Spring 5-2005

Potential use of Chitosan for antimicrobial control of Eschevichia coli 0157:H7 in apple cider

Carol Lynette White
University of Tennessee - Knoxville

Potential use of chitosan for antimicrobial control of *Escherichia coli O157:H7* in apple cider

Submitted by: Carol Lynette White
Faculty Mentor: Dr. David A. Golden
University of Tennessee
College of Agricultural Sciences and Natural Resources
Department: Food Science and Technology

ABSTRACT

This University Honors Program Senior Project consisted of laboratory research analyzing the antimicrobial activity of chitosan in unpasteurized apple cider. This research specifically investigated the antimicrobial activity of chitosan against *Escherichia coli O157:H7*, a pathogen sometimes found in unpasteurized apple cider products. First, the most effective concentration of chitosan to use throughout the experiment was determined. The two concentrations used in this study were 0 (control) and 0.1%. Next, the ability of chitosan to serve as an antimicrobial agent against *E.coli O157:H7* was observed. The effects of chitosan were tested in filtered (0% insoluble solids) and unfiltered (considered as 100% insoluble solids) apple cider inoculated with *E.coli O157:H7*. Duplicate flasks and media plates were prepared to ensure consistency of results in the experiment. Data were collected for seven days after the initial inoculation. The expected results included using chitosan to reduce pathogens in the apple cider product to achieve a 5-log reduction in population, thus creating a new standard in cider production. However, the results from this experiment show that the antimicrobial effect of chitosan in the cider product is insignificant. Nevertheless, this research is relevant in the search for more effective antimicrobials that can be obtained from products that would otherwise be considered waste. While the results did not display the expected effects, it is predicted that chitosan may enhance the effects of other antimicrobials. Since chitosan is a positively charged compound and most microorganism are normally negatively charged, natural binding of chitosan with microbial cells could alter membrane permeability, thereby improving the efficacy of other added preservatives. In addition, chitosan still has many relevant uses within the food and health industries and research on this natural polymer will continue.

INTRODUCTION

Consumers are now demanding food products containing fewer synthetic additives while desiring increased safety, higher quality, and extended shelf life. These demands have led to greater research in the use of natural antimicrobials to aid in the preservation of food products. Although there are a number of potential antimicrobials, very few are suitable for use in particular food products. This study was conducted to evaluate antimicrobial effects of chitosan in unpasteurized filtered and unfiltered apple cider inoculated with *Escherichia coli O157:H7*.

Chitosan (poly-ß-1, 4-glucosamine) is a derivative of chitin, a polysaccharide found in the exoskeletons of crustaceans and arthropodsan in fungal cell walls. Thus, it may be extracted from products that are otherwise discarded, such as shellfish and mushroom processing wastes. Recent studies have shown that chitosan can form a seal that can stop uncontrolled bleeding (Fleming-Michael 2003). Thus, the strength and antimicrobial properties of chitosan may prove beneficial to the military for controlling hemorrhaging on the battlefield. Current research includes testing the efficacy of chitosan as an enhancer of other antimicrobials in food products. Chitosan works extremely well at lower, more acidic pH, making it an ideal antimicrobial for ciders and other acidic products. Thus, this study was conducted to examine the ability of chitosan to serve as an antimicrobial in a cider product.

Escherichia coli (E. coli) O157:H7 is a member of the enterohemorrhagic *E. coli* (EHEC) and causes hemorrhagic colitis in humans. *E. coli* O157:H7 illness symptoms often include severe abdominal cramps and diarrhea (sometimes bloody), and can lead to death. In children under the age of five, infection can cause a complication known as hemolytic uremic syndrome (HUS), which results in red blood cell destruction, renal failure, and complications in the central nervous system. *E. coli* O157:H7 can cause disease at a low infectious dose (10 - 100 cells). This low

infectious dosage can be attributed to the tolerance of the *E.coli O157:H7* to low pH, which promotes passage through the stomach and colonization in the intestinal tract.

Apple cider is commonly manufactured locally at small cider mills, where the apples are crushed in presses; and the resulting cider is frequently not pasteurized. In October 1996, unpasteurized apple cider and cider were associated with two outbreaks of *E. coli* O157:H7. The first outbreak occurred in the Western United States and was associated with the consumption of unpasteurized apple cider. This outbreak caused 66 human illnesses and one death (CDC 1996). The second outbreak occurred in the Northeastern United States and involved contaminated apple cider, which resulted in illness to 14 people and one case of HUS (CDC 1996). An outbreak of *E. coli* O157:H7 infections in 1991 occurred at a cider mill whose orchards served as a grazing field for cattle (Besser et al. 1993). The cattle grazed nearby the mill, resulting in manure inadvertently contacting the apples (Besser et al. 1993). Although the exact mechanism for contamination is not known in most apple cider-related outbreaks, manure was suspected to have contaminated the apples used in cider making (CDC 1996). In many instances, apples that have already fallen from the tree are used to make apple cider. The apples can become contaminated if they come in contact with manure that is contaminated with the pathogen.

Since *E. coli* O157:H7 has been linked with foodborne disease outbreaks related to consumption of produce, it serves as a good model pathogen for testing the efficacy of antimicrobial processing of fruits and vegetables. If a process is to become employed in the food industry, it must first be tested and validated in food production and processing systems. Many times, an antimicrobial process may be very effective *in vitro* and have must less efficacy when evaluated in food. Since the effects of chitosan as a food antimicrobial have not been extensively tested in a food system, research must be conducted in this area.

MATERIALS AND METHODS

Preparation of Chitosan Solution. A 200 mL stock solution of 0.1% medium molecular weight (MMW) chitosan in 0.1% acetic acid in 0.1 M NaCl solution was made first. To prepare this solution, 0.2 g of MMW chitosan was added to 160 mL of 0.1M NaCl. This solution was heated to boiling, cooled, and 2 mL of 10% acetic acid was added to improve chitosan solubility. The solution was stirred overnight, and the weight of the solution was then adjusted to 200g with 0.1 M NaCl. The solution was stirred for two hours before each use and refrigerated after each use.

Preparation of Innoculum. For this study, *E.coli O157:H7* strains 43888, 43889, 43890 were mixed in equal proportions and inoculated into apple cider. Before inoculation, cider was vacuum-filtered with No. 1 Whatman filter paper, and flasks containing filtered cider (0% insoluble solids) and unfiltered cider (considered as 100% insoluble solids) were prepared. For the inoculum, 2.5 mL of mixed culture suspension were added to flasks containing 225 mL of cider and 25 mL of chitosan.

Plating technique. The technique used to plate all samples, including duplicates for each flask, was spiral plating. For each treatment and duplicate flask, 10-fold serial dilutions were made. The undiluted, 10^{-3}, and 10^{-6} dilutions were plated on two different types of media. Duplicate plates were prepared to provide consistency to the results.

Media Used. For this laboratory experiment, trypsic soy agar (TSA) and modified eosin methylene blue (MEMB) agar were used.

Time involved. This study was conducted over a period of 7 days from the initial inoculation.

RESULTS

Results from this experiment show that the antimicrobial effect of chitosan in cider product was insignificant. Duplicate flasks were prepared to ensure the continuity of results for each treatment. Tables 1 and 2 show the average $\log_{10}$ CFU *E. coli* O157:H7/ml of filtered and unfiltered cider with 0 or 0.1% chitosan concentrations. When the data points were graphed, the effects of the chitosan in the cider, filtered or unfiltered, were indistinguishable. In Figures 1 and 2, the only differences noted in the effects of 0.1% chitosan on *E.coli O157:H7* in filtered apple cider (0% insoluble solids) and unfiltered cider (considered as 100% insoluble solids) were between the media types. In Figure 2, the 100% solids, 0.1% chitosan treatment plated on the MEMB media showed a rapid decline between days 6 and 7. This rapid decline could be the result of the natural decline of the organisms. In Figures 3 and 4, survival of *E. coli* O157:H7 on TSA and MEMB in filtered (0% insoluble solids) and unfiltered (considered as 100% insoluble solids) apple cider with 0 or 0.1% chitosan, seemed to be unaffected by chitosan or solids content.

Time (days)	**0% solids, 0% chitosan, TSA**	**0% solids, 0.1% chitosan, TSA**	**0% solids, 0% chitosan, MEMB**	**0% solids, 0.1% chitosan, MEMB**
0	6.35	6.51	6.42	6.42
1	6.01	5.83	5.02	5.18
6	4.50	4.70	3.72	3.95
7	4.24	4.53	3.34	3.68

Table 1: Average $\log_{10}$ CFU *E. coli* O157:H7/ml of filtered cider with 0 or 0.1% chitosan

Time (days)	**100% solids, 0% chitosan, TSA**	**100% solids, 0.1% chitosan, TSA**	**100% solids, 0% chitosan, MEMB**	**100% solids, 0.1% chitosan, MEMB**
0	6.35	6.51	6.42	6.42
1	5.03	5.39	4.50	4.90
6	4.17	4.44	3.44	3.88
7	4.03	3.20	3.29	2.30

Table 2: Average $\log_{10}$ CFU *E. coli* O157:H7/ml of unfiltered cider with 0 or 0.1%chitosan

Figure 1: Effect of 0.1% chitosan on survival of *E. coli* O157:H7 in filtered apple cider (0% insoluble solids)

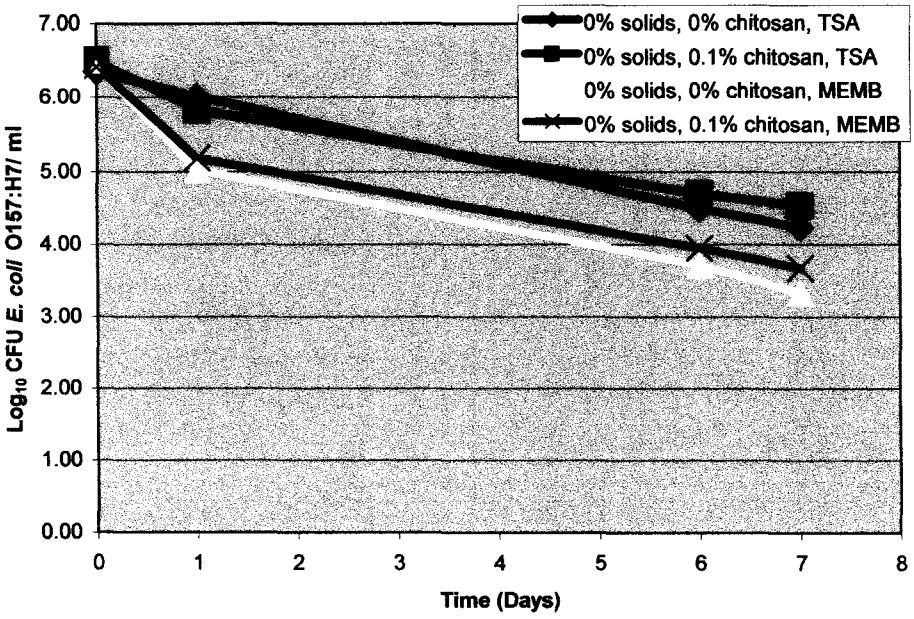


Figure 2: Effect of 0.1% chitosan on survival of *E.coli* 0157:H7 in unfiltered apple cider (considered as 100% insoluble solids)

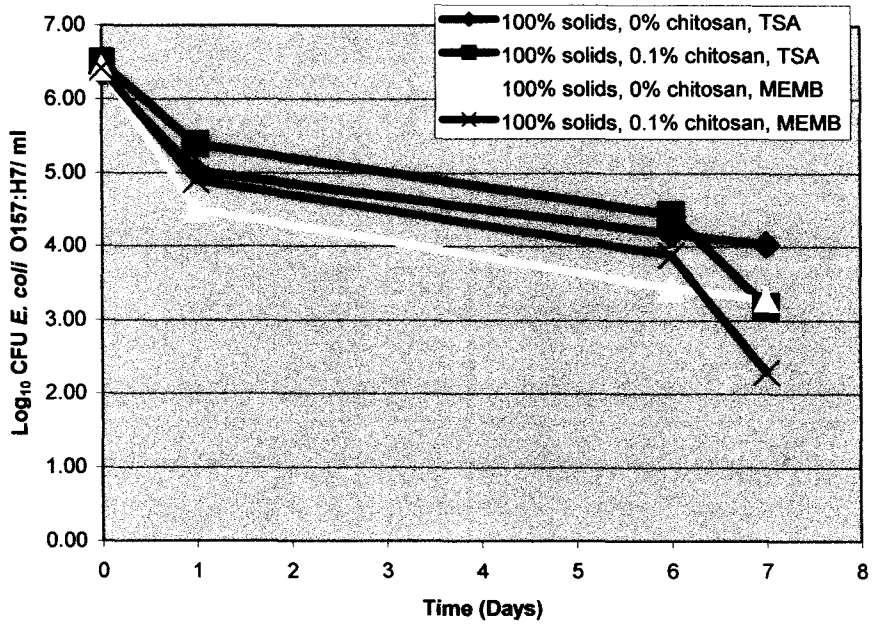


Figure 3: Recovery of *E.coli* O157:H7 on TSA in filtered (0% insoluble solids) and unfiltered (100% insoluble solids) apple cider with 0 or 0.1% chitosan

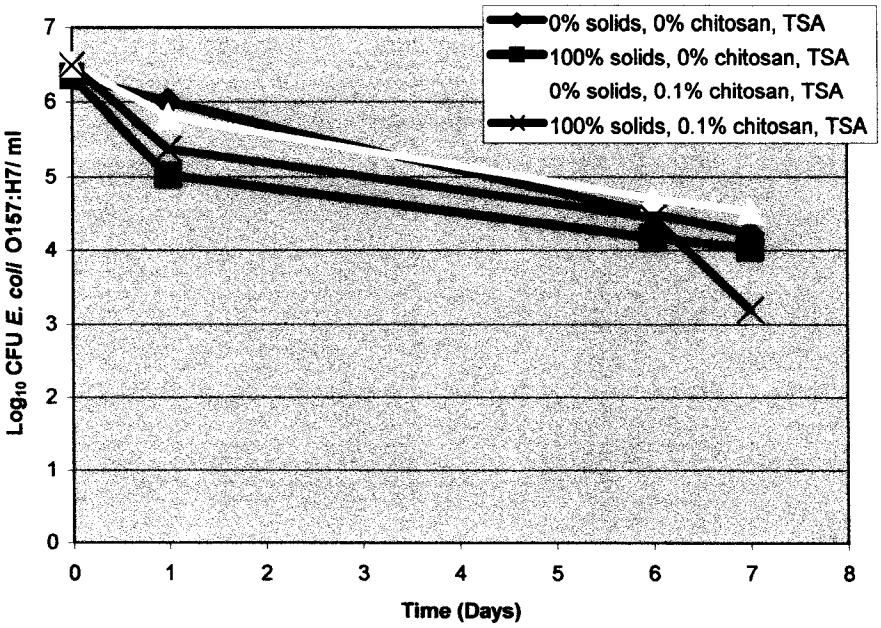
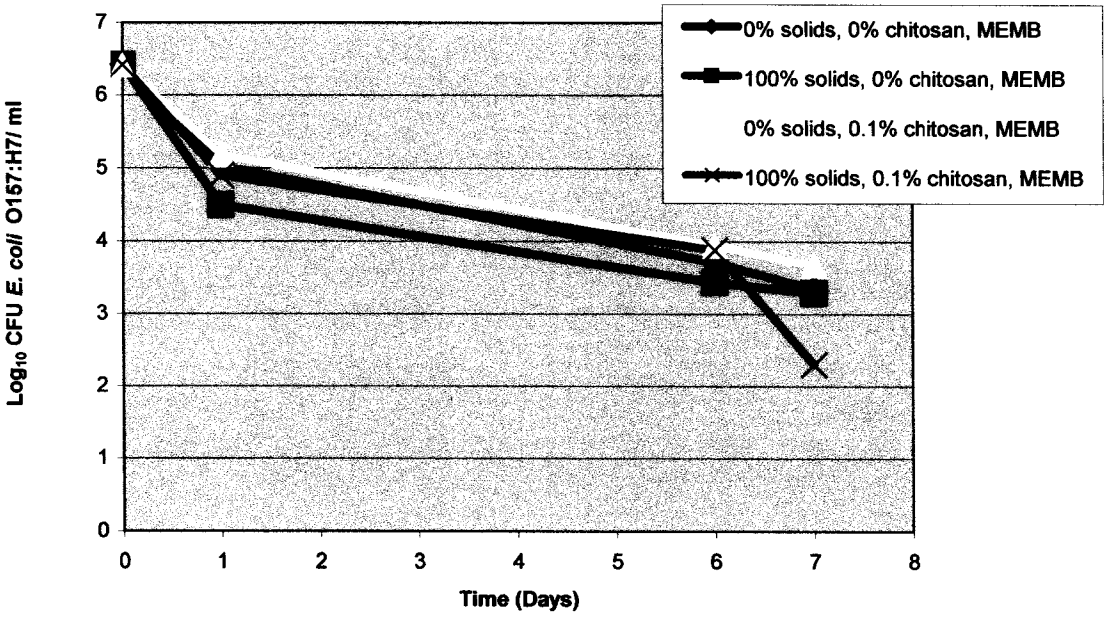


Figure 4: Recovery of *E. coli* O157:H7 on MEMB in filtered (0% insoluble solids) and unfiltered (considered as 100% insoluble solids) apple cider with 0 or 0.1% chitosan



DISCUSSION

The purpose of this senior honors research project was to determine the effects of chitosan as an antimicrobial agent against *E. coli* O157:H7 in apple cider. Several different treatments were used in this experiment. Two levels of chitosan concentration, 0 and 0.1% were tested in filtered (0% insoluble solids) and unfiltered (considered as 100% insoluble solids) apple cider. Apple cider was filtered with No. 1 Whatman filter paper. Duplicate flasks of each treatment were prepared to insure consistency of results. The different samples were plated on TSA and MEMB agar, and duplicate plates were prepared for each treatment. The data were collected over a seven day time period.

The results indicate that chitosan was not an effective antimicrobial agent against *E. coli* O157:H7. The decreases in survival over time displayed the natural decline of *E. coli* O157:H7 in the apple cider environment, rather than the significant log reduction of the pathogen that was originally expected. There are several possible reasons for this outcome. For example, in analyzing the effects of chitosan against *E. coli* O157:H7 in filtered versus unfiltered cider, all of the flasks were thoroughly mixed before each plating procedure. Chitosan has also shown to serve as a natural flocculating agent, and by mixing the treatments prior to plating, some of the effects of the chitosan on the cider and pathogen might have been disrupted. To eliminate this issue, the samples should have been allowed to flocculate and plated without mixing the sample. In addition, *E. coli* O157:H7 will not grow in apple cider, due to its low pH (~3.6), making it difficult to observe the actual effects of the chitosan. Possible solutions would be to test the chitosan against *E. coli* O157:H7 in a different environment or test against a different pathogen. However, chitosan may be more beneficial as an enhancer of other antimicrobial agents. A study from South Bank University in London noted that chitosan may be useful as an adjunct in the potentiation of

the antimicrobial efficacy of antimicrobial compounds such as benzoates (Sagoo et al. 2002). In addition, a study conducted at Clemson University concluded that, while chitosan is still not approved as a food additive, the usage of chitosan as an antimicrobial additive in food packaging films could be advantageous (Park et al. 2002).

In conclusion, the purpose of this senior honors research project was to analyze the effects of chitosan as an antimicrobial agent against *E. coli* O157:H7 in apple cider. The results indicated that chitosan was not an effective antimicrobial agent in this study. However, chitosan has many valuable uses within the food industry. There is great potential for chitosan as a powerful antimicrobial against other pathogens, an enhancer of additional antimicrobials, or as an additive in food packaging films. As research continues, more uses for this substance will be discovered.

REFERENCES

Besser, R.E., S.M. Lett, and J.T. Webber. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA. 269:2217-2220.

CDC (Center for Disease Control and Prevention).1996. Outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpastuerized commercial apple cider—British Columbia, California, and Washington, October 1996. MMWR. 45:975.

Fleming-Michael, Karen. New bandage stanches battlefield hemorrhage. Fort Detrick Standard. 21 Feb. 2003. http://www.dcmilitary.com/army/standard/8_04/national_news/21677-1.html

Johnson, Faith Michelle. 2004. Atmospheric plasma inactivation of foodborne pathogens on fresh produce surfaces. M.S. Thesis University of Tennessee, Knoxville, accessed at http://idserver.utk.edu/?id=200400000007700.

Park, S. I., Marsh, K. S., Dawson, P. L., Acton, J. C., Han, I.2002. Antimicrobial activity of chitosan incorporated polyethylene films. Clemson Univerisity, Clemson, South Carolina, accessed at http://ift.confex.com/ift/2002/techprogram/paper_14373.htm.

Roller, Sibel. 2003. Natural Antimicrobials for the Minimal Processing of Foods. Culinary and Hospitality Industry Publications Services. http://www.chipsbooks.com/natural.htm

Sagoo, SK, Board R, Roller S. 2002. Chitosan potentiates the antimicrobial action of sodium benzoate on spoilage yeasts. Letters in Applied Microbiology. March 2002. 34(3):168-72.