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Undergraduate Research Articles

Inhibitory activity against *Listeria monocytogenes* by soy-protein edible film containing grape seed extract, nisin, and malic acid

Brittany Adams, T. Sivarooban†, N.S. Hettiarachchy§, and M.G. Johnson‡*

ABSTRACT

The frequent outbreaks of food-borne illness necessitate development of intervention strategies, including the use of natural antimicrobials. *Listeria monocytogenes* is one of the most important bacterial pathogens that recently has caused a significant number of outbreaks. With the aim of finding potent natural agents that can minimize pathogen contamination concerns, this study evaluated the inhibitory activities against *L. monocytogenes* of grape seed extract (GSE), malic acid (M), nisin (N), and combinations thereof incorporated into soy-protein edible films. Soy-protein films with/without addition of antimicrobial agents (GSE: 1%, Nisin: 10,000 IU/g, Malic acid: 1%, and their combinations) were prepared and evaluated for anti-listerial activities. The highest inhibitory activity after 1 h incubation at 25°C was found in the treatment containing GSE, nisin, and malic acid, which produced reductions of log 3.7 colony-forming units (CFU)/ml as compared to control film without the addition of antimicrobial agents. These data demonstrated that the GSE, nisin, and malic acid combination incorporated into soy-protein edible films is very effective in inhibiting *L. monocytogenes* growth at 25°C and has potential for applications on a variety of food products to help prevent *L. monocytogenes* contamination and growth.

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MEET THE STUDENT-AUTHOR

Brittany Adams

I graduated from Jonesboro High School in 2003 and enrolled at the University of Arkansas in the fall as a food science major. I was awarded the Chancellor's scholarship as well as the Governor's Distinguished Scholarship and since then, I have also been awarded an Institute of Food Technologists scholarship. I am a member of the Food Science Club and the Institute of Food Technologists. I am also an active member of the IFT college bowl team for the University of Arkansas.

Spring semester of my freshman year I began working for Dr. Hettiarachchy conducting research in the area of utilizing proteins and anti-microbial plant extracts to inhibit pathogens, which led me to this research project. I have competed twice in the Gamma Sigma Delta undergraduate research competition and received 1st place twice in the poster category and 2nd place in the oral category. I also competed in the Ozark Food Processors Association undergraduate poster competition and received 1st place for my research in anti-microbials for food safety. I plan to continue with graduate studies after completing my B.S. in food science and become a director of research in R&D.

INTRODUCTION

Food-borne pathogens, which cause illness, death, and great economic losses, are of great concern to consumers and the food industry. The annual economic loss associated with food-borne disease is approximately $5-6 billion (Murphy et al, 2003). *Listeria monocytogenes* is one of the most significant pathogens that has caused numerous outbreaks (Janes et al., 2002). *L. monocytogenes* is a cold-loving (psychrophilic) pathogen that causes an average of 2,500 illnesses and 500 deaths each year (Mead et al., 1999; CDC, 2003). In 2003 and 2004, respectively, 45,251 and 502,845 pounds of refrigerated ready-to-eat (RTE) meat products including chicken, turkey, and beef meats were recalled due to *L. monocytogenes* contamination (FSIS/USDA, 2005).

L. monocytogenes is of particular concern for the elderly, unborn fetuses, and those who are immuno-compromised (Lorber, 1990). Food products can be cross contaminated with *L. monocytogenes* during processing. This causes the most problems in RTE products such as luncheon meats, which require no further treatment post-packaging. These RTE meat products become prime candidates for food-borne disease transmission if they are contaminated during processing, and thus can cause illness in consumers.

The food industry of late has been showing an increased interest in edible films to control various spoilage challenges of foods and increase shelf life. Research regarding these films has centered on casein, collagen, corn zein, gelatin, soy protein, wheat gluten, calcium alginate, and methylcellulose (Eswaranandam et al., 2004; Gennadios and Weller, 1991; Guilbert, 1997; Hoffman et al., 1998; Janes et al., 2002; Lungu and Johnson, 2005; Were et al., 1998&1999). These edible films can be used as carriers for various beneficial compounds including antimicrobials. A slow release of these antimicrobials from the film to the food product would control bacterial growth, and thus increase the shelf life of the products.

There is an increasing interest in natural antimicrobial compounds, especially those of plant origin, since they are considered as safe and economical. Phenolic compounds in plant extracts are responsible for antimicrobial and antioxidant activities. Grape seeds have been recognized to have health benefits for human beings. Hettiarahchy et al., (2003a and 2003b) and Adams et al., (2004) have demonstrated the antimicrobial and antioxidant qualities of grape seed extract. Grape seed extract is commercially available and is currently used in a variety of food products.

Organic acids and their salts are also promising antimicrobial agents because of their established acceptance in food products and low cost (Miller et al., 1995). Organic acids are either naturally present in fruits and vegetables or synthesized by microorganisms. Eswaranandam et al., (2004) reported that malic acid incorporated into a soy protein film was very effective in controlling *L. monocytogenes*.

Nisin is a hydrophobic protein containing 34 amino acids. It is a bacteriocin produced by Lactococcus lactic sub sp. Lactis (Jung et al., 1991; Montville et al., 2001). It is recognized as a safe biological food preservative by the Food and Drug Administration and is effective in controlling a wide range of gram-positive bacterial growth, including *L. monocytogenes*. Currently, nisin is approved for use in the U.S. for soft cheeses to control *Clostridium botulinum* growth.

No literature is available on the antimicrobial activity of grape-seed and tea extracts, nisin, and their combinations with malic acid-incorporated edible films. Therefore, the objective of this study was to evaluate the inhibitory effects at 25°C against *L. monocytogenes* of grape seed extract (GSE) and the combined effects of grape seed extract with nisin and malic acid incorporated into soy-protein edible films.

MATERIALS AND METHODS

Pathogenic *L. monocytogenes* (strain V7 serotype 1/2a) was obtained from the Food Microbiology Research Laboratory in the Department of Food Science at the University of Arkansas, Fayetteville. *Listeria* Selective Agar (Oxford formulation) and malic acid were obtained from EM science, (EM Industries, Gibbstown, N.J.) Glycerol was purchased from Sigma Chemical Co. (St. Louis, Mo.) Soy protein isolate (AR-DEX®) was obtained from Archer Daniel Midland, Decatur, Ill. Commercial grape seed extract (GSE) powder was obtained from MegaNatural Inc. (Madera, Calif.) Nisin (Nisaplin®) was obtained from Aplin and Barrett Ltd (Dorset, U.K.)

Antimicrobial testing of grape seed extract / nisin / malic acid combinations in BHI broth model system

The anti-microbial effects of grape seed extract, nisin, and malic acid were evaluated for their effectiveness against *L. monocytogenes* in a model system. One loop of the organism was transferred from the frozen stock at -70°C to 10 mL of Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 h. Thereafter, 10 µL of this culture was transferred into 10 mL of fresh BHI and incubated for activation at 37°C for 18 h after which 20 _L of the culture was added to 10 mL BHI for the tests (approximately 106 log C F U / ml). Precisely 0.5 mL of this culture was combined with 0.5 mL of a test solution (GSE, nisin, malic acid, or a combination) and incubated at 37°C for 24 h. The samples were diluted in phosphate buffer solution (PBS) and spread plated onto Listeria Selective Agar and incubated for 48 h at 37°C and the colonies were counted.

Preparation of soy protein films

To prepare the films, 10 g of soy protein were added to and mixed well with 90 g of de-ionized water in each sample (GSE, nisin, malic acid, combinations, and control). A concentration of 3.5% glycerol (plasticizer) was added and stirred with a magnetic stirrer for 30 min. The solutions were then heated and stirred in an 85°C water bath for 30 min and were then allowed to cool to room temperature. After cooling, GSE (1%w/w), nisin (10,000 IU/g), malic acid (1%w/w), or one of their combinations were added to the film solutions. A control film was prepared which contained soy protein isolate and glycerol with no additional substances.

Casting films

The resulting film solutions prepared were cast onto 19 x 28 cm^2 mylar plastic sheets coated with silicone (Richard Mistler, Inc., Morrisville, Penn.) by way of draw-down equipment (Paul N. Gardner Co., Inc.,Pompano Beach, Fla.) The solutions formed a film with uniform thickness and were then dried for 4 h in a controlled humidity chamber (Hot Pack, Philadelphia, Penn.) at 50°C and 40% RH. The films were then removed from the chamber and placed between sheets of wax paper and into a 5°C, 50% RH desiccator over a NaBr-saturated solution (Sanplatec Corp., Osaka, Japan) for storage until testing of antimicrobial activity.

Thickness of films

At three different locations on each of the films, a small section was measured for thickness. The three values were averaged to determine the thickness of each film. These measurements were performed with a micrometer (Model 2804 – 10, Mitutoyo, Japan) and determined to the nearest 2.5 mm.

Puncture strength of films

Puncture strength measures the ability of the film to hold up under various stresses after being applied to the food product. This measurement was taken using a texture analyzer (TA-XT2I, Texture Technologies Corp., Scarsdale N.Y.) The film samples were allowed to reach room temperature and a RH of 50% for 48 h before the test was conducted. A 30-mm piece of film was placed on a 10-mm film-testing rig (TA- 108S Mini) and punctured with a 2-mm probe (TA-52). The puncture strength of the film (given as a force in Newtons) was measured at the point when the probe pierced the film.

Antimicrobial testing of soy protein films

To determine the killing effect of each film, bacterial cultures were inoculated onto a film disc and total plate counts were enumerated. The bacterial suspensions were prepared once more and diluted in PBS to yield approximately 1.5 x 10^6 bacteria. Thereafter, 15 µL of this suspension was inoculated onto each film disc (1 cm diameter). The inoculated discs were then transferred to stomacher bags and 985 µL of PBS were added to each bag. These bags were stomached for 2 min to completely dissolve the film. The resulting mixture was diluted up to a 10^6 dilution with PBS and spread plated onto *Listeria* Selective Agar plates. These plates were incubated at 37°C for 48 h and the colonies were counted.

RESULTS AND DISCUSSION

Anti-Listerial activities of grape seed extract, nisin and malic acid, and their combinations in BHI broth medium at 37°C

It was found that an initial *L. monocytogenes* inoculation level of about 6.7 log CFU/ml grew to 8.9 log CFU/ml after 24 h at 37°C in BHI (control) medium (Fig. 1.) In the presence of GSE or nisin alone at the same initial inoculation level of 6.7 log CFU/ml, *L. monocytogenes* changed to 4.4 and 8.6 log CFU/ml, respectively, (Fig. 1.) The growth was reduced to non-detectable levels after 6 h incubation in the presence of malic acid alone. The highest inhibitory activity was found in the treatment with GSE combined with nisin and malic acid or GSE combined with malic acid or malic acid combined with nisin, (Fig. 1.) The logs CFU/ml at 0 h and 3 h for treatments with GSE with malic acid / malic acid with nisin / GSE, nisin, and malic acid were 6.7 and non-detectable levels, respectively. No growth was observed in these extracts after incubation of 3 to 24 h.

Thickness and puncture strength of films

Incorporation of GSE increased the thickness and puncture strength of soy protein film, (Fig. 2 and 3.) Higher molecular-weight individual phenolic constituents including catechin, genestic acid, epicatechin, and syringic acid; and gallic acid, protochatechuic, and caffeic acid (Rababah et al., 2004) present in the GSE might have contributed to the thickness and puncture strength of soy protein films. The higher molecular-weight, hydrophilic nature of these phenolics and their hydroxyl groups may participate to increase the protein-protein inter-chain interactions and reduce the plasticizing effect in the film-forming solution.

When nisin was incorporated with soy protein film, thickness of the film significantly increased ($p<0.05$), (Fig. 2.) The nisin molecules could have contributed to hydrophobic and electrostatic interactions with protein molecules. No significant differences were observed in the addition of nisin (10,000 IU/g) on puncture strength of soy protein film, (Fig. 3.)

Incorporation of malic acid reduced the thickness and puncture strength of soy protein film, (Fig. 2 and 3.) The lower molecular-weight and plasticizing properties of malic acid may have disturbed the higher molecular-weight protein-polymer chain interactions. The addition of malic acid to the film solutions also decreased the pH of the film.

Anti-Listerial activities of soy-protein edible film containing grape seed extract, nisin, malic acid, and their combinations

The counts of *L. monocytogenes* were approximately 6.6 log CFU/ml after 1 h incubation at 25°C in the soy-protein edible film which had no addition of antimicrobial agents (control), (Table 1). The count of *L. monocytogenes* was reduced to log 5.7 CFU/ml in the presence of GSE alone (Table 1). Of all possible combinations of the compounds, the highest inhibitory activity was found in the combination of GSE, nisin, and malic acid, with reductions of 3.7 log CFU/ml compared to the soy-protein film control, (Table 1) The hydroxyl groups present in the phenolic compounds of GSE were likely responsible for its antimicrobial activity. Nisin has the ability to form pores in the cell membrane of the bacteria, thereby allowing a free flow of ions into and out of the cell, which causes an imbalance and eventually cell death. Malic acid has a very small molecular weight and can easily enter the bacterial cell. This helps disrupt the internal pH of the cell and reduce the protein motive force, thereby causing death. Nisin and malic acid enhanced the effectiveness of anti-listerial activities of GSE. The killing mechanism of these three natural antimicrobial compounds incorporated into soy protein film was very effective against *L. monocytogenes*.

These findings will have applications for controlling *L. monocytogenes* contamination in various food products including raw and ready-to-eat poultry and beef products as well as fresh, whole, and minimally processed vegetables and fruits.

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Table 1. Effect of grape seed extract, nisin, malic acid and their combinations incorporated in soy-protein edible film against *Listeria monocytogenes* at 25°C

Edible film	*L. monocytogenes* (Log CFU/ml)
Control (SPI)	6.6 ± 0.1^{a}
GSE (1% w/w)	5.7 ± 0.1^{b}
N	4.8 ± 0.2^{de}
M	5.1 ± 0.1^{cd}
GSE + N	4.6 ± 0.1^{e}
GSE + M	5.3 ± 0.1^{c}
N + M	4.0 ± 0.2^{f}
GSE + N + M	2.9 ± 0.1^{g}

Film disc (1cm diameter) was inoculated (appoximately10^{6} CFU/ml) and incubated at 25°C for 1 h. Log numbers of *L. monocytogenes* in control film was 6.6 ± 0.1 CFU/ml.

All means were measurements of three separate experiments in duplicates. Means within a column followed by same superscript are not significantly different ($p<0.05$).

Abbreviation of treatments are as follows: SPI=soy protein film, GSE=grape seed extract (1% w/w), N=nisin (10,000IU/g), M=malic acid (1% w/w).

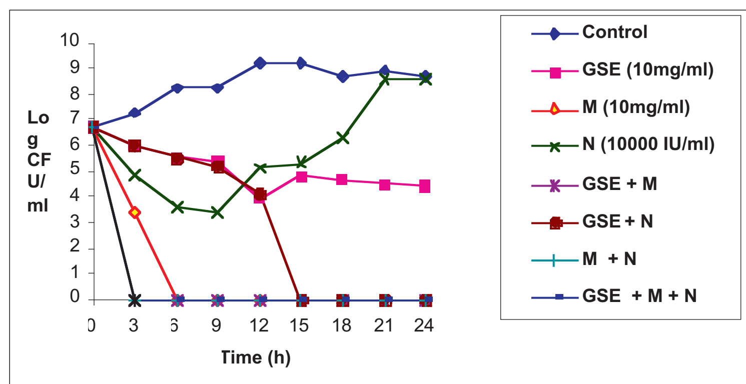

Fig. 1. Anti-listerial activities of grape seed extract (GSE), nisin (N), malic acid (M), and their combinations in BHI broth medium at 37°C. (Values represent means of three separate experiments.)

Abbreviation of treatments are as follows: SPI=soy protein film, GSE=grape seed extract (1% w/w), N=nisin (10,000IU/g), M=malic acid (1% w/w).

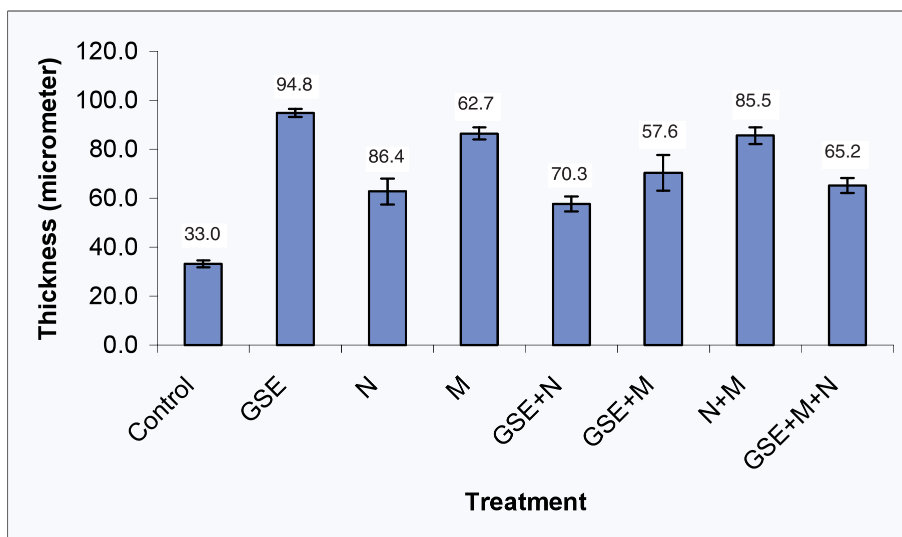

Fig 2. Thickness of soy protein films containing grape seed extract (GSE), nisin (N), and malic acid (M) and their combinations

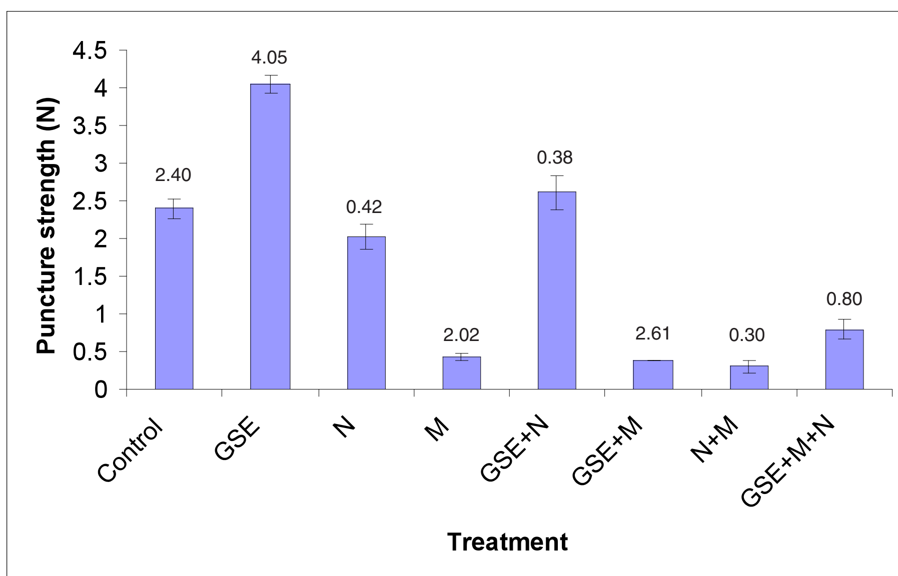

Fig 3. Puncture strength of soy protein films containing grape seed extract (GSE), nisin (N), and malic acid (M) and their combinations

Abbreviation of treatments are as follows: SPI=soy protein film, GSE=grape seed extract (1% w/w), N=nisin (10,000IU/g), M=malic acid (1% w/w).