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Amanda Bettasso
University of Arkansas, Fayetteville

Navam Hettiarachychy
University of Arkansas, Fayetteville

Vidya Chitturi
University of Arkansas, Fayetteville

Michael Johnson
University of Arkansas, Fayetteville

Investigating the effectiveness of malic acid, nisin, and grape seed extract incorporated into whey-protein coatings to inhibit the growth of *Listeria monocytogenes* on ready-to-eat poultry

Amanda Bettasso[*], *Navam Hettiarachchy*[†], *Vidya Chitturi*[§], *and Michael Johnson*[‡]

ABSTRACT

The ability to control growth of *Listeria monocytogenes* on ready-to-eat poultry products with the antimicrobials nisin, malic acid, and grape seed extract incorporated into whey-protein coatings was evaluated. The antimicrobials were incorporated into the coating solution alone and in combinations. One gram pieces of turkey frankfurters were coated with the coating solutions and then inoculated with *L. monocytogenes* and stored at 4°C for 28 days. The inhibitory effect of the coatings on turkey frankfurter pieces was evaluated on d 0, 7, 14, 21, and 28. Coatings containing 2% malic acid, 3% malic acid, and the combination of nisin (6,000 IU/g) and malic acid (1%) were the most effective in inhibiting the growth of *L. monocytogenes*. Malic acid at 2 and 3% concentrations reduced *L. monocytogenes* population by 2.0 log cycles compared to the control after 28 d. Combination of 1% nisin and 1% malic acid reduced the population of *L. monocytogenes* by 2.7 log cycles compared to the control after 28 d of storage at 4°C. Grape seed extract did not inhibit the pathogen effectively when used alone or in combination with malic acid or nisin. Results of this investigation demonstrate synergistic effects of nisin and malic acid, which can be effectively incorporated into whey-protein coatings to control the post-processing contamination of *L. monocytogenes* in ready-to-eat poultry products.

[*] Amanda Bettasso is a senior majoring in food science.

[†] N. Hettiarachchy, faculty mentor, is a university professor in the Department of Food Science.

[§] V. Chitturi, technical advisor, recently completed a doctorate at the University of Arkansas.

[‡] M. Johnson, a committee member, is a professor in the Department of Food Science.

MEET THE STUDENT-AUTHOR

Amanda Bettasso

After I graduated from Joplin High School in Joplin, Mo., in 2004, I began my studies at the University of Arkansas. I am a senior food science major and have recently completed the AFLS honors program. I have been the recipient of various scholarships while attending the UofA, such as: University Scholarship, Food Science Scholarship, and the OFPA scholarship. I have been a resident assistant for University Housing for the past two years. I am an active member of the Food Science Club and am currently serving as the treasurer. I am a member of the Institute of Food Technology and the Research Chefs Association. I began doing research under Dr. Navam Hettiarachchy during my junior year in the area of food safety. I plan to get my master's degree in food science and eventually obtain a job in research and development.

INTRODUCTION

Listeria monocytogenes is a psychrotrophic, Gram-positive, facultative intracellular organism widely distributed in the environment. It affects pregnant women, immunocompromised patients, newborns, and very old people. *L. monocytogenes* specifically causes listeriosis, which can give rise to meningitis, encephalitis, septicaemia, endocarditis and cause spontaneous abortion, premature birth, stillbirth, abscesses, and lymphadenitis (Kiss et al, 2004). *L. monocytogenes* can survive at temperatures ranging from 2–45°C and in low pH and high osmotic stress, resulting in many challenges for its control in the food industry (Gandhi and Chikindas, 2007). On an average there are 2500 reported cases of listeriosis annually, resulting in about 500 deaths according to the Center for Disease Control (CDC, 2005). *L. monocytogenes* contamination is especially a problem in the ready-to-eat (RTE) meat industry, often causing massive recalls. In February 2007 approximately 52,650 pounds of RTE chicken, processed in South Carolina, was recalled (FSIS, 2007a). There was an outbreak in May 2007 in ready-to-eat turkey products from a poultry ranch in California (FSIS, 2007b). These outbreaks, along with many others, have lead to increased regulations in RTE meats and increased interest in improving the current preservation techniques to inhibit the growth of *L. monocytogenes*.

There are several existing hurdle technologies that attempt to inhibit the growth of *L. monocytogenes* in RTE meats. Thermal processing techniques, although effective, pose the problem of decreasing product quality due to an edge-heating effect that is typically associated with microwave heating (Huang and Sites, 2007). Organic acids are often used in conjunction with other antimicrobials to prevent contamination in food products and have proven to be effective in bacterial inhibition (Palumbo and Williams, 1994; Murphy et al., 2006). Antimicrobial peptides, such as nisin, are used as food preservatives to inhibit growth of several foodborne pathogens and spoilage bacteria in various food products (Zuoxing et al., 2006). Recently, increased interest in plant extracts has grown due to their antioxidant and antimicrobial activities. In addition, there is increasing evidence of antimicrobial properties of phenolic constituents of grape seed extract. Grape seed extract is effective in inhibiting *Escherichia coli* O157:H7, *Salmonella* typhimurium, and *Listeria* (Ahn et al., 2004). Dr. Hettiarachchy's research team focuses on developing protein-based antimicrobial edible films and coatings. This research team showed that the combination of nisin with the natural extracts in edible films could help

increase protections against *L. monocytogenes*, specifically at refrigerated storage temperatures (Theivendran et al., 2006). Heat-denatured whey protein produces bland, flexible, water-based edible films with excellent oxygen, aroma and oil-barrier properties (Perez-Gago and Krotcha, 2001).

With outbreaks occurring as recently as June 2007, resulting in recall of 2,768 pounds of contaminated RTE chicken products out of Tennessee, there is still a call for effective pathogen hurdle technologies (FSIS, 2007c). Hence the objectives of this study are to 1) develop whey-protein edible films incorporated with natural antimicrobials such as grape seed extract, nisin, and malic acid and 2) evaluate their effectiveness in inhibiting *Listeria monocytogenes* in turkey frankfurter systems.

MATERIALS AND METHODS

Listeria monocytogenes was obtained from the Food Microbiology Research Laboratory at the University of Arkansas, Food Science Department. Nisin (N) was purchased from Aplin and Barret Ltd., Dorset, United Kingdom. Grape seed extract (GSE) powder was obtained from Mega Natural Inc., Madera, California. Malic acid (MA) was purchased from Baker, Phillipsburg, N.J. Whey protein was purchased from Land O'Lakes Food Ingredients Division, Arden Hills, Minn., and glycerol plasticizer was purchased from the Sigma Chemical Company, St. Louis, Mo. Turkey frankfurters were obtained from Hettiarachchy's lab, previously made by her team at the University of Arkansas.

Determination of nisin activity. During refrigerated storage nisin loses its activity, hence the activity was determined. *Lactobacillus plantarum* NCDO 955, a standard indicator organism, was used to determine the activity of nisin. Activity was considered as a measure of inhibitory effect of nisin against *L. plantarum* on MRS soft agar plates. Frozen culture of *L. plantarum* was activated by inoculating 10 µl of the culture in MRS medium and incubating at 30°C for 24 h. Approximately 6 ml of MRS soft agar (preboiled at 45°C and cooled) were inoculated with 10 µl of *L. plantarum* and mixed gently. Inoculated soft agar was then poured onto MRS agar plates and allowed to solidify for 30 min under laminar flowing conditions. Stock solution of 0.01 g/ml nisin was prepared in sterilized de-ionized water. Serial dilutions from 1:2 to 1:256 were prepared from the stock and 5 µl of serial dilution were transferred into the surface of the agar by a micropipette tip. The spotted plates were dried for 30 min under the laminar hood and then incubated at 30°C for 24 hours. The activity of nisin was determined using the inverse value of the highest dilution (D) that produced at least a 2mm zone of inhibition. Nisin activity was calculated using the formula:

$$\frac{\text{Amount of sample (5 µl) spotted}}{\text{Inverse of the highest dilution (D)}}$$

Whey-protein film preparation. Nine grams of whey protein were added to 91 grams of water and stirred on a magnetic stirrer continuously until the protein dissolved. Then, 45% glycerol (w/w of protein) was added during continuous stirring for 30 min. The solution was heated at 85°C to denature the protein and to cross link glycerol with the denatured protein to form a polymer. Solutions were cooled to ambient temperature. Nisin (N) was added at 6000, 12,000, and 18,000 IU/g; malic acid (MA) or grape seed extract (GSE) was added at 1%, 2%, and 3% concentrations alone. Concentration of these treatments below 1.0% was not effective in inhibiting the pathogen (Theivendran et al., 2006). Concentrations more than 3.0% resulted in formation of gels in the solution, which is not desirable for coating the food products. Hence the concentrations selected in this study reflect optimum levels for film formation. The combinations of nisin (6000 IU/g), MA (1%), and GSE (1%) were incorporated and stirred until the ingredients were completely solublized. The coating solutions were then stored at 4°C until the meat samples were ready to be coated.

Activation of frozen L. monocytogenes for inoculation of meat samples. The strain V7 serotype 1/2a of *L. monocytogenes* was used in this project. A loopful of *L. monocytogenes* from stock frozen to -70°C was placed in 10 mL of BHI broth and incubated at 37°C for 24 h. A subculture was prepared with 10µL from the first culture in 10mL of BHI and incubated at 37°C for 18 h. Then 1000 µL of this culture were centrifuged for 10 minutes at 7000 xg. Serial dilutions of the cell suspensions were made to obtain an inoculums size of 107 (CFU/ml).

Turkey frankfurter preparation. Turkey breast halves were obtained from Cargill Inc. Minneapolis, Minn. Composition of the turkey frankfurters was as follows: Meat (52.35%), fat (21%), salt (1.25%), phosphate (0.4%), and water (25%). All the dry ingredients, mea,t and water were blended under vacuum for 30 min at 45 rpm with a 220-kg Keebler mixer (Keebler Engineering, Inc., Chicago, Ill.) to form a meat emulsion batter. The meat batters were subsequently stuffed into non-permeable casings (4.6 cm in diameter) and cooked in water tanks for 2 h at 85°C until products reached an internal temperature of 74°C. The frankfurters were chilled in a 4°C cooler. After chilling, the frankfurters were stripped of their casings and sliced into 1-gram pieces (1x1x1cm cubes), packed in sterile Whirl-Pak® bags, and stored at -20°C until used.

Preparation of meat samples. To initiate the studies, the turkey frankfurters were thawed, then dipped in the whey-protein coating (WPI) solutions and dried under laminar flow conditions. A control sample without WPI coating and a control sample with WPI coating with no added antimicrobials were also included. A total of 225 samples were prepared (15 treatments X 3 replications X 5 sampling days). The concentrations and treatments are given in Table 1. After the protein-coated meat pieces were completely drip-dried, they were inoculated with *L. monocytogenes* by dipping them into the cell suspensions in phosphate-buffered saline (PBS) (approximately 10^7 CFU/ml) for one min and dried under laminar flow conditions. Then the meat pieces were packed in sterile Whirl-Pak® bags and incubated for 28 d at 4°C.

L. monocytogenes count determinations in turkey frankfurter samples. The samples were analyzed for survivors of *L. monocytogenes* on d 0, 7, 14, 21, and 28. Samples were crushed in a stomacher with 9.0 ml of PBS. Serial dilutions of the stomached samples were spread-plated on *Listeria*-selective agar. The plates were incubated at 37°C for 48 h and colony-forming units were counted. The plate counts were then analyzed to determine if there were log reductions in *L. monocytogenes* growth.

RESULTS AND DISCUSSION

Inhibitory effect of whey-protein films. At zero hour, mean population of *Listeria* on the frankfurter samples was 5.0 log CFU/g. As shown in Figure 1, microbial counts, in the control without whey-protein coating (C) and in the control with whey protein coating without antimicrobials (C1), increased over the 28 d study. The control sample started with a listerial level of approximately 5.0 log CFU/g at zero hour and increased to 7.85 log CFU/g on d 28. Control 1 had the same initial listerial level as C and grew to 8.12 log CFU/g after 28 d.

WPI coatings with nisin (6000 IU/g, N1) on frankfurter pieces did not inhibit the pathogen effectively when compared to the controls. As shown in Figure 1, the listerial count started at the same level as the control (C) and increased to 7.9 log CFU/g after 28 d. WPI coating with nisin (12,000 IU/g, N2) on frankfurter pieces lowered the *Listeria* population to 4.7 log CFU/g on d 14. This was a 3.7 log reduction compared to the control (C). However, after d 14 the *L. monocytogenes* counts steadily increased to 7.0 log CFU/g after storage for 28 d at 4°C. This can be explained as the development of resistance to the nisin by the *L. monocytogenes* population. The WPI coating with nisin (18,000 IU/g, N3) had a similar pattern to N2, but was more effective in inhibiting the pathogen. The population of *L. monocytogenes* in N3 samples increased over the first 7 d to 7.1 log CFU/g, and then decreased over the next 14 d (from d 7 to d 21) to 4.3 log CFU/g. The L. monocytogenes population developed resistance to the nisin at this concentration and increased to 5.8 log CFU/g at 28 d. The N2 (12,000 IU/g) and N3 (18,000 IU/g) treatments lowered the *Listeria* population by 0.8 and 2 log CFU/g, respectively, compared to the control (C) after 28 d of storage.

Figure 2 shows the response of *L. monocytogenes* in turkey frankfurters coated with WPI films containing malic acid at 1.0% (MA1), 2.0% (MA2), and 3.0% (MA3) concentrations. Listerial counts on frankfurter samples with MA1 steadily increased over the 28 d period to 7.7 log CFU/g. Listerial counts on frankfurter samples containing MA2 and MA3 increased to 7.1 log CFU/ml on d 7 and the decreased over the next 21 d to 5.5 and 5.3 log CFU/g in MA2 and MA3, respectively. There was approximately a 2.0 log CFU/g reduction after 28 d in comparison to the control (C) in frankfurter samples coated with WPI containing 2% and 3% malic acid.

In contrast to nisin and malic acid, the samples containing grape seed extract (GSE) did not inhibit the pathogen. All the GSE treatments at 1.0, 2.0, and 3.0% concentrations (GSE1, GSE2, and GSE3, respectively) had similar growth patterns as the control. The population of *L. monocytogenes* on turkey frankfurter samples coated with WPI containing GSE at 1.0, 2.0, and 3.0% concentrations increased to 8.53, 8.58, and 8.61 log CFU/g, respectively, as shown in Figure 3. These results suggest that grape seed extract is not effective in inhibiting *L. monocytogenes* on turkey frankfurters coated with the WPI films. This can be explained as the availability of protein molecules as nutrients and availability of oxygen conditions, which provide a conducive environment for the growth of *L. monocytogenes*. Previous studies by Dr. Hettiarachchy's team have demonstrated inhibitory effects of grape seed extract incorporated edible films in pre-processing contamination conditions (inoculation of the pathogen on meat samples and then coating the samples with antimicrobial edible films) (Theivendran et al 2006; Chitturi, 2008). However, grape seed extract incorporated WPI films are not effective in controlling post-processing contamination of *L. monocytogenes* on turkey frankfurters.

Figure 4 shows growth of *L. monocytogenes* in turkey frankfurters coated with WPI films containing the combination of N, MA, and GSE. Treatments GSE+MA and N+GSE had similar listerial counts as the controls, C and C1. Both these treatments had listerial counts starting at 5.0 log CFU/g at zero hour and grew to 8.0 and 8.1 log

CFU/g in samples containing GSE+MA and N+GSE, respectively, after 28 d of storage. Listerial levels in the samples containing N+MA increased from 5.0 log CFU/g at zero hour to 6.3 log CFU/g on d 21. After d 21, the listerial level decreased to approximately 5.2 log CFU/g. There was a 2.7 log CFU/g reduction compared to the controls after 28 days of storage. The WPI coatings containing a combination of N and MA were more effective in inhibiting the pathogen than when either was used alone. This can be explained due to the improved stability of nisin at acidic pH. Similar observations were made by Ko et al. (2001), who demonstrated that nisin-incorporated edible films have more potency at lower pH conditions. Moreover, pore formation by nisin on the membrane of *L. monocytogenes* facilitates the lower molecular-weight compound malic acid in penetrating inside the cell and lowering the pH of the cell. This leads to a reduction in listerial levels.

The combination of all three antimicrobials, N+MA+GSE, resulted in a decrease in listerial levels over the first 7 d from 5.0 log CFU/g to 3.7 log CFU/g. The listerial levels then steadily increased over the 28 d storage to 6.8 log CFU/g. There was a 1.0 log CFU/g reduction in the *Listeria* population in comparison to the control after 28 d of storage. Incorporation of grape seed extract with N and MA did not further enhance antimicrobial activities. This could be explained due to the lack of inhibition from the grape seed extract.

In summary, nisin (12000 IU and 18000 IU) and malic acid (2.0 and 3.0%) reduced the *L. monocytogenes* population by 2.0 log CFU/g in turkey frankfurters after 28 d of storage as compared to the controls. Grape seed extract did not inhibit the pathogen at all when used alone or in combination with nisin and malic acid and compared to the control. The most effective treatment in the study was the nisin and malic acid combination (N+MA), which lowered the population of *L. monocytogenes* by 2.7 log CFU/g, compared to the control, after 28 d of storage. Edible films can be an effective hurdle technology against foodborne pathogens and could be used in the ready-to-eat poultry and meat industry.

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Table 1. Composition and concentrations of treatments used in whey protein edible films

#	Treatment	Abbreviation	Concentration (%)		
			N	MA	GSE
1	Control with no WPI film, no antimicrobial additives	C	-	-	-
2	Control with WPI film, no antimicrobial additives	C1	-	-	-
3	WPI film + nisin	N1	6,000 IU/g	-	-
4	WPI film + nisin	N2	12,000 IU/g	-	-
5	WPI film + nisin	N3	18,000 IU/g	-	-
6	WPI film + grape seed extract	GSE1	-	-	1.0
7	WPI film + grape seed extract	GSE2	-	-	2.0
8	WPI film + grape seed extract	GSE3	-	-	3.0
9	WPI + malic acid	MA1	-	1.0	-
10	WPI + malic acid	MA2	-	2.0	-
11	WPI + malic acid	MA3	-	3.0	-
12	WPI + Malic acid + grape seed extract	MA+ GSE	-	1.0	1.0
13	WPI + nisin + malic acid	N + MA	6000 IU/g	1.0	-
14	WPI + nisin + grape seed extract	N + GSE	6000 IU/g	-	1.0
15	WPI + nisin + grape seed extract + malic acid	N + GSE + MA	6000 IU/g	1.0	1.0

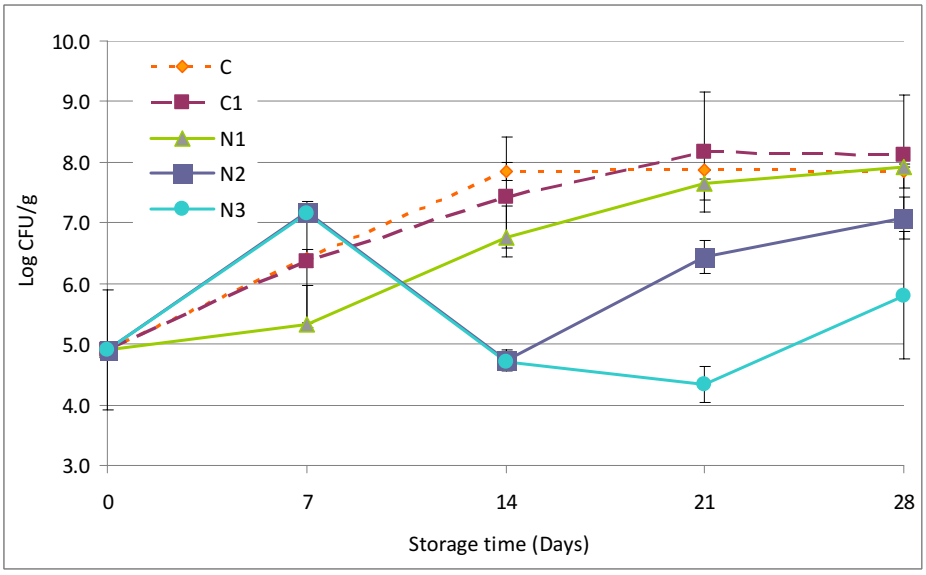

Fig. 1. Inhibitory activity of N-incorporated whey-protein coatings on turkey frankfurters, inoculated with *L. monocytogenes* stored at 4 °C for 28 d. C: Control without WPI films, C1: control with WPI film and without antimicrobials, N: WPI film with nisin at 6,000 IU/g = N1, 12,000 IU/g = N2, and 18,000 IU/g = N3. Values in figure are means of three different replications, error bars represent standard error.

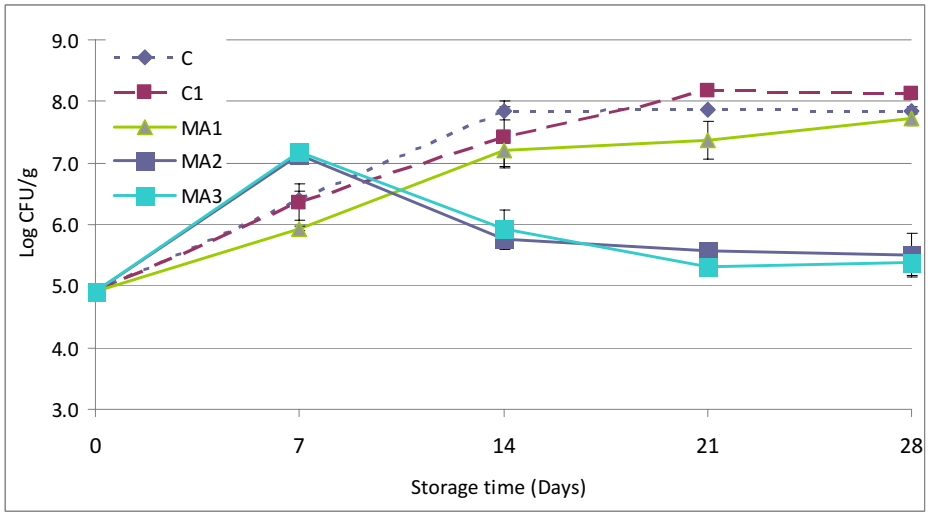

Fig. 2. Inhibitory activity of MA-incorporated whey protein coatings on turkey frankfurters, inoculated with *L. monocytogenes* stored at 4 °C for 28 d. C: Control without WPI films, C1: control with WPI film and without antimicrobials, MA: WPI film with malic acid at 1% = MA1, 2% = MA2, and 3% MA3. Values in figure are means of three different replications, error bars represent standard error.

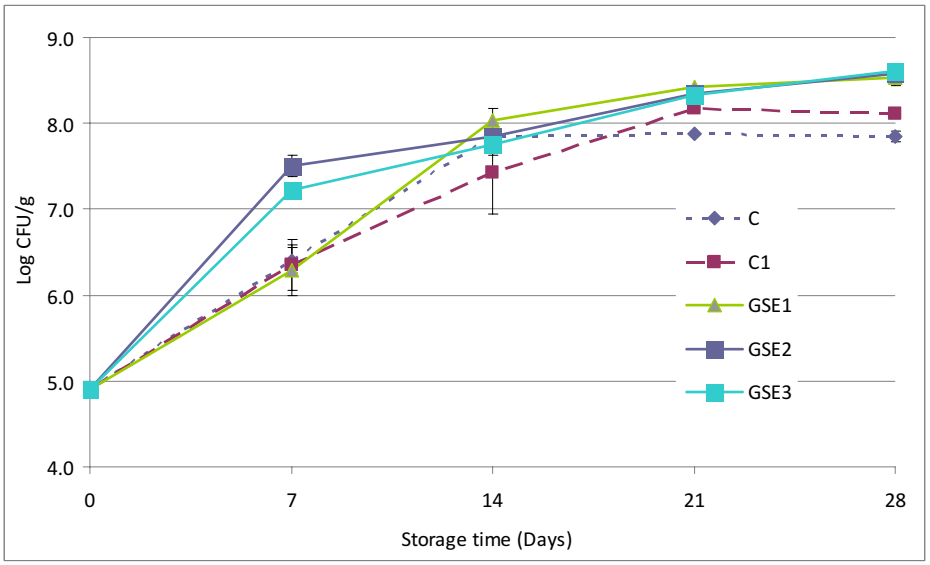

Fig. 3. Influence of GSE **incorporated into whey protein coatings on turkey frankfurters, inoculated with** *L. monocytogenes* **stored at** 4 **°C for** 28 **days. C: Control without** WPI **films, C1: control with** WPI **film and with out antimicrobials**, GSE: WPI **film with grape seed extract at 1**% = GSE**1**, 2% GSE2, **and** 3% GSE3**.** Values in figure are means of three different replications, error bars represent standard error

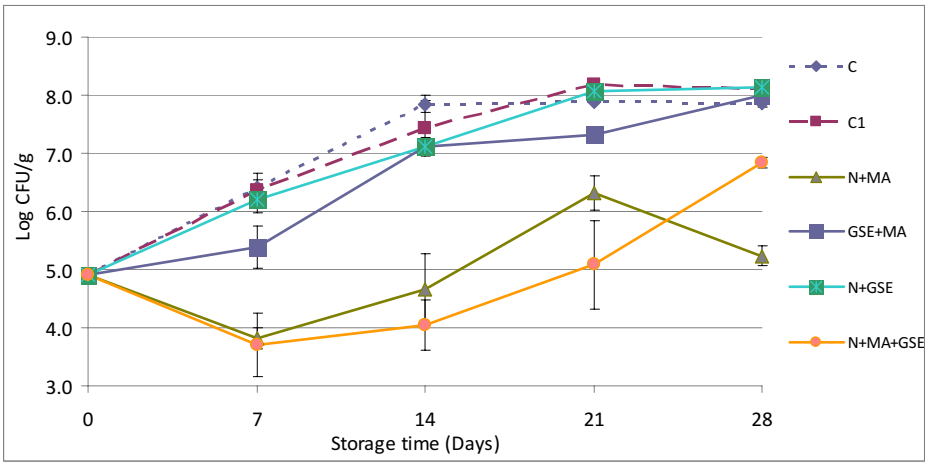

Fig. 4. Inhibitory activity of combinations of GSE,N,and MA into whey protein coatings on turkey frankfurters, inoculated with *L. monocytogenes* stored at 4 °C for 28 d. C: Control without WPI films, C1: control with WPI film and without antimicrobials. C: Control without WPI films, C1: control with WPI film and with out antimicrobials, GSE: grape seed extract, MA: malic acid, N: nisin Values in figure are means of three different replications, error bars represent standard error.