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Research Article

Single and combined effects of oregano leaf and clove oils on the reduction of *salmonella* enteritidis on chicken egg shells

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Abstract

Table eggs are versatile foodstuffs used in various food products. Eggs are often consumed or incorporated into other food products to enhance taste and nutrition and act as an emulsifying agent. Too often the consumption of contaminated eggs leads to foodborne illnesses in people. When whole eggs or egg products come into contact with contaminated surfaces or other contaminated eggs, pathogens can spread from one egg to another. Research has been conducted to uncover ways to protect whole eggs and their products from contamination by coating the egg's exterior with edible coatings. Mineral oil, among other edible oils, is one such oil shown to be effective in reducing the bacteria in eggs when applied to the egg's exterior. With the average consumer becoming more aware of the chemicals used in the preservation of food, some researchers have started to explore more natural solutions to minimize the contamination of food products. The use of essential oils to prevent contamination has been studied and analyzed for their antimicrobial properties in the inhibition of the growth of foodborne pathogens. The oils used in this current study are Oregano and Clove Oil. Both oils used individually and combined compared to a positive control showed no differences ($p > 0.05$). Interestingly, when the oils were combined 1:1 there was a difference of 0.66 log in the survival bacterial counts compared to the positive control. However, this difference was also not significant ($p > 0.05$).

Introduction

Eggs are common staples for many people across the globe. They can be consumed raw, boiled, scrambled, and in many other ways. Wardy, et al. [1], stated that eggs are an important and inexpensive source of high protein, vitamins, and minerals. Eggs are also added to combinations of ingredients to produce goods like brownies, angel food cakes, and even mayonnaise. Because eggs are perishable; during pre-harvest and post-harvest an egg becomes more susceptible to bacteria that can penetrate the eggshell and lead to spoilage [2]. Contaminated eggs pose a threat when they are consumed raw or undercooked. Bacteria-infected eggs are associated with gastroenteritis; which often results in diarrhea and abdominal pain [3]. Contaminated eggs are involved in cross-contamination. Cross-contamination is associated with fruits and vegetables when stored together. Dirty or inadequately washed eggs lead to cross-contamination and the spread of bacteria, like *Salmonella*, to other eggs and food products. Prevention is a widespread practice used to control egg contamination. Egg sampling, grading, and washing during post-harvest can reduce contamination before the eggs leave the processing plants. Testing is done to identify any outbreaks of bacterial contamination. Eggs become contaminated when the shell meets a contaminated surface [4]. *Salmonella* Enteritidis is a pathogen that readily contaminates eggs and leads to illness in humans [5]. Thousands of illnesses are reported yearly due to Salmonella-contaminated foods. Contamination of 2 eggs by *Salmonella* has become a major concern in the food industry. Bio-contaminate protocols are enforced to identify and prevent the spread of any pathogen before foods enter the marketplace [5]. In past studies, researchers have had success in using essential oils such as clove and oregano oil coating on the outside of the eggshell to not only prevent bacterial contamination but also to extend shelf life. Oregano oil and clove oil are essential oils known for their numerous health

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benefits and uses. According to [6] Oregano, particularly the species *Origanum vulgare* (Greek Oregano), contains compounds such as thymol and carvacrol. These compounds exhibit anti-pathogenic properties, which can help combat the emergence of antibiotic-resistant strains and drug-resistant biofilms associated with infections, particularly as populations grow and immunity levels vary. Oregano oil is also noted for its antioxidant, anti-inflammatory, analgesic, and antimicrobial properties, making it valuable for food stability and health. It has been linked to cardiovascular disease reduction, stroke prevention, and potential cancer reduction, and is approved by the USA as a spice and natural flavoring to reduce oxidation.

Veenstra, et al. [7] Clove oil, derived from *Syzygium aromaticum*, consists of phenylpropanoids and various aliphatic and cyclic volatile terpenes. Known for its strong antimicrobial properties, clove oil is often used for its therapeutic benefits, which include pain relief and anti-inflammatory effects.

Waimaleongora-Ek, et al. [8], reported that mineral oil coating reduced the weight loss of eggs by more than 10 times and extended the shelf life of eggs. Food-grade mineral oil is commonly used as a coating for table eggs because it is odorless, tasteless, and colorless [8]. Though the egg has a natural barrier that lies on the outside of the egg (the cuticle) that protects it, the mineral oil protects the egg from contamination by covering the pores of the eggshell and creating a barrier so that pathogens cannot penetrate the egg. Immediately before oviposition the cuticle is secreted over the shell and acts as a natural defense barrier for the egg [9,10]. During the cleaning process, the cuticle can be damaged or even washed away leaving the egg unprotected [11-13]. Along with keeping pathogens from entering the egg mineral oil coatings also reduce weight loss of the egg and preserve the interior quality of the egg [8]. Other types of edible coatings, such as essential oils, have been applied to the eggshell to prevent bacterial contamination and deterioration of the egg. Essential oils derived from plants are known to possess antibacterial properties. It was hypothesized that a coating applied to the outside of the egg can block the ability of foodborne pathogens to penetrate the egg. The study's objective was to examine the antimicrobial effects of 2 essential oils (Oregano Leaf and Clove); used independently and combined, on the reduction of Salmonella Enteritidis into table eggs.

Materials and methods

Bacterial strain

A lyophilized pelleted isolate of *S.* Enteritidis (Kwik-Stik™, Microbiologics®, St. Cloud, MN, USA) was purchased and stored under refrigeration at 4 °C upon arrival in the poultry research laboratory (Department of Agriculture, Nutrition and Human Ecology, Prairie View A&M University, Prairie View, TX, USA) and stored until it was ready to be used. Once the study began the pelleted bacteria housed in the Kwik-Stik™ tube was removed from its foil pouch and allowed to acclimate to room temperature. The lyophilized pellet was hydrated by pinching the fluid ampule at the top of the Kwik-Stik™ and allowing the hydrating fluid to flow through the shaft and collect at the opposite end where the pellet and swab were contained. The pellet was homogenized by gently squeezing the outside end of the tube to crush and mix the pellet and fluid together. The swab now containing homogenized fluid and pellet was removed from the tube and swabbed onto brilliant green agar supplemented with Nalidixic acid (100 mcg/mL) and Novobiocin (15 mcg/mL) (BGA NA/NO, Northwest Laboratory Services, Winslow, ME, USA) and incubated for 24 hours at 37 °C. A three-quarter turn method was used to spread the bacteria on BGA to isolate colonies. Following the 24-hour incubation period, an isolated colony was selected using a sterile 10 µl loop and placed in a 10 mL test tube of sterile tryptic soy broth (TSB, Becton, Dickinson and Co., Franklin Lakes, NJ, USA), and vortexed to ensure all contents were homogenized. The tube was then placed in a 37 °C incubator for 24 hours. After incubation, the tube was removed and vortexed to ensure that settled bacteria at the bottom of the tube were thoroughly mixed. The bacteria were passed by submerging a sterile loop into the incubated tube's contents and then into a new test 10 mL sterile tube of TSB. Once transferred the tube was vortexed to homogenize the contents and then was incubated overnight at 37 °C. The isolation was passed for three days to ensure the strength of the bacteria. TSB used for passing the isolate was stored in the refrigerator at 4 °C and was only taken out and allowed to acclimate to room temperature when needed.

Bacterial lawn

After the bacteria were passed for three days the bacteria were plated on tryptic soy agar (TSA, Becton, Dickinson and, Co., Franklin Lakes, NJ, USA) using a bacterial lawn method of spreading. Sterile loops were dipped into the test tube culture and applied across the entire surface of the plate. The culture was spread from top to bottom, left to right, and diagonally.

Zones of inhibition

To test zones of inhibition sterile blank discs (Thermo Fisher Scientific™, Waltham, MA, USA) were used. Food-grade Oregano leaf oil with a dry herb strength of 1:3 (333 mg/mL) with grain alcohol and water and Clove oil with a dry herb strength of 1:4 (250 mg/mL) with organic alcohol and water were purchased online from iHerb.com. The treatment groups consisted of; Group 1, negative control (no oil, no bacteria), Group 2, positive control (no oil), Group 3 Oregano leaf oil, Group 4, Clove oil, and Group 5, a combination of both Oregano and Clove oil in a 1:1 ratio. Sterile discs were soaked in each oil and plated onto the bacterial lawn. To prevent interaction between each treatment group only discs from the same treatment group were plated on one plate. No data was collected for Group 1 because this treatment group contained no oil or bacteria and was treated as the negative control. For Group 2, classified as the positive control, sterile discs were not soaked in any media and placed on the bacterial lawn. Once the discs adhered to the lawn surfaces plates were incubated for 24 hours. The plates were not inverted while in the incubator to prevent the oil-soaked discs from falling from the agar surface. After 24 hours the plates were removed and analyzed for zones of inhibition around the oil-containing discs. Areas of inhibited growth around the sterile discs were measured by measuring the diameter in millimeters around the sterile discs.

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Egg treatment

Sixty eggs were obtained from Brookshire's Food & Pharmacy© (Prairie View, TX, USA) for the experiment. Food-grade Oregano leaf oil with a dry herb strength of 1:3 (333 mg/mL) with grain alcohol and water and Clove oil with a dry herb strength of 1:4 (250 mg/mL) with organic alcohol and water was purchased online from iHerb.com. Before the experiment, the eggs were stored under refrigeration at 4 °C. For experimentation, the eggs were allowed to adjust to room temperature at 25 °C before use. After which, the eggs were washed individually for 30 seconds in a plastic bag containing phosphate-buffered saline (PBS, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) to remove coatings from the egg's exterior. The eggs were then removed from the plastic bag and allowed to dry for 10 minutes at room temperature. After drying the eggs were placed in the incubator for 16 hours at 37 °C to create a pressure differential to draw the bacteria through the shell once the egg was immersed in the inoculum. The 0.1 mL for the wash solutions from 12 eggs were collected and spread plated individually on BGA to test for the presence of *Salmonella* on the exterior of the egg's shell. After which the plates were incubated for 24 hours at 37 °C. The following day plates were observed for any growth.

Application of the treatments

After being taken out of the incubator the sixty eggs were divided into treatment groups with 12 eggs per treatment group. Oregano leaf and Clove oils were applied individually and combined using a sponge ensuring that the eggs' surfaces were covered. Care was taken to prevent cross-contamination from one treatment group to the other by cutting the sponge into pieces. Only one piece of sponge per egg was used. After the oil coatings were applied, the eggs could dry at room temperature in a clean carton with the air cell end up.

Inoculating the eggs

Once dried the eggs were immersed immediately in a cold-water bath, at 5 °C, containing 1.96 x 10^6 cfu/mL of *S.* Enteritidis in 500 mL of water for 5 minutes according to the procedure outlined by Padron [12]. After 5 minutes the eggs were removed from the water bath and stored with the air cell ending up in a carton for 10 minutes to dry. Once dry the eggs were placed in the incubator for 24 hours.

Microbial examination

After 24 hours, the eggs were removed from the incubator and their contents were aseptically removed. Using a sterile needle a hole was made in the small end of the egg to allow for the tip of a syringe to enter. A different needle was used for each egg and discarded after it was used. A sterile syringe was inserted into the opening and the contents of the eggs were removed by suction. A new syringe was used for each egg and then discarded. Once the contents of each egg were removed 0.5% sterile saline was transferred into the egg with a pipette. Excess material inside the egg was removed. The rinse was removed using a transfer pipette and the egg was placed open end down to remove any remaining rinse from the egg. The empty eggs 20 were filled with molten Tergitol-7 agar (Thermo Fisher Scientific™, Waltham, MA, USA) at 50 °C. The agar contains Triphenyl tetrazolium chloride (TTC) that acts as an indicator dye for the presence of *Salmonella*. *Salmonella* has the ability to change the TTC into formazan dye and turn red [14]. The eggs were filled completely, and the openings were sealed with molding clay. After the agar was set the eggs were stored again with the air cell end up and placed in the incubator for 24 hours at 37 °C. Once 24 hours had elapsed the agar was observed by removing the eggshell.

Dilution of the sample

The presence of any red colonies was an indication of contamination. A section, no larger than a microscope cover slip, of the inner and outer egg membrane from each egg was removed from the shell using aseptic techniques. It was then placed in a test tube containing 9 mL of PBS, vortexed, and then diluted in 1:10 dilutions. Each sample was diluted out to a 1:1,000,000 (10^6) dilution. Once diluted 0.10 mL from the 10^{-4}, $10^{-5,}$ and 10^{-6} dilution tubes were spread separately on BGA plates that contained NA/NO for all the samples in each treatment group. The plates were then placed in the incubator for 24 hours at 37 °C.

Bacterial enumeration

After 24 hours the plates were removed and with a plate counter the number of colonies present was enumerated and recorded. Colonies that appeared after incubation were tested using a *Salmonella* Agglutination Latex test (Thermo Fisher Scientific™, Waltham, MA, USA) to confirm the presence of Salmonella. If there were no colonies an enrichment test would be performed. A sterile loop was used to streak across the agar surface where there were no colonies seen and placed into 10 mL of tetrathionate broth (Neogen®, Lansing, MI, USA) to recover any colonies that were dormant. The samples were incubated for 24 hours at 37 °C. After exposure to the tetrathionate enrichment, a 10µl sample was streaked onto a BGA plate and incubated overnight at 37 °C.

Experimental design

To examine the interactions between the two oil treatments independently and combined the experimental design was a 2 x 2 factorial. There were two levels of Oregano leaf oil (0 and 166.5 mg), two levels of Clove oil (0 and 125 mg), and a combination of Oregano oil (166.5 mg) and Clove oil (125 mg).

Data analysis

The bacteria count for each treatment group was transformed into a Logarithm. The data were subjected to Analysis of Variance (ANOVA) using SAS Institute Software, Inc., Cary, N.C. The treatment means were partitioned with a *p* - value of less than 0.05 to indicate statistical significance. The percentage of recovery of *Salmonella* was compared using the Chi-square test of Independence. All group combinations were applied to determine significant differences in the treatments.

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Results

Zones of inhibition created by the oils

Zones of inhibition for each treatment group are presented in Table 1.

For each oil treatment of Oregano Leaf and Clove oil used single and combined, there were visible areas around the sterile discs where no bacteria could grow. Zones of inhibited growth were seen for the Oregano Leaf, Clove, and Combinations of oil treatments. Of the oil treatment groups, the combination of the two oils had an average diameter of 1.3 mm. Followed by the Clove oil with an average diameter of 1.2 mm, then the Oregano leaf oil with an average diameter of 1.1 mm. The positive control had no zones of inhibition.

Oil treatments on the egg

The effects of Oregano Leaf oil on the reduction of *S.* Enteritidis are presented in Figure 1.

Compared to the positive control, which had the bacteria, but without any Oregano oil, there was no significant difference with the application of the oil. The positive control with the bacteria had 6.57 log (cfu/mL) compared to 6.63 log (cfu/mL) with the addition of the Oregano leaf oil, an increase of 0.06 log.

Figure 2 examined the effects of Clove oil on the reduction of *Salmonella* Enteritidis on eggshells. The results showed that the coating of Clove oil did not reduce the growth of the pathogen. Without the Clove oil, the bacteria load on the eggshell was 6.57 log (cfu/mL) which increased to 6.62 log with the treatment of the oil.

The combination of both oils, Oregano and Clove, was applied to the eggshell to determine the effect on *S.* Enteritidis (Figure 3). The bacteria load with the combined treatments was 5.91 log (cfu/mL) which was lower than 6.63 log for the single application of Oregano oil, and 6.62 log for the Clove oil.

A comparison was made between the effect of Oregano oil and the combination of both oils (Figure 4). The results showed that the bacteria count for the combination was 0.66 log (cfu/mL), lower than the straight treatment of Oregano oil.

Finally, Figure 5 shows the effect of the combination of both oils with the single treatment of Clove oil. The combined treatment of Clove and Oregano oils was 0.66 log less than the single application of Clove oil; 5.91 log for the combined treatment and 6.62 log (cfu/mL) for the individual application of Clove oil.

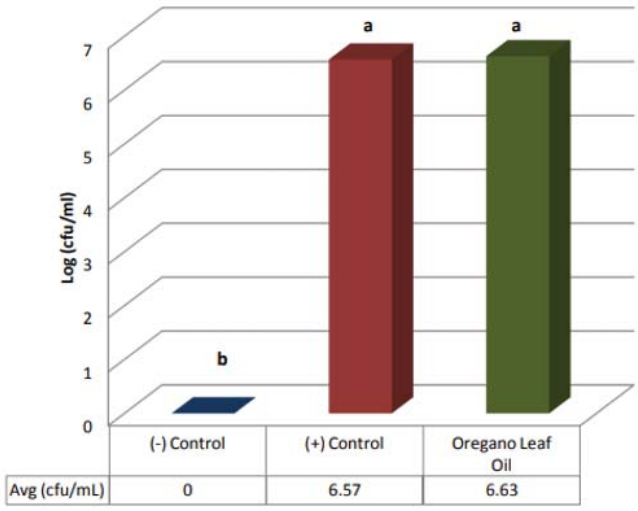

Figure 1: Effects of Oregano Leaf Oil Coating on Penetration of *Salmonella Enteritidis.*
[a-b]Bars with the same letters are not significantly different ($p > 0.05$).
(-) Control represents negative control with no bacteria and no treatment;
(+) Control represents positive control with bacteria and no treatment.

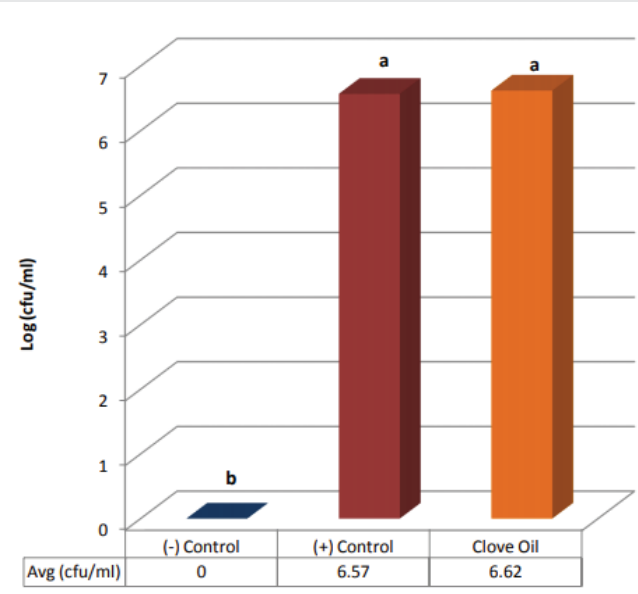

Figure 2: Effects of Clove Oil Coating on Penetration of *Salmonella Enteritidis.*
[a-b]Bars with the same letters are not significantly different ($p > 0.05$).
(-) Control represents negative control with no bacteria and no treatment.
(+) Control represents positive control with bacteria and no treatment.

Table 1: Zones of Inhibition Created by Single and Combined Use of Oregano Leaf and Clove Oil.

Treatments	Discs				Avg
(+) Control	1	0	0	0	0
Oregano Leaf Oil	1	1	1.3	1.2	1.1
Clove Oil	1	1.2	1	1.4	1.2
Combination of both Oils	1	1.5	1.6	1.3	1.4

The table illustrates the effectiveness of Oregano Leaf and Clove oil used single and combined to inhibit growth of *Salmonella enteritidis.* The diameter of the zones created around the discs was measured in millimeters and recorded in the table.

Potential reasons for lack of significance

Despite the promising properties and benefits of essential oils like oregano and clove oil, the current body of research remains incomplete. Although initial studies have highlighted their potential in combating antibiotic-resistant strains, reducing inflammation, and providing antioxidant effects, more comprehensive and rigorous research is necessary. Further studies are needed to fully understand their mechanisms of action, optimal dosages, long-term effects, and potential

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interactions with other treatments. Continued research will help to substantiate these preliminary findings and could pave the way for their broader application in medical and health contexts.

Implication of the results with practical application

If oregano and clove oils prove effective in practical applications, they could revolutionize the food industry, particularly in reducing microbial contamination in the meat industry. Utilizing these essential oils as natural alternatives to synthetic preservatives and harmful chemicals could enhance food safety and extend shelf life without the adverse health effects associated with some conventional methods. Their natural antimicrobial properties could offer a safer, more sustainable solution for maintaining meat quality and preventing spoilage, ultimately benefiting both consumers and producers by providing a healthier, more environmentally friendly option.

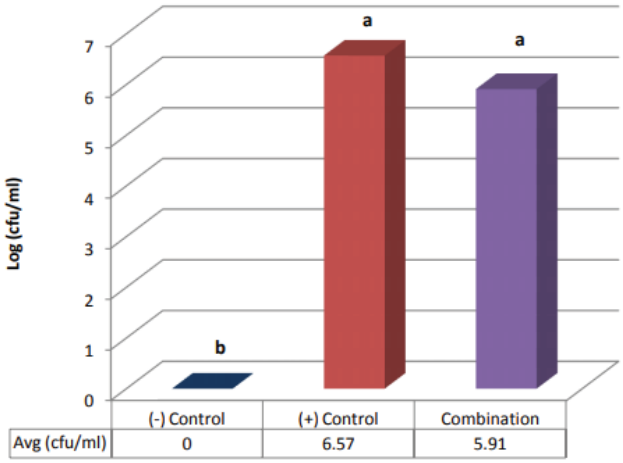

Figure 3: Effects of combined Oil Coating on Penetration of *Salmonella Enteritidis*.
[a-b]Bars with the same letters are not significantly different ($p > 0.05$).
(-) Control represents negative control with no bacteria and no treatment.
(+) Control represents positive control with bacteria and no treatment.

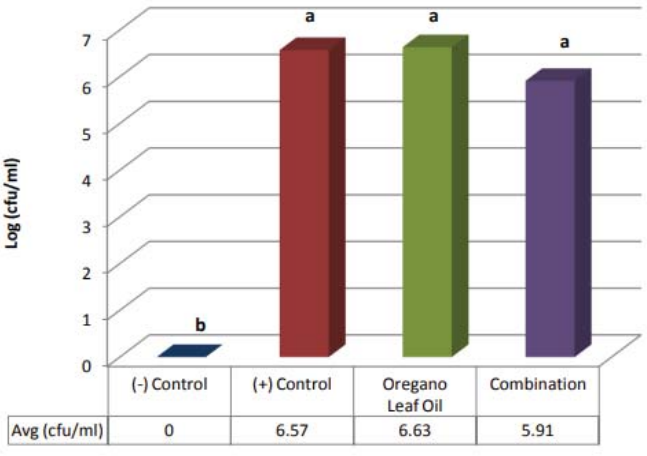

Figure 4: Comparing the Effects of Oregano Leaf and the combination of Oregano leaf and Clove Oils and Bacterial Penetration.
[a-b]Bars with the same letters are not significantly different ($p > 0.05$).
(-) Control represents negative control with no bacteria and no treatment.
(+) Control represents positive control with bacteria and no treatment.

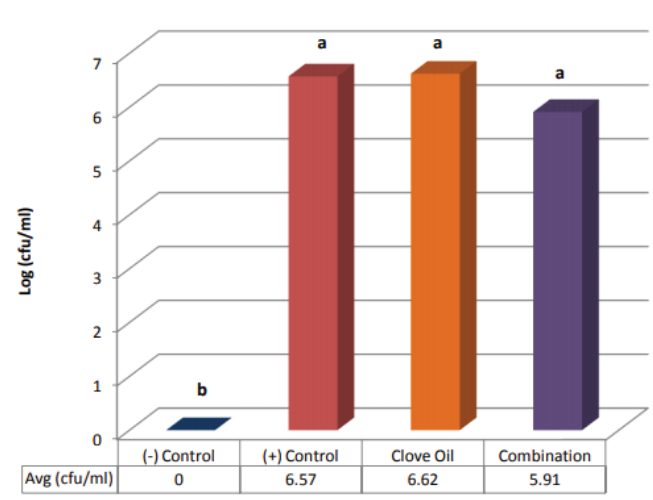

Figure 5: Comparing the Effects of Oregano Leaf and the combination of Oregano leaf and Clove Oils and Bacterial Penetration.
[a-b]Bars with the same letters are not significantly different ($p > 0.05$).
(-) Control represents negative control with no bacteria and no treatment.
(+) Control represents positive control with bacteria and no treatment.

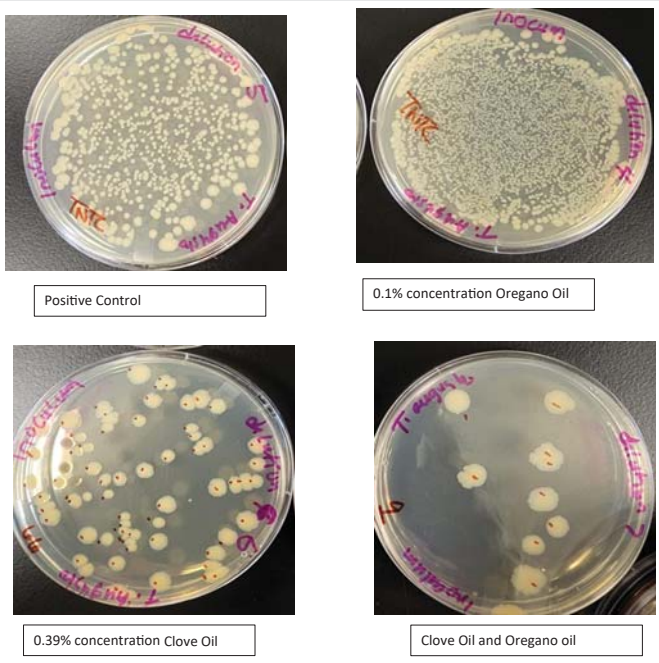

Figure 6: Microbial Growth.

Discussion

Table eggs, which are consumed worldwide and are incorporated into many different food products, are very sensitive to *Salmonella* Enteritidis contamination. Bacterial contamination through the shell of table eggs increases spoilage and shortens shelf life. Egg spoilage is a major economic problem for producers, processors, and consumers. For the producers, contaminated eggs lead to fewer eggs that are marketable; which translates to less revenue for the farmer. Pressure is placed on production, collection, and storage techniques to lower the number of contaminated eggs and to prevent cross-contamination. Consumers take notice of the visual quality of the eggs, as broken, cracked eggs are

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undesirable. To avoid loss, post-harvest treatment of eggs after laying becomes a paramount practice for the farmer. However, not all eggs are delivered straight to the consumers, as most of the eggs consumed in the U.S. are delivered to processors who repackage, store, and distribute the eggs through supermarkets and convenience stores for the convenience of the consumers [15]. From the producers, who are the farmers, the processors take the eggs and package them before the eggs are sold. For example, products like liquid eggs or egg whites are sold in many grocery stores. Parts of the egg are incorporated into food products as protein ingredients. For example, egg yolk is a good emulsifying agent and is an ingredient in mayonnaise. Many attempts have been made to reduce the loss and extend the shelf-life of eggs. One method that has been tried is the coating of the shell with oils [16]. Mineral oil is frequently used to coat eggshells to extend the shelf-life of 30 table eggs [16]. The current study examined the effects of Oregano leaf and Clove oils, single and combined, as a coating for the eggshells. Oregano has been shown to have antimicrobial activity both in vitro and in food [17]. Both carvacrol and thymol, which are found in Oregano leaf oil, have been shown to disintegrate the cell membranes of *Salmonella* [18]. In a study by Skandamis, et al. [19] it was reported that in conjunction with vacuum packaging, Oregano oil provided complete elimination of *S.* Typhimurium from meat after 8 days of storage at 5 °C. Clove oil has been effective against *S.* Enteritidis full-fat cheese [20]. During laying, the outer covering of the eggs is coated with a natural secretion called the cuticle. The cuticle is a wax-like covering that serves as a protective coating to seal the spores of the shell. The cuticle, the outermost covering, is a part of the egg's natural defense to protect the egg once it is laid. The eggshell contains millions of minute microscopic openings that allow for the exchange of oxygen and CO_2 for the developing embryo [21]. Before eggs are packaged for marketing, they are often washed to remove fecal stains. During washing the outer covering is removed leaving the spores open for the entrance of bacteria [3]. The most common bacteria to invade the shell through the spores is Salmonella; with the most common serotype being *Salmonella* Enteritidis. *Salmonella* is a foodborne pathogenic bacterium, and when ingested affects the health of consumers, and even causes death. The findings from this research did not support the data reported by other researchers (Figure 6). Neither Oregano nor Clove oils prevented the penetration of *Salmonella* entering the shell, whether single or combined. An explanation could be the viscosity of the oil which did not increase the thickness of the covering.

Conclusion

It has been proposed by Burt that the physical structure of food may limit the antibacterial properties of essential oils [22]. For example, a study of the relative performance of oregano oil against *S.* Typhimurium in gelatin revealed that the gel matrix reduced the inhibitory effects of the oil. In some food products, essential oils are effective in inhibiting bacteria growth. Essential oils used in vegetables have shown effectiveness in inhibiting growth, which is believed to be due to the low-fat content of vegetables. The results of the current study showed that the bacterium was able to penetrate the shell despite the oil coating. The count in the positive control eggs, without any treatment, was 6.57 log (cfu/mL). The application of either Oregano leaf or Clove oil did not protect the shell from the invasion of *Salmonella* Enteritidis. Neither the Oregano oil nor the Clove oil was effective in protecting the shell from the pathogen. Interestingly, the combination of both oils did reduce contamination some, but not enough to be statistically different. When the oils were examined individually, or when combined, the effect of Oregano although not significantly different from the position control, was less effective than the Clove oil. The application of clove oil did change the bacteria number compared to the control or the single treatment. There was a slight additive or synergistic effect with the combination of both oils with a 5.91 log (cfu/mL).

Acknowledgment

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Ethical consideration

All ethical considerations were in place for this research.

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