several benefits of *Streptococcus thermophilus* with other yogurt bacteria. In infants to reduce the acute diarrhea and it can reduce the level of nasal colonization of pathogenic bacteria *Streptococcus pneumonia*, *Staphylococci aureus,* and β haemolytic *Streptococci*. Furthermore, a study showed that *Streptococcus thermophilus* along with *L. bulgaricus* have the ability to help to reduce cholesterol assimilation *(9)*. Both bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* can improve lactose digestion and eliminates symptoms of lactose intolerance*(3)*. The consumption of lactic acid bacteria has several benefits, more importantly, enhance the immune system, improving intestinal tract, reducing symptoms of lactose intolerance and reducing the risk of certain cancers *(8)*.

The traditional way of taking myrrh as a medicine to treat sore throat and cold was used. By adding 4 oz. boiled water to a third tea spoon of myrrh. This followed by consuming a tea spoon of myrrh dispersion every day.

To our knowledge there has not been much research performed on myrrh in foods.

Myrrh could play a role in safer and increased shelf life yogurts. Whether myrrh has antimicrobial effect on yogurt culture bacteria in pure form and on culture bacteria in yogurt matrix is not known.

The objective of this research was to study the effect of myrrh on survivability of pure yogurt cultures; *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, and viability of these yogurt culture bacteria in the matrix of a plain yogurt.

3.2 Materials and methods

3.2.1 Preparation of the myrrh

The myrrh powder (Eritera, packed by Embalee mond trading CC, LOT 319458) was dissolved in boiling distilled water to make 10% (wt/vol) dispersion. The suspension of myrrh was set for an hour at room temperature to cool down. The suspension was centrifuged (3677 g, 5 min, 20°C; Centrifuge Fisher Scientific Accuspin 400, Osterode, Germany). Myrrh is composed of 97% insoluble matter that remained in the bottom of the tube and 3% soluble in the supernatant which was used. Myrrh supernatant was diluted to make 1% (v/v) dispersion. The control had no myrrh.

3.2.2 Concentration of myrrh dispersion

Several dispersions of myrrh (0.1%, 0.5%, 1%, 2%, 3%, 5%, and 10% v/v) have been added to each of yogurt culture bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in order determine the best concentration for yogurt treatment. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* showed full growth and survivability at 0.1 % and 0.5 % of myrrh dispersions for 8 hours which indicated weakness of the Myrrh dispersion. At 1% of Myrrh dispersion showed the best treatment on each of the yogurt culture bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* with stability on *Streptococcus thermophilus* growth and slightly decrease in *Lactobacillus bulgaricus .* At 2%, 3%, of Myrrh dispersion on each of yogurt culture bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* did not survive more than 4 h and at high elevated myrrh dispersions of 5%, and 10%, on each of yogurt culture bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus*

were completely killed after 2 h. Therefore, myrrh dispersion at 1% v/v was the best treatment and used for the growth, viscosity, pH, titratable acidity and color experiments.

3.2.3 Preparation of peptone buffer

Peptone water (0.1%) was prepared by dissolving 1 g of peptone powder (Bacto Peptone, Difco) in 1 L of distilled water. Followed by dispensing 9 ml of the peptone water in a tube. All tubes were autoclaved at 121°C for 15 min prior to use.

3.2.4 Preparation of the media for the pure peptone water

The M-17 agar (Oxoid, Basingstike,UK) was prepared by adding 45 gram to 950 ml distilled water. The lactose solution (Oxoid, Basingstike,UK) was prepared by adding 5 g in 50 ml distilled water to make 10% (wt/vol). Both M-17 and lactose solution were separately boiled and autoclaved. The M-17 and lactose solutions were cooled to 55 °C then mixed together before being poured onto petri dishes (Corning™ Falcon™, 100 x 15, Durham, NC). The MRS agar was prepared by adding 70g (Difco, Beton,Dickinson and Co.m Sparks, MD) to 1 L distilled water. Both Media and lactose solution were autoclaved separately at 121 °C for 15 minutes.

3.2.5 Inoculation of myrrh with *Streptococcus thermophilus* and *Lactobacillus bulgaricus.*

A 0.3 ml of the myrrh dispersion was added to 27.6 ml of 0.1 % peptone water and 3 ml of freshly thawed pure cultures of *Streptococcus thermophilus* ST-M5 *and Lacobacillus bulgaricus* LB-12 separately to make up to 1% of myrrh dispersion in 30 ml final volume. The inoculated myrrh dispersion was evaluated at 0, 2, 4, 6, and 8 hours.

This was followed by pour plating 1 ml of each dilution on M17 agar for *Streptococcus thermophilus* and MRS agar for *Lactobaciulls bulgaricus* in duplicate. All M17 plates were incubated aerobically at 37 °C for 24h whereas, all MRS for *Lactobaciulls bulgaricus* plates were incubated anaerobically at 42 °C for 72 h. After incubation, a Quebec Darkfield Colony Counter (Leica Inc., Buffalo, NY, USA) was used to assist in enumerating the colonies.

3.2.6 Preparation of yogurt

Two sanitized 17 L pails contained two gallon of whole milk in each pail. The pails were placed in water bath and were heated up to 85 °C for 30 minutes. Pails were transferred immediately and placed into an ice water bath. Once the temperature reached 46.6 °C, 2 ml of freshly thawed pure cultures of *Streptococcus thermophilus* ST-M5 and *Lactobaciulls bulgaricus* LB-12 respectively were added. This was followed by adding 0% (control) and 1% of myrrh dispersion with agitation. After mixing, yogurt mixes were poured into 355ml Reynolds RDC212–Del-Pak Combo-Pak containers (Alcoa, Inc., Pittsburgh, PA, USA) and incubated at 40 °C to pH 4.6 before cooling to 4 °C. Control yogurt had no myrrh. The preparation of yogurt were in triplicate

3.2.7 Preparation of the Media for the yogurt matrix

3.2.8 Preparation of *Streptococcus thermophilus* agar

The appropriate proportion of ingredients used to make *Streptococcus thermophilus* agar by weight 10 g of sucrose (Amersco, Solon, OH), 10 g of Bacto Tryptone (Becton, Dickinson and Co., Sparks, MD), 2 g of K_2HPO_4 (Fisher Scientific, Fair Lawn, NJ), and 5

g of Bacto yeast extract (Bacto, Dickinson and Co., Sparks, MD) in 1 L of distilled water. All ingredients were mixed with heating until boiled. The pH was adjusted to 6.8 by adding approximately 15 drops of 6 N HCl. This was followed, by adding 6 ml of 0.5% bromocresol purple solution and 12 g of agar were added to the mixture. The media was autoclaved at 121 °C for 15 minutes.

3.2.9 Preparation of MRS agar pH 5.2

Difco lactobacillis MRS broth (Becton, Dickinson and Co.m Sparks, MD) was prepared by weighing 55 g of the powder and suspended in 1 L of distilled water. MRS broth was adjusted to pH 5.2 by adding approximately 40 drops of 6 N HCl before sterilization followed, by adding 15 g of agar. The media was heated to boiling and was stirred to completely dissolve the MRS powder and agar which were autoclaved at 121 °C for 15 minutes.

3.2.10 Influence of myrrh on yogurt cultures

One g of yogurt was diluted in 9 ml of 0.1% (wt/vol) peptone water .An ml of each dilution was aseptically pipetted onto petri dishes and pour plated using *Streptococcus thermophilus* agar for *Streptococcus thermophilus* and MRS agar pH 5.2 for *Lacobacillus bulgaricus*. Plating was conducted in duplicate at 0, 1, 2, 3, 4, and 5 weeks after yogurt manufactured. All *Streptococcus thermophilus* agar plates were incubated aerobically at 37 °C for 24h whereas. All MRS plates were incubated anaerobically at 42 °C for 72h. After incubation period, a Quebec Darkfield Colony Counter (Leica Inc., Buffalo, NY, USA) was used to assist in enumerating the colonies.

3.2.11 pH measurements

The pH of the yogurts were measured with an UltraBasic pH/mV Meter (Denver Instrument Co., Arvada, CO, USA). The pH electrode was calibrated with pH 7.00 and 4.00 buffer solutions (VWR International, West Chester, PA, USA) prior to use. The pH of the yogurts at 5 °C at 0, 1, 2, 3,4 , and 5 week of storage.

3.2.12 Titratable acidity

The Titratable acidity was measured by weighing 9 g of yogurt in crucible and adding 3 drops of phenolphthalein indicator. This was followed by stirring and titrating 0.1 N NaOH. Once the color changed to pink and remained pink for 30s the titration was stopped. Each batch was tested weekly for 5 weeks.

3.2.13 Apparent viscosity

The apparent viscosity was measured for myrrh and control (Brookfield model DV-II and Helipath Stand in the down direction; Brookfield Engineering Laboratories Inc., Stoughton, MA). (Temperatures ranged from 3°C – 8°C). A T-C spindle was used at 30 rpm and a hundred data points were averaged per replication. Three replications were conducted. Using The computer and Wingather 32 software (Brookfield Engineering Laboratories Inc.).

3.2.14 Color measurements

The L* (whiteness to blackness), a* (redness to greenness), and b* (yellowness to blueness), C* (chrome) and h* (hue angel) values of the yogurts were determined with a HunterLab MiniScan XE Plus spectrophotometer (HunterLab, Reston, VA, USA)

using Universal software (HunterLab, Reston, VA, USA). The spectrophotometer was calibrated with white and black tiles D65/10°. Five measurements were taken and averaged for each sample at approximately 5 ± 1°C. Color measurements were obtained at 0, 1, 2, 3, 4, and 5 week of storage.

3.3 Statistical analysis

Data were analyzed as a randomized block design with repeated measures using and Proc GLM of SAS (version 9.4, SAS Institute Inc., Cary, NC). Differences of least squares means were used to determine significant differences at $P < 0.05$ for main effects (myrrh and time of exposure) and the interaction effect (myrrh * time of exposure).

3.4 Results

3.4.1 Microbial counts in peptone water

Streptococcus thermophilus

The microbial counts of *Streptococcus thermophilus* ST-M5 in peptone water as influenced by myrrh at 0, 2, 4, 6, and 8 hours is shown in Figure 2. Microbial counts for myrrh and control remained steady around 9.9 Log CFU/ ml. There was no significant difference between control and myrrh on the interaction effect of myrrh * time. Also, the main effect Myrrh and main effect time were not significant. The myrrh concentration of 1% was not strong to kill the *Streptococcus thermophiles* ST-M5 bacteria.

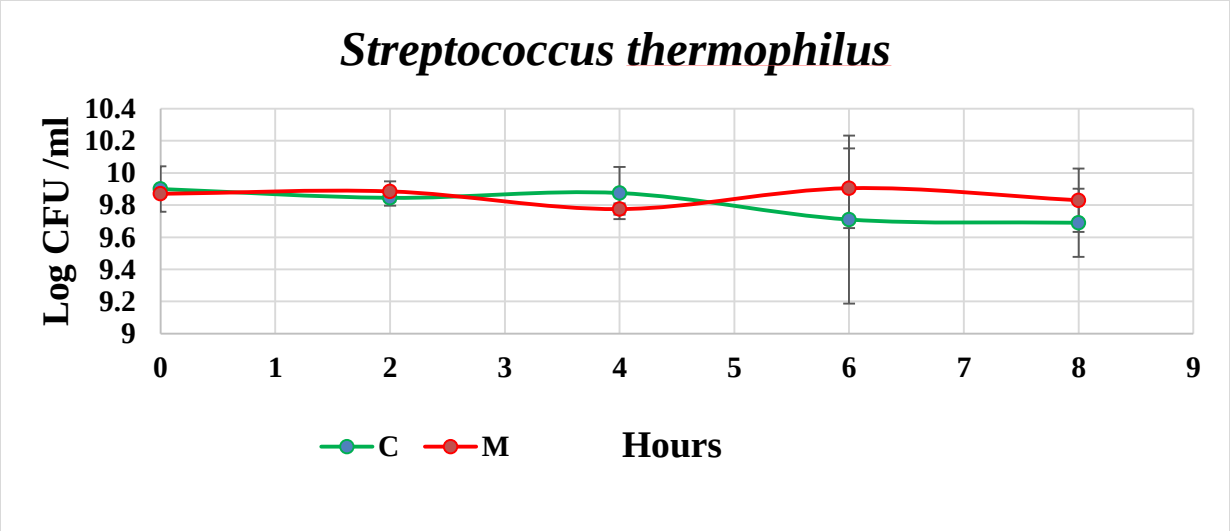

Figure 2. *Streptococcus thermophiles* ST-M5 counts influenced by myrrh and control in peptone water

Lactobacillus bulgaricus

The microbial counts of *Lactobacillus bulgaricus* LB-12 in peptone water as influenced by myrrh at 0, 2, 4, 6, and 8 hours is shown in Figure 3. Microbial counts for myrrh and control had slightly decreased from 9.4 to 9.0 log CFU/ ml. The interaction effect of treatment * time was not significant. Also, the main effect treatment and main effect time were not significant. The myrrh concentration of 1% was not strong to kill the *Lactobacillus bulgaricus* LB-12 bacteria.

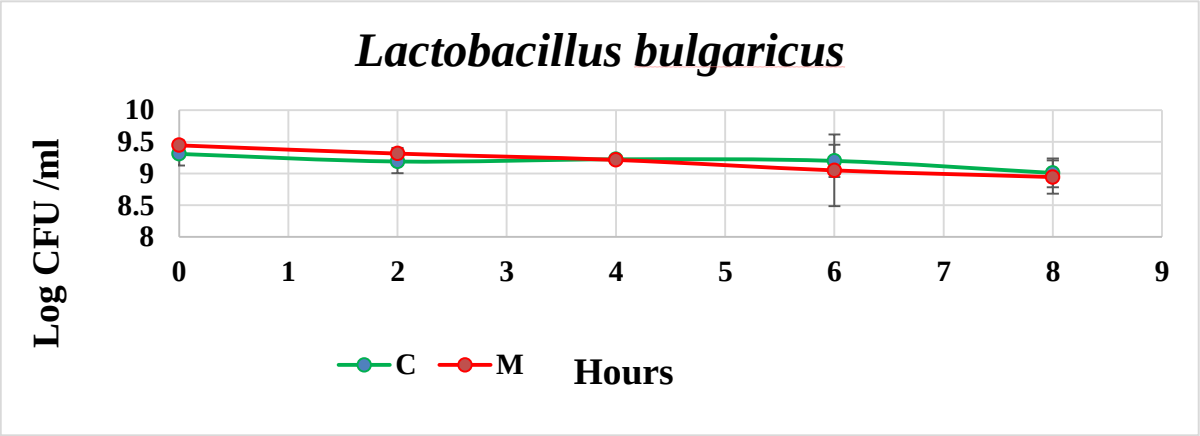

Figure 3.*Lactobacillus bulgaricus* LB-12 counts influenced by myrrh and control in peptone water

3.4.2 Microbial counts in yogurt matrix

Streptococcus thermophilus

The microbial counts of *Streptococcus thermophilus* ST-M5 as influenced by myrrh for shelf life study throughout 5 weeks is shown in Figure 4. Microbial counts for myrrh and control increased 0.71 and 1.01 log CFU/g respectively over time from 0 to 5 weeks is shown Figure 4. There was no significant difference on the interaction effect of treatment * time. Also, the main effect treatment was not significant. There was a significant ($P < 0.001$) time effect Table 10. Microbial counts for *Streptococcus thermophilus* in yogurt containing myrrh (5.4 log cfu/ml) were not significantly different than *S. thermophilus* counts in the control yogurt (5.3 log cfu/ml). The myrrh dispersion of 1% was not strong to kill the *Streptococcus thermophilus* ST-M5 bacteria. Furthermore, yogurt starter culture *S. thermophilus* and *L. bulgaricus* have a symbiotic relationship in the milk matrix to grow and multiply. The *S. thermophilus* obtained the advantage and had a slight increase of a log.

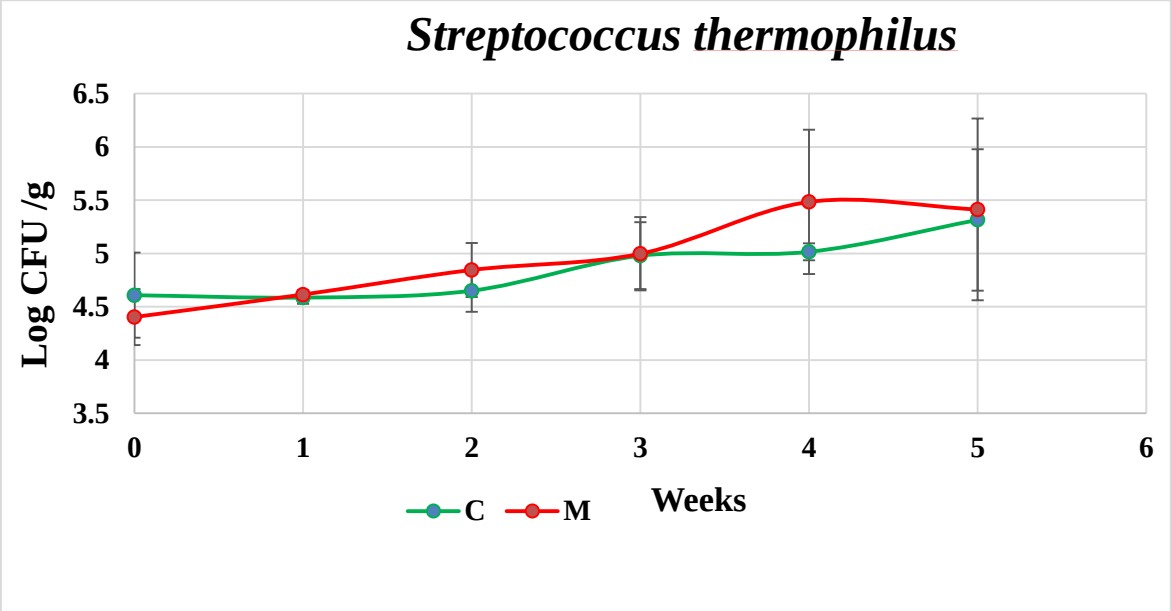

Figure 4. *Streptococcus thermophilus* counts influenced by myrrh and control in yogurts.

Table 10. Means for *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 counts in yogurts for various storage times.

Week	0	1	2	3	4	5
S.thermophilus	4.54 [c]	4.60 [bc]	4.74 [bc]	4.98 [ab]	5.25 [a]	5.36 [a]
L. bulgaricus	6.79 [a]	6.65 [a]	6.12 [a]	5.05 [b]	4.09 [c]	2.94 [d]

[abcd] Means not containing a common letter are significantly different ($p > 0.05$)

Lactobacillus bulgaricus

The microbial counts of *Lactobacillus bulgaricus* LB-12 as influenced by myrrh for shelf life study over 5 weeks is shown in Figure 5. Microbial counts for myrrh and control decreased from 3.93 and 3.77 log CFU/g respectively over time from 0 to 5 weeks Figure 5. There was no significant difference in interaction effect(treatment * time). There was significant time effect $P < 0.001$ and myrrh effect $P < 0.046$. Although *L. bulgaricus* log counts were significantly lower for the myrrh yogurts than for the control, the log counts remained within a log of each other during 5 weeks of storage. Moreover, yogurt starter culture *S. thermophilus* and *L. bulgaricus* have a symbiotic relationship in the milk to grow and multiply, the significant reduction of the *L. bulgaricus* microbial counts due to the slight increasing of *S. thermophilus.* The myrrh concentration of 1% was not strong to kill the *Lactobacillus bulgaricus* LB-12 bacteria.

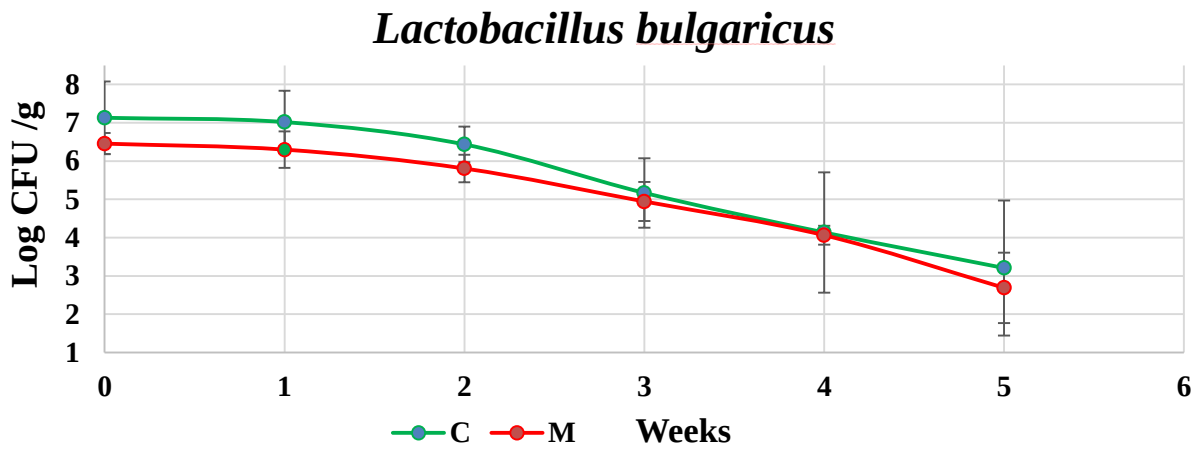

Figure 5. *Lactobacillus bulgaricus* LB-12 counts influenced by myrrh and control in yogurts.

Table 11. Means for *Lactobacillus bulgaricus* LB-12 for yogurts containing myrrh and control.

Treatment	Means
Control	5.5162 [a]
Myrrh	5.0433 [b]

[ab] Means not containing a common letter are significantly different ($p < 0.05$)

3.4.3 Measurements

pH

The pH of yogurt during its 5 weeks shelf life study is shown in Figure 6. There was no significant difference in the interaction effect of treatment * time. Also, there was no significant difference in time effect. The main effect myrrh was significant ($P < 0.043$). The pH of the yogurts containing myrrh was significantly higher than the control yogurt, However, their pH values were within 0.1 pH units of each other at any given week. This may be due to the addition of the free hydroxyl ions originating from myrrh/ water dispersion. Free hydroxyl ions may have neutralized some of the lactic acid produced by *Streptococcus thermophilus* ST-M5 and for *Lactobacillus bulgaricus* LB-12 bacteria resulting in a slight increase of the yogurt's pH compared to the control. Also, dairy products are able to bind or release ions causing a small change in pH upon addition of acid or alkali substance. Furthermore, generally, dairy products have high protein content ensuring a relative high buffering capacity due to the amino groups' buffering effect *(7)*. According to Mohamed et. Al (2016) the pH values increased with increasing of essential oil (EO) concentration. This increase may be due to the action of EO inhibition of acidity forming and the alkaline effect of EO itself. During storage, pH values slightly decreased in all treatments. The decrease in pH during storage could be related to the hydrolysis that occurred in emulsifying salt and their interactions with proteins *(1)*

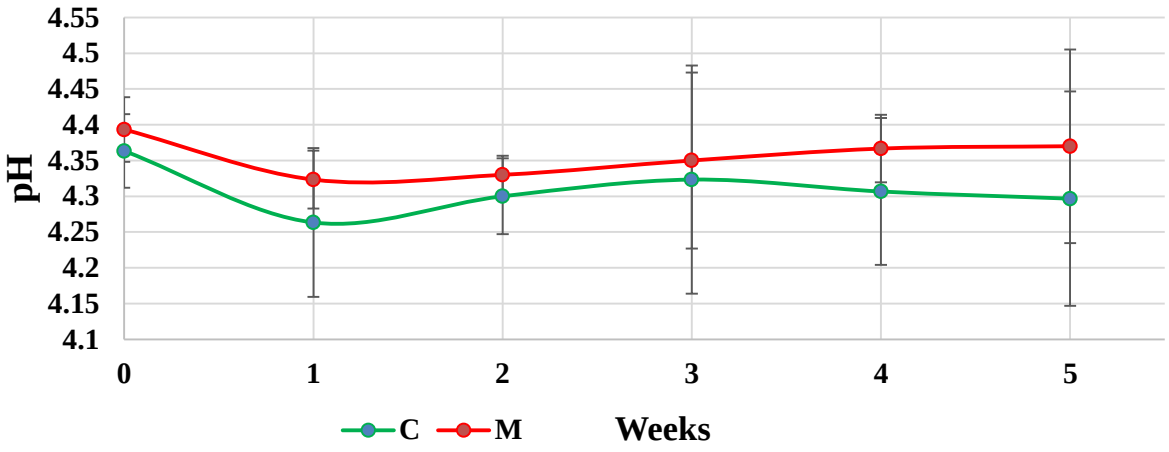

Figure 6.The pH of yogurts containing myrrh and control.

Table 12. The p values for (myrrh, time and interaction) for the pH of yogurts.

Effect	pH	TA
Myrrh	0.0430	0.3575
Time	0.3774	0.2112
Interaction (myrrh* time)	0.9802	0.8260

Table 13. Means of the pH yogurts containing myrrh and control.

Treatment	Means
Control	4.35556 [a]
Myrrh	4.30889 [b]

[ab] Means not containing a common letter are significantly different ($p < 0.05$)

Titratable Acidity (TA)

Titratable Acidity values for myrrh and control yogurts throughout 5 weeks is shown in Figure 7. The TA values of myrrh remained unchanged compared to the control. Myrrh did not alter the TA values of the yogurt. The titratable acidity values remained steady around 1.2 % expressed as lactic acid for myrrh and control yogurt types throughout the 5 weeks storage period. There was no significant difference between control and myrrh on the interaction effect of myrrh * time. Also, the main effect myrrh and main effect time were not significant.

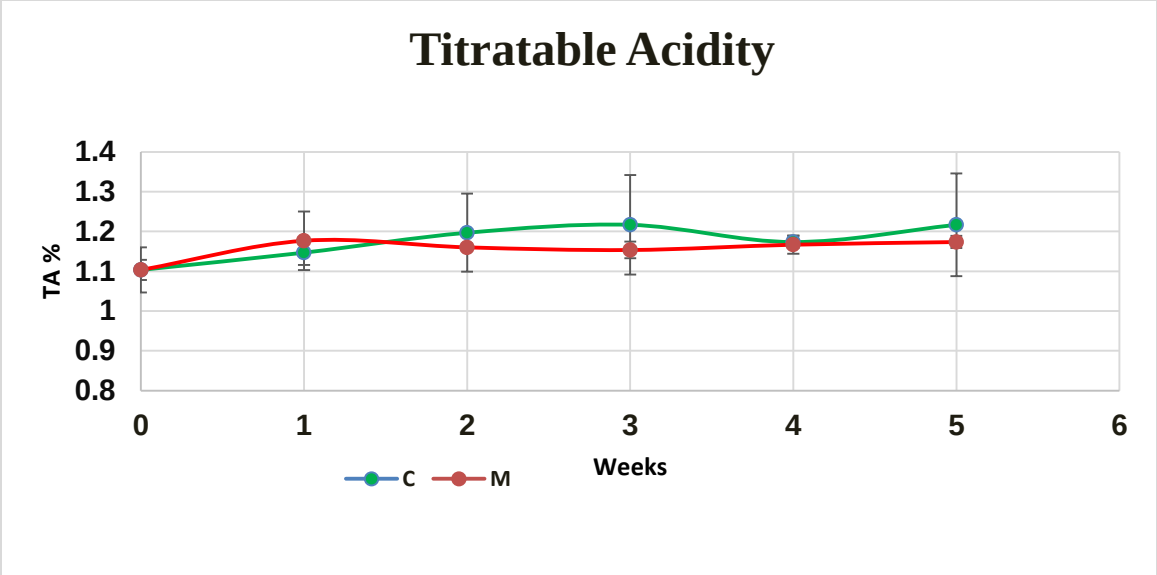

Figure 7.The Titratable Acidity of yogurts containing myrrh and control.

Apparent viscosity

The apparent viscosity values for myrrh and control for shelf life study throughout 4 weeks is shown in Figure 8. Viscosity values of myrrh and control yogurts slightly increased from around 5100 to 6600 cPs. Both yogurt types for myrrh and control throughout the storage period had no significant difference between control and myrrh for the interaction effect (myrrh * time). Also, the main effect of myrrh and main effect time were not significant.

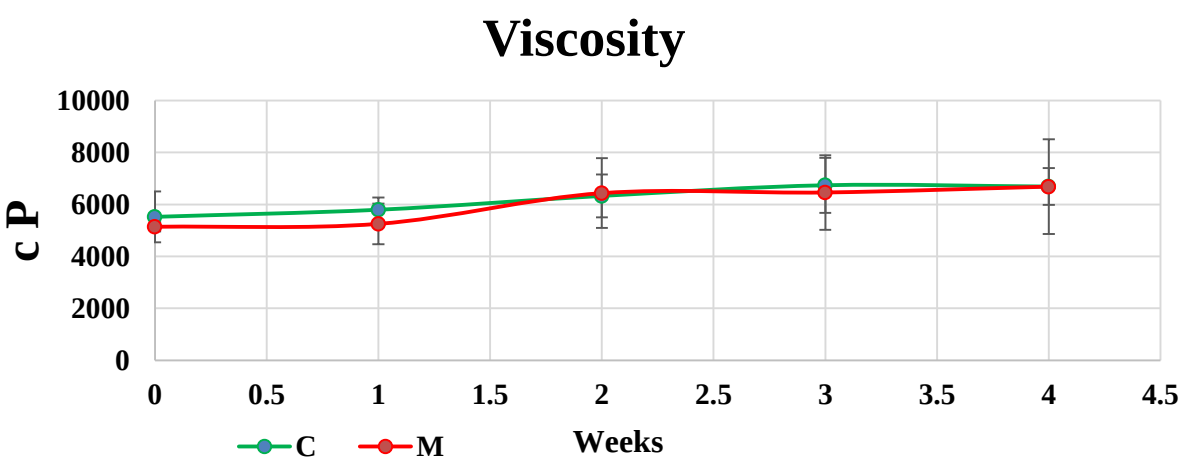

Figure 8. The Viscosity of yogurts containing myrrh and control.

3.4.4 COLOR

L* (Lightness to darkness)

The *L** (lightness) of the myrrh and control yogurts throughout 5 weeks storage is shown in Figure 9. The control yogurt remained steady throughout the 5 weeks while the myrrh yogurt increased by 2 units through the 5 weeks. There was no significant difference on interaction (myrrh * time) and time effect. There was a significant ($P < 0.003$) effect for treatment (Table 14) and (Table 15).

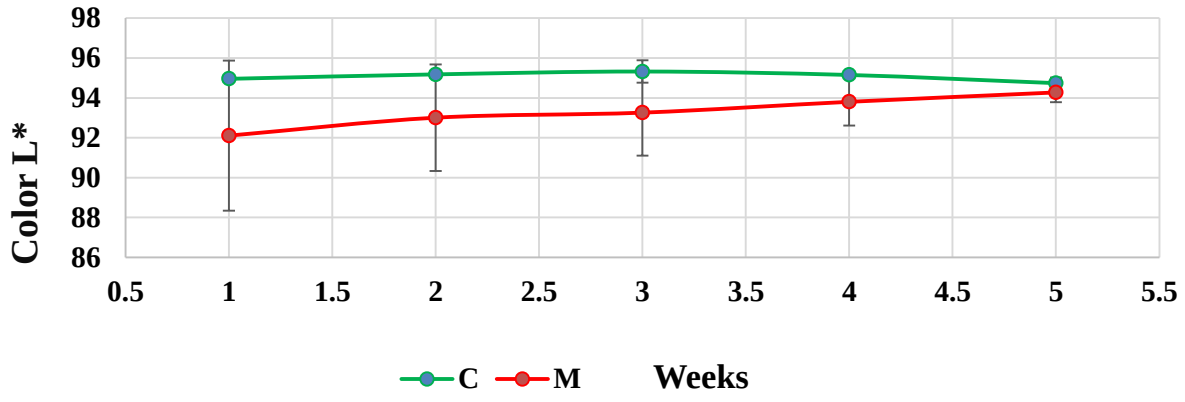

Figure 9. Measurement of L* color for yogurts containing myrrh and control during storage time for 5 weeks.

Table 14. The p values for (myrrh, time and interactions) for the colors of yogurts containing myrrh and Control.

Effect	L*	a*	b*	C*	h*
Myrrh	0.0032	0.0001	0.3505	0.4662	0.0034
Time	0.7631	0.1851	0.9820	0.9743	0.4221
Interaction (myrrh * time)	0.6688	0.2219	0.6869	0.6850	0.5992

Table 15. Means for the colors of myrrh and control as influenced by myrrh.

Effect	L*	a*	b*	C*	h*
Control	95.0700 [a]	-0.8980 [a]	10.2300 [a]	10.2773 [a]	94.9080 [a]
Myrrh	93.2900 [b]	-0.5760 [b]	10.3673 [a]	10.3847 [a]	92.4947 [b]

[ab] Means not containing a common letter are significantly different ($p < 0.05$)

a* (Redness to greenness)

The *a** (greenness) of the myrrh and control yogurts throughout 5 weeks storage is shown in Figure 10. The control yogurt remained steady at -0.8 to -1.0 throughout the 5 weeks whereas the myrrh yogurt decreased -0.14 to -0.58 units from week 1 to week 5. There was no significant difference for the interaction effect (myrrh * time) and main effect time. There was a significant ($P < 0.001$) effect for treatment myrrh Table 14 and Table 15.

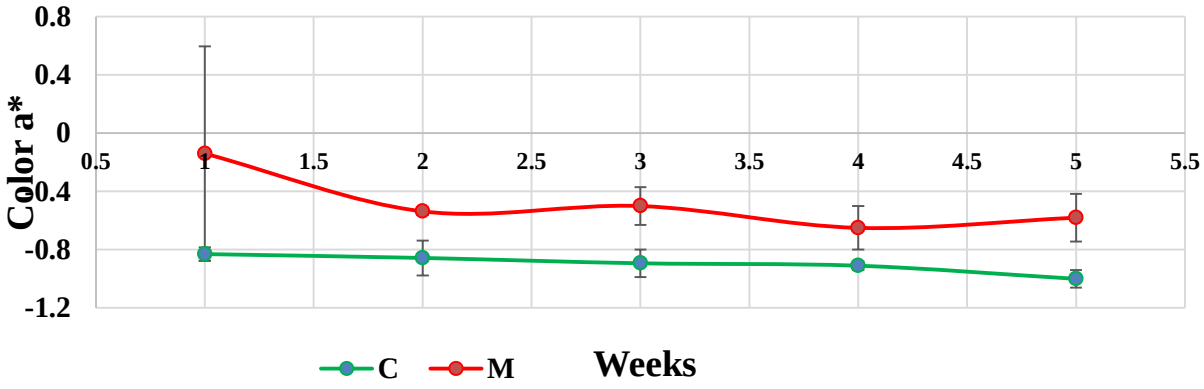

Figure 10. Measurement of a* color for yogurts containing myrrh and control during storage time for 5 weeks.

b* (Yellowness to blueness)

The *b** (yellowness) of the myrrh and control yogurts throughout 5 weeks storage is shown in Figure 11. The myrrh and control yogurts fluctuated throughout the 5 weeks storage. There was no significant difference between control and myrrh on the interaction effect of myrrh * time also, the main effect myrrh and main effect time were not significant

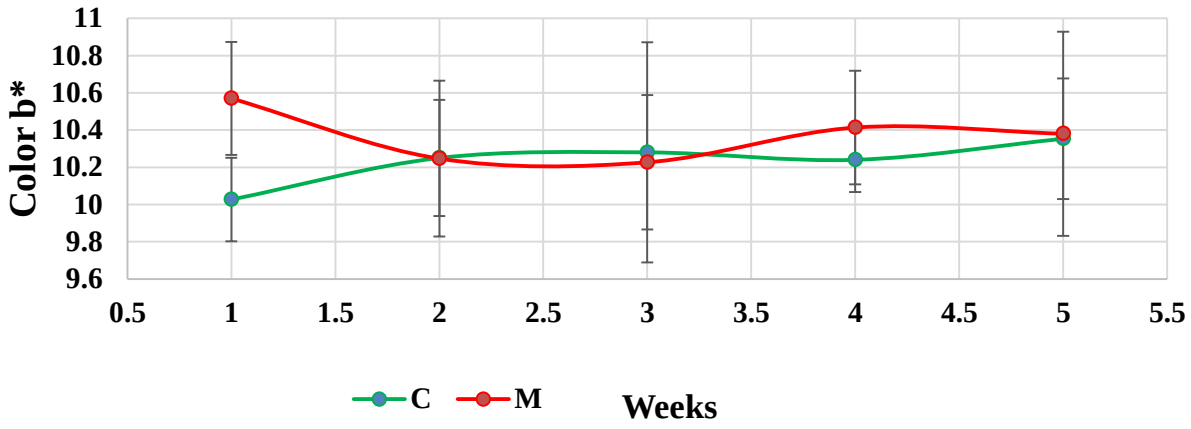

Figure 11. Measurement of b* color for yogurts containing myrrh and control during storage time for 5 weeks.

C* (Chroma/saturation)

The *C** (Chroma) of the myrrh and control yogurts throughout 5 weeks storage is shown in Figure 12. The myrrh and control yogurts fluctuated throughout the 5 weeks storage. There was no significant difference between control and myrrh on the interaction effect of myrrh * time also, the main effect myrrh and main effect time were not significant

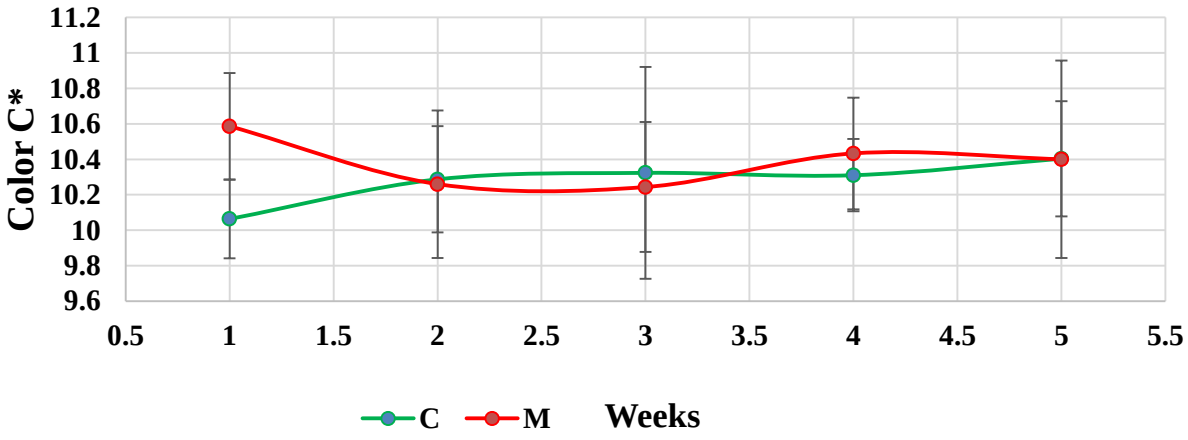

Figure 12. Measurement of C* color for yogurts containing myrrh and control during storage time for 5 weeks.

h* (hue)

The *h** (hue) of the myrrh and control yogurts throughout 5 weeks storage is shown in Figure 13. The control yogurt remained steady at 94.73 to 95.52 throughout the 5 weeks whereas the myrrh yogurt increased from 89.98 to 93.18 units from week 1 to week 5. There was no significant difference on the interaction effect (myrrh * time) and time effect. There was a significant on the effect for treatment ($P < 0.003$) in Table 14 and Table 15.

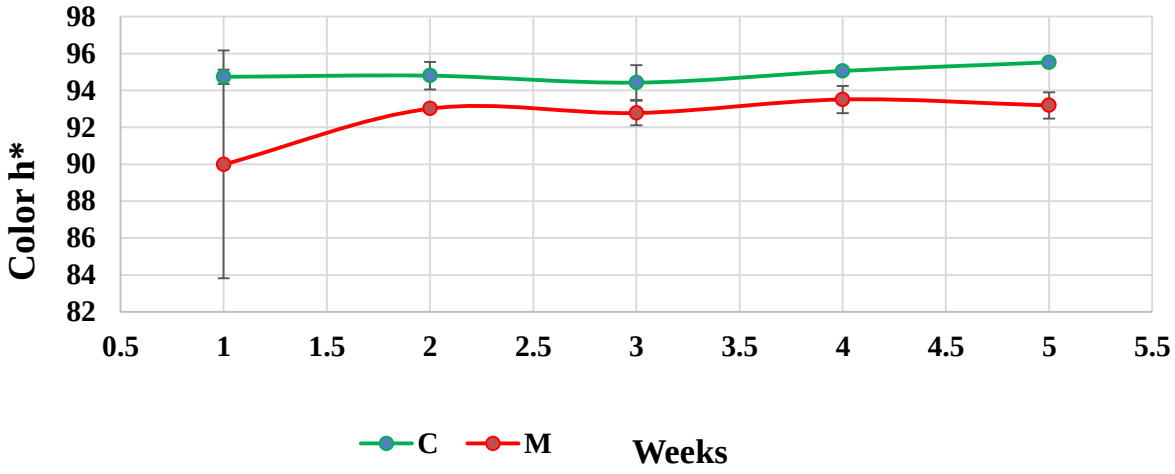

Figure 13. Measurement of h* color for yogurts containing myrrh and control during storage time for 5 weeks.

The brownish color of yogurt was obtained from the myrrh gum which contains yellowish to brownish colorant. The lightness of the control in yogurt when it is fresh and throughout 5 weeks had gained higher values of whiteness degree and during time storage than myrrh treatment. The Lightness of myrrh treatment slightly increase which indicates degradation of myrrh dispersion throughout 5 weeks storage time. According to Mohammed et al. (2016) myrrh Essential oil with cheese in control treatment showed the highest whiteness when it is fresh and during storage time compared to other treatments. *(1)*. The greenness for the control remained stable with a slight decrease, whereas the myrrh treatment showed significant decrease in the first weeks, then remained stable for the rest of 4 weeks. This indicates that degradation occurred in the first week. Although the myrrh dispersion has yellowish to brownish color, there was no significant difference in the b* parameter. Whiteness decreased with the increase of myrrh ratios. According to Mohammed et. al. (2016) reported that the yellowish parameter (b) was higher than the reddish parameter (a) and this was due to the myrrh

crystals *(1)* . The C* parameter kept fluctuating during 5 weeks storage time without significant difference. The hue showed significance increasing in the first week then remained steady for the rest of 5 weeks storage time.

3.5 Conclusions

Myrrh is a natural flavoring and a safe substance that is approved by FDA as a food flavor. Myrrh has antibacterial and antifungal properties against pathogens. Myrrh at 1% (v/v) had no kill effect on *S.thermophilus* and *L. bulgaricus* in pure culture form and in the yogurt matrix. The pH of yogurts containing myrrh was higher than the control yogurt within 0.1 pH units. The titratable acidity values remained steady throughout the 5 weeks at around 1.2%. There was no significant difference between myrrh yogurt and control for the titratable acidity and viscosity. Although there was some statistical significance in some color parameters, the color of myrrh yogurt was similar to the control yogurt. Yogurt culture bacteria can survive in the presence of myrrh in the pure form and in the yogurt matrix.

3.6 References

1. A.G. Mohamed, H. M. A., Jihan M. Kassem, Walid A. Gafour and A.G. Attalah 2016. Impact of Myrrh Essential Oil as a Highly Effective Antimicrobial Agent in Processed Cheese Spreads. *International Journal of Dairy Science*. 11 (2):41 - 51.

2. Barbaros H. Öze, G. A. E. 2014. Dairy Microbiology and Biochemistry CRC press.

3. de Vrese, M., A. Stegelmann, B. Richter, S. Fenselau, C. Laue, and J. Schrezenmeir. 2001. Probiotics—compensation for lactase insufficiency. *The American journal of clinical nutrition*. 73:421s-429s.

4. Hanuš, L. O., T. Řezanka, V. M. Dembitsky, and A. Moussaieff. 2005. Myrrh-commiphora chemistry. *Biomedical papers*. 149:3-28.

5. Kim, S. Y., D. H. Kang, J. K. Kim, Y. G. Ha, J. Y. Hwang, T. Kim, and S. H. Lee. 2011. Antimicrobial activity of plant extracts against *Salmonella* Typhimurium, Escherichia coli O157: H7, and Listeria monocytogenes on fresh lettuce. *Journal of food science*. 76:M41-M46.

6. Lotfy, M., G. Badra, W. Burham, and F. Alenzi. 2006. Combined use of honey, bee propolis and myrrh in healing a deep, infected wound in a patient with diabetes mellitus. *Br J Biomed Sci*. 63:171-173.

7. Maher M. Al-Daabbas, K. A.-I. a. B. M. A.-A. 2011. Effect of chemical composition on the buffering capacity of selected dairy products. *Jordan Journal of Agricultural Sciences*. 7:690-700.

8. Parvez, S., K. A. Malik, S. Ah Kang, and H. Y. Kim. 2006. Probiotics and their fermented food products are beneficial for health. *Journal of applied microbiology*. 100:1171-1185.

9. Sharma, R., B. Bhaskar, B. S. Sanodiya, G. S. Thakur, P. Jaiswal, N. Yadav, A. Sharma, and P. S. Bisen. 2014. Probiotic Efficacy and Potential of Streptococcus thermophilus modulating human health: A synoptic review. *J Pharm Biol Sci*. 9:52-8.

10. Weerathilake, W., D. Rasika, J. Ruwanmali, and M. Munasinghe. The evolution, processing, varieties and health benefits of yogurt. *International Journal of Scientific and Research Publications*. 4:1-10.

11. White, C. H., A. Kilara, Y. Hui, and R. C. Chandan. 2008. Manufacturing yogurt and fermented milks. John Wiley & Sons.

12. Zhu, N., S. Sheng, S. Sang, R. T. Rosen, and C. T. Ho. 2003. Isolation and characterization of several aromatic sesquiterpenes from Commiphora myrrha. *Flavour and fragrance journal*. 18:282-285.

Vita

Mohammed Alhejaili, born and raised in Medina Saudi Arabia, started studying Microbiology in 2005 and he received the bachelor's degree in 2008 from Taibah University in Medina, Saudi Arabia. In 2009 Mohammed Alhejaili moved to the United Kingdom and obtained his masters' degrees in Food Science, Safety and Health in 2011 from Heriot Watt University. To pursue his passion in food safety, he moved to the United State.

He enrolled in the Ph.D. program of Food Science at Louisiana State University in 2014, and conducted his research on the isolation and detection of *Salmonella* and generic *E.coli* in irrigation water. Furthermore, Mohammed studied the influence of myrrh as an antimicrobial on the shelf life of yogurt.

He is a candidate to receive his Doctorate of Philosophy in December 2017 and plans to begin his professional career in Dairy production. Mohammed's future aspirations include becoming a professor in academia.