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Arzu Cagri

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Elliot T. Ryser
Major professor

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ANTIMICROBIAL WHEY PROTEIN ISOLATE-BASED EDIBLE CASINGS

By

ARZU CAGRI

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ABSTRACT

ANTIMICROBIAL WHEY PROTEIN ISOLATE-BASED EDIBLE CASINGS

By

ARZU CAGRI

Methods were developed to optimally produce low pH (5.2) antimicrobial whey protein isolate (WPI) edible films containing sorbic acid (SA) or p-aminobenzoic acid (PABA). Solutions of lactic acid and acetic acid (1:0, 1:1, and 2.3:1) were used to acidify the film solution. Films containing either antimicrobial agent inhibited the growth of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 on trypticase soy agar. Increased concentrations of PABA or SA in WPI film increased the percent elongation (%E) and water vapor permeability (WVP). While tensile strength (TS) of WPI films decreased with increasing levels of SA, TS of films was not affected addition of PABA.

Antimicrobial properties of films containing PABA, SA, or PABA:SA were tested over 21 days of refrigerated storage on slices of summer sausage and bologna inoculated to contain 10^6 *L. monocytogenes*, *E. coli* O157:H7 or *S.* Typhimurium DT104 CFU/g. These films decreased populations of the test pathogens and retarded the growth of mesophilic aerobic bacteria, lactic acid bacteria and mold/yeast throughout this period of storage. Contact with the slices decreased TS of the films, but increased % E.

In the last objective, heat-cured WPI films containing PABA were heat-sealed to form casings. A commercial-type hot dog batter was stuffed into WPI, collagen, or natural casings. After cooking, the hot dogs were surface-inoculated with *Listeria*

monocytogenes, vacuum packaged, and examined for numbers of *L. monocytogenes,* mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB), and yeast/mold during 42 days of storage at 4°C. *Listeria* populations on hot dogs prepared with WPI-PABA casings remained relatively unchanged; however, numbers of *Listeria* on hot dogs prepared with WPI, collagen, and natural casings increased during 42 days of refrigerated storage. Populations of MAB, LAB, and mold on WPI-PABA casings were lower compared to other casings. Physical (e.g. purge loss, color), mechanical (shear force), chemical (e.g. moisture, fat, protein content, pH, TBA) and sensory properties of hot dogs prepared with different casings were also analyzed. Taste panelists ranked flavor, juiciness, and overall acceptability higher for hot dogs prepared with WPI-PABA casings compared to hot dogs prepared with WPI, collagen or natural casings. Hence, WPI casings containing PABA appear to be a promising means to prevent growth of *Listeria* on hot dogs and extend shelf-life.

To my parents

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ABBREVIATIONS

AA	Acetic acid
ASTM	American Society for Testing and Materials
B	Bologna slices
CDC	Centers for Disease Control and Prevention
CMC	Carboxymetylcellulose
Coll	Collagen
% E	Percent elongation
FDA	Food and Drug Administration
Gly	Glycerol
GRAS	Generaly recognized as safe
HPC	Hydroxypropyl cellulose
HPMC	Hydropropionate methylcellulose
LA	Lactic acid
LAB	Lactic acid bacteria
MAB	Mesophilic aerobic bacteria
MC	Methylcellulose
OP	Oxygen permeability
PABA	p-aminobenzoic acid
PEG	Polyethylene glycol
PW	Peptone water
RH	Relative humidity
SA	Sorbic acid
SAS	Statistical Analysis System
SDS	Sodium dodecyl sulfate
Sol	Sorbate
SPI	Soy protein isolate
SS	Summer sausage
TBA	2- Thiobarbutiric acid
TS	Tensile strength

TSA	Trypticase soy agar
TSAYE	Trypticase soy agar + yeast extract
TSB	Trypticase soy broth
TS	Trisodium phosphate
WG	Wheat gluten
WPI	Whey protein isolate
WVP	Water vapor permeability

INTRODUCTION

Microbial susceptibility of the food surface is a major determinant of product quality and safety during refrigerated storage and distribution since most Class I product recalls in the U.S. result from post-processing contamination during subsequent handling and packaging. The increasing rate at which ready-to-eat meat products are becoming contaminated with *Listeria monocytogenes* after processing has raised new safety concerns. From April 1998 to December 2001 over 75 *Listeria*-related Class I recalls involving more than 100 million pounds of cooked ready-to-eat meats were issued (USDA-FSIS, 2001a). In 1998, more than 35 million pounds of hot dogs and luncheon meats contaminated with *L. monocytogenes* were voluntarily recalled by one Michigan manufacturer in response to a listeriosis outbreak that resulted in 101 cases in 22 states, including 21 fatalities (CDC, 1998). Another outbreak involving 29 cases in 10 states (including 7 fatalities) prompted the recall of approximately 14.5 million pounds of turkey and chicken delicatessen meat with the product again becoming contaminated with *L. monocytogenes* after processing (CDC, 2000). In 2000, almost 2,300 cases of food-borne listeriosis were reported in the United States at an estimated cost of $2.33 billion (~$ 1 million /case), making *L. monocytogenes* the second most costliest food-borne pathogen known to date after *Salmonella* ($ 2.38 billion) (USDA-FSIS 2001b).

Two other pathogens have emerged as major public health concerns. *Escherichia coli* O157:H7 has been highly publicized due to outbreaks of illness associated with ground beef (Ostroff et al., 1990, Bell et al., 1994, Mead et al., 1997, Tuttle et al., 1999) and dry-cured salami (Tilden et al., 1996). Unlike most food-borne pathogens, *E. coli* O157:H7 shows high tolerance to acidic environments. Transfer of *E. coli* O157:H7 from

contaminated meat or utensils to other foods such as fresh fruits and vegetables is also a major concern (CDC, 1995). The annual cost of the 62,458 *E. coli* O157:H7 infections reported in 2000 was estimated at $659.1 million (~$10,500/case) (USDA-FSIS, 2001b).

Salmonella is the second most frequently reported cause of foodborne illness in the United States after *Campylobacter*. An estimated 1.34 million cases of salmonellosis occur annually, resulting in 600 deaths. The annual economic cost of foodborne salmonellosis was estimated at 2.38 billion dollars (USDA-FSIS 2001b). The incidence of salmonellosis appears to be rising both in the U.S. and other industrialized nations. *S. enteritidis* isolations from humans increased dramatically during the early to mid 1990's particularly in the northeast United States (6-fold or more). *Salmonella* is most commonly found in meat, poultry, eggs and water, with kitchen surfaces also a common breeding ground for *Salmonella.*

Salmonella Typhimurium DT104, a multiantibiotic resistant strain, is also emerging as a serious threat to public health (Glynn et al., 1998). Food-borne transmission of *S.* Typhimurium DT104 has been documented for several meat-related outbreaks; suspected vehicles included roast beef, ham, pork sausage, salami sticks, cooked meats, frozen sausage samples, and chicken legs (Davies et al., 1996; Anonymous, 1996). In England, 17% of 786 fresh or frozen sausage samples yielded *Salmonella* spp., including *S.* Typhimurium DT104 (Nichols and de Louvois, 1995). Sharma et al. (2001) also recovered *S.* Typhimurium DT104 at several points in pork production. These findings indicate that meat products may pose a serious health risk if not cooked and handled properly.

Post-processing pasteurization is one means for minimizing post-processing contaminants on meat products. Using this strategy, packaged products are individually pasteurized by heat or other means (e.g. high pressure, UV) to inactivate surface bacteria. However, some of these treatments makes the products organolepticaly unacceptable to consumers (Farber and Peterkin, 1999). Alternatively, various antimicrobial dips and sprays have been applied to ready-to-eat foods to minimize microbial growth (Robach and Sofos, 1982; Cunningham, 1979). However, their effectiveness over time is limited because the preservatives diffuse into the food, allowing surface organisms to grow. Therefore, it is important to prevent or control migration of these preservatives. Guilbert and his research group have assessed the ability of edible films to control the diffusion of sorbic acid and potassium sorbate (Guilbert, 1988; Giannakopoulus and Guilbert, 1986; Guilbert et al., 1985). In another study, lactic acid-treated casein films containing sorbic acid (Guilbert, 1988) retained 30% of the sorbic acid at the surface after 30 days of storage at 95% RH, whereas all of the sorbic acid with no casein film application on control samples diffused after 24 hours of storage. They confirmed that the edible film matrix entrapped antimicrobials or other food additives and reduced diffusion during storage.

Research on edible films as packaging materials has increased because of two potential that these films may improve overall food quality, extend shelf life, and improve economic efficiency of packaging materials. In addition, environmental concerns regarding disposal of fluid whey and increased production of non-biodegradable packaging materials have prompted interest in the development of edible films from whey protein isolate and whey protein concentrate as well as wheat gluten, soy protein

isolate, zein protein and others. Previously, coating foods with edible films provided only barrier and protective functions. However, incorporating various substances into packaging material can improve its functionality. In addition to acting as a barrier against mass diffusion (moisture, gases, and volatiles), edible films can also be used as carriers for a wide range of food additives such as antimicrobial agents, flavoring agents, antioxidants, and colorants. Incorporating antimicrobial compounds such as nisin, pediocin, benzoic acid, sorbic acid/propionic acid, or lysozyme into edible films or coatings is another means of enhancing the safety and shelf-life of products subject to surface contaminants such as ready-to-eat meats and cheeses. For example, soy protein and corn zein films containing pediocin or nisin and calcium alginate coatings containing organic acids were shown to inhibit the growth of pathogenic (e.g. *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7*)* and spoilage bacteria (*Lactobacillus plantarum*) on meat surfaces (Sirugusa and Dickson, 1993; Dawson et al., 1997; Padget et al., 1998). In addition, coating with an antimicrobial whey protein solution containing propionic/sorbic acid prevented growth of *L. monocytogenes* on frankfurters (McDade et al., 1999). However, non-uniformity of the coating on frankfurters after dipping, draining and drying would likely produce a less effective antimicrobial barrier as compared to pre-cast films. Whey protein isolate (WPI) edible films containing antimicrobials would be uniform in thickness. Consequently, these films would be better suited to inhibit post-processing surface contaminants such as *L. monocytogenes.*

The underlying hypothesis for this research was that low pH whey protein-based edible films (pH 5.2) containing sorbic acid (SA) or p-aminobenzoic acid (PABA) could be developed to inhibit *L. monocytogens*, *E.coli* O157:H7 and *S. Typhimurium* DT104

both in a model system and on hot dogs when used as heat sealed tubular casings and extend product shelf-life. The specific objectives of this research were as follows:

(1) Develop a series of low pH whey protein isolate (WPI) edible films (pH 5.2) containing p-aminobenzoic acid or sorbic acid and determine water vapor permeability and mechanical (tensile strength and elongation) properties.

(2) Test the antimicrobial properties of these films against *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium DT104 on a laboratory medium.

(3) Compare the barrier and mechanical properties of these films to commercial collagen and cellulose films and modify the mechanical and barrier properties if needed using heat curing.

(4) Assess the ability of these films to retain their desirable antimicrobial and mechanical properties while in direct contact with slices of bologna and summer sausage during 21 days of refrigerated storage.

(5) Develope WPI hot dog casings containing PABA, SA, and PABA:SA by heat-sealing the aforementioned films into a tubular shape in order to:

   a) Test the ability of WPI casings containing PABA, SA or PABA:SA to inhibit *L. monocytogenes* on surface-inoculated hot dogs.

   b) Determine the ability of the WPI casings to retain their desirable mechanical properties during hot dog manufacture.

   c) Assess the impact of different casings on storage stability of hot dogs based on numbers of total aerobic mesophilic bacteria, lactic acid bacteria, yeast and mold, 2-thiobarbutiric acid (TBA) values, purge loss, shear force, color, pH, chemical analysis and sensory evaluation.

CHAPTER 1

LITERATURE REVIEW

1.1. EDIBLE FILM

1.1.1. Definition and historical background

Edible films or coatings are defined as continuous matrices that can prepared from proteins, polysaccharides, and/or lipids. These films and coatings have the potential to increase food quality and reduce food-packaging requirements since they act as mass transfer barriers to moisture, oxygen, lipids and solutes and can also carry a wide range of food additives including vitamins, colorants, flavoring agents, preservatives and antimicrobial agents.

Historically, yuba, the first freestanding edible film, was developed in Japan from soymilk during the 15th century and used for food preservation (Guilbert and Biquet, 1996). Edible coating for food products date back even further with waxes applied to oranges and lemons in China to retard water loss during the twelfth century (Hardenberg, 1967). During the sixteenth century, coating food products with fat (e.g. lard) was used to control moisture loss in foods in England (Labuza and Contrereas-Medellin, 1981). Hot melt paraffin waxes have been used to coat citrus fruits in the United States since the 1930s, and carnauba wax and oil-in-water emulsions have been used for coating fresh fruits and vegetables since the 1950s (Kaplan, 1986). Currently, edible films and coatings find use in a variety of applications, including casings for sausages, and chocolate coatings for nuts and fruits.

1.1.2. Formation of edible films

1.1.2.1. Components of edible films

Edible films typically contain three major compounds; proteins, polysaccharides, and lipids. Proteins used in the edible film include wheat gluten, collagen, corn, soy, peanut, casein and, whey protein (Kester and Fennema, 1986). Polysaccharides incorporated in edible films have included alginate, dextrin, pectin, carrageenan and cellulose derivatives (Greener and Fennema, 1994). Suitable lipids include waxes, acylglycerols, and fatty acids (Greener and Fennema, 1993; Park et al., 1994; Debeaufort and Voiley, 1995). In addition, composite films containing both lipid and hydrocolloid components have been developed.

Plasticizers can be added to film-forming solutions to enhance properties of the final film. Plasticizers will decrease brittleness and increase flexibility of the film, which is important for packaging applications. Common food-grade plasticizers include sorbitol, glycerol, mannitol, sucrose, and polyethylene glycol. Plasticizers used for protein-based edible films decrease protein interactions, and increase both polymer chain mobility and intermolecular spacing (Lieberman and Gilbert, 1973). Plasticizers must be small molecules with low molecular weights and high boiling points that are highly compatible with the polymers (Banker, 1966). The type and concentration of the plasticizer influences the properties of protein films (Gennadios et al., 1994a; Cuq et al., 1997; Gueguen et al., 1998). When large amounts of plasticizer are used, mechanical strength, barrier properties, and elasticity decrease (Gontard et al., 1993; Park et al., 1994; Cherian et al., 1995; Debeaufort et al., 1997; Galietta et al., 1998).

Crosslinking agents are used to improve water resistance, cohesiveness, rigidity, mechanical strength and barrier properties (Guilbert, 1995; Marquie et al., 1995; Remunan-Lopez et al., 1997). Commonly used covalent crosslinking agents include glutaraldehyde, calcium chloride, tannic and lactic acids. Exposure to ultraviolet light will increase the cohesiveness the protein films through the formation of crosslinks (Brault et al., 1997). Alternatively, enzymatic crosslinking treatments with transglutaminases or peroxidases can be used to stabilize films.

1.1.2.2. Film Formation Techniques

Many techniques have been developed for forming films. These include coacervation, thermal gelation, solvent removal and solidification of melt. In coacervation, two solutions of oppositely charged hydrocolloids are combined, causing interactions and precipitation of the polymer complex. Solvent removal is another common means of forming hydrocolloid edible films. In this process, a continuous structure is formed and stabilized by the interactions between molecules through the action of various chemical or physical treatments. Macromolecules in the film-forming solution are dispersed in a solvent medium such as water, ethanol or acetic acid that contains several additives (plasticizers, crosslinking agents, solutes). The film forming solution is then cast in a thin layer, dried, and detached from the surface.

In preparing some types of protein films (whey protein, casein, soy protein, wheat gluten), the macromolecule solution is heated for protein gelation and coagulation which involves denaturation, gelification or precipitation, followed by rapid cooling of hydrocolloid solution. Intramolecular and intermolecular disulfide bonds in the protein

complex are cleaved and reduced to sulfhydryl groups during protein denaturation (Okamota, 1978). When the film-forming solution is cast, disulfide bonds are reformed which link the polypeptide chains together to produce the film structure with hydrogen and hydrophobic bonds also contributing to film structure.

Another common film-forming technique is melting followed by solidification. Solidification of the melt by cooling is commonly used to prepare lipid films. Wax films can also be formed by casting molten wax on a dried film of methylcellulose and then dissolving away the methylcellulose film (Donhowe and Fennema, 1993).

1.1.3. Protein-Based Edible Films

The proteins that have been used most extensively for production of films and coatings include whey protein, casein, corn zein, soy protein, gluten, collagen, and gelatin.

1.1.3.1. Whey Protein Films

1.1.3.1.1. Composition and Structure

Whey protein is the protein that remains in milk serum after acid/rennet coagulation of fluid milk (de Wit, 1989; Morr and Ha, 1993). Whey protein consists of five different proteins: β-lactoglobulin, α-lactalbumin, bovine serum albumin, proteose-peptones, and immunoglobulins (Kinsella and Whitehead, 1989).

β-Lactoglobulin (β-Lg) which comprises 50-75% of whey protein, is a globular protein with a molecular mass of approximately 18,300 Da (Eigel, 1984). β-Lg consists of 162 amino acid residues and contains two disulfide groups, one free sulfhydryl group,

and several hydrophobic groups located in the interior of the globular structure (Brunner, 1977). The thiol group is particularly important since it facilitates molecular thiol-disulfate interchange reactions which allow formation of intermolecular disulfate-bonded dimers and polymers upon heating (Kinsella, 1984). The structure of β-Lg is pH dependent, with this protein existing as a dimer in solutions above pH 5.2. Below pH 3.5 and above 7.5, the dimer dissociates to a monomer, and between pH 3.5 and 5.2 the dimer polymerizes to an octomer. β-Lg undergoes time and temperature dependent denaturation reactions at temperatures above 65°C, which result in a general molecular expansion, exposure of the internal SH group, and hydrophobic and ε-NH_2 groups (Brunner, 1977; Kinsella, 1984). Structurally, β-Lg has about 15% helix, 43% β-sheet, and 15-20% β-turns (Papiz et al., 1986).

α-Lactoalbumin (α-La) which comprises about 19% of total whey protein (Dybing and Smith, 1991), is another globular protein that contains 123 amino acid residues and four disulfide bonds. It has a molecular weight of 14,000 Da. The secondary structure of this molecule is composed of 30% α- helix, 9% β-sheet, and 61% unordered structure (Alexandrescu et al., 1993). Conformational changes occur in α-La at pH 4 with the molecule losing Ca^{+2} which is tightly bound at higher pH. α-La is denatured at 65.2°C and pH 6.7 with 80 to 90% of this denaturation reversed after cooling. This reversibility is lost if the native S-S bonds are broken, for example, by heat-induced thiol-disulfate interchange reactions between α-La and β-Lg (de Wit, 1989).

1.1.3.1.2. Film Formation

Film production requires heat denaturation since the hydrophobic and SH groups are deep within the globular whey protein. Denaturation changes the three-dimensional protein structure and exposes internal SH and hydrophobic groups (Shimada and Cheftel, 1998), which promote formation of new intermolecular S-S and hydrophobic bonds during drying (McHugh and Krochta, 1994a). Film formation is favored in more alkaline film solutions since SH reactivity increases at pH > 8 (Kella and Kinsella, 1988; Banerjee and Chen, 1995). Glycerol, sorbitol and polyethylene glycol have been commonly used as plasticizers to produce whey protein films that are transparent, bland, and flexible, and have excellent oxygen, aroma and oil barrier characteristics. However these films are poor moisture barriers due to their hydrophilic character (Table 1.1). Incorporation of lipids, including acetylated monoglycerides, waxes, fatty alcohols and fatty acids, into WPI film decreases water vapor permeability (WVP) by increasing hydrophobicity (McHugh and Krochta 1994b; Sherwin et al., 1998; Kim and Ustunol, 2001). Addition of beeswax and fatty acids to WPI film improves moisture barrier properties more than fatty alcohols with the longer chains more effectively reducing WVP (McHugh and Krochta, 1994c). Sherwin et al. (1998) reported that particle size in the emulsion film increased with increasing fatty acid chain length from C_{12} to C_{22}. McHugh and Krochta (1994c) showed that incorporation of large lipid particles decreased WVP of emulsion WPI film due to interactions at the protein-lipid interface or the dispersed phase.

WPI film properties are affected by the extent of intermolecular disulfide bonding and plasticizers competing for protein chain-to-chain hydrogen bonding. Plasticizer content and relative humidity (RH) both have an exponential effect on film permeability.

Table 1.1. Water vapor permeability (WVP) and oxygen permeability (OP) of edible films.

Film	WVP g.mm/m^2.d.kPa	OP cm^3.µm/m^2.d.kPa	References
Coll	-	<0.04[a]	Lieberman and Guilbert (1973)
Zein	12-24[a]	11.8[c]	Gennadios et al. (1993), Parris and Coffin (1997)
WG	53[b]	3.9-6.1[d]	Gennadios et al. (1993)
SPI	72-154[a]	1.6-4.5[c]	Park and Chinnan (1990), Gennadios et al. (1993)
WPI	62.0- 70.2[c]	18.5 –76.1[e]	Krochta (1994)
Casein	45.2[d]	1.8[a]	Chick and Ustunol (1998)
Alginate	42.2[e]	-	Parris et al. (1995)
Chitosan	1.6[f]	90.2[c]	Caner et al. (1998)
MC	7.7[f]	187.4[c]	Park and Chinnan (1994)
HPMC	9.5[f]	297.3[c]	Park and Chinnan (1994)
Pectin	41.4[f]	57.0[c]	Wong et al. (1992)
Starch	220[g]	2.9[c]	Allen et al. (1963)
Beeswax	0.05[f]	1.3[c]	Kester and Fennema (1989)
Candelilla	0.02[f]	0.3[c]	Kester and Fennema (1989)
Carnauba	0.03[f]	0.2[c]	Kester and Fennema (1989)
Microcrystalline	0.03[f]	2.2[c]	Kester and Fennema (1989)
Acetylated monoglycerides	20.04 – 53.7[f]	-	Lovegren and Feuge (1954)

Coll: Collagen, HPMC: Hydropropionate methylcellulose, MC: Methylcellulose, WG: Wheat gluten, WPI: Whey protein isolate, SPI: Soy protein isolate

WVP: Temp. (°C), RH (%) a= 25°C, 50/100% RH, b= 21°C, 85/0% RH, c= 25°C, 0/79% RH, d= 37.8°C, 100/90% RH, e=30°C, 0/100% RH, f=25°C, 0/100% RH, g=38°C, 30/100% RH

OP: Temp. (°C), RH (%), a= 23°C, 0% RH, b = 25°C, 63% RH, c= 25°C, 0% RH, d=23-38°C, 0% RH, e= 23°C, 50% RH

At film compositions where glycerol- and sorbitol-plasticized WPI films have equal mechanical properties, sorbitol-plasticized films have lower oxygen permeability (OP) (McHugh and Krochta, 1994d, e). Inhibition of intermolecular disulfide bond formation with sodium dodecyl sulfate (SDS) increases WPI film solubility, extendibility and flexibility, while having little effect on WVP. Inhibition of sulfhydryl-disulfide interchange with N-ethyl maleimide reduces WPI film solubility and elongation, with little effect on other film mechanical properties or WVP. Reduction of disulfide bonds with cysteine has no effect on WVP of WPI films.

Tensile strength of WPI films is comparably higher than most other protein-based films; with the opposite true for elongation (Table 1.2). Heat curing is commonly used to cross-link synthetic polymers and improve mechanical and barrier properties (El-Hibri and Paul, 1985; Perkins, 1988). Miller et al. (1997) also showed that heat curing of glycerol-plasticized WPI films increases film strength, while decreasing film extendibility, flexibility and WVP. Exposing free sulfhydryl groups promotes intermolecular disulfide bond formation to produce insoluble films.

1.1.3.2. Corn zein

1.1.3.2.1. Composition and structure

Zein, the prolamin (soluble in 70% ethanol) fraction of corn gluten, comprises approximately 70% of corn gluten. This protein is soluble in aqueous ethanol and insoluble in water due to higher levels of nonpolar amino acids (i.e. leucine, proline, and

alanine) (Shewry and Miflin, 1985). Zein is also rich in glutamine, the amide derivative of glutamic acid, which promotes protein association by hydrogen bonding (Reiners et al., 1973; Wall and Paulis, 1978). Corn zein can be fractionated into three distinct classes: α-zein, β-zein, and γ-zein (Esen, 1987). α-Zein and β-zein constitute 75-85 and 10-15% of total zein and are comprised of polypeptides with molecular masses of 21,000-25,000 and 17,000-18,000, respectively. Finally, γ-zein is made up of a proline-rich polypeptide of 27,000 and constitutes 5-10% of total zein.

Zein is produced commercially by extracting corn gluten with 80-90% aqueous isopropyl alcohol containing 0.25% sodium hydroxide at 60-70°C (Reiner, 1973). The centrifugally clarified extract is chilled to precipitate the zein with additional extractions and precipitation used to increase purity.

1.1.3.2.2. Film Formation

Preparation of zein films generally involves casting alcohol solutions on inert, flat surfaces. Formed films are peeled after the solvent has evaporated (Gennadios and Weller, 1990). Films also have been prepared from acetone solutions (Yamada et al., 1995). Zein films are naturally brittle, and therefore, plasticizers such as oleic acid (Kanig and Goodman, 1962), glycerol (Mendoza, 1975; Aydt and Weller, 1988) and lactic acid (Wu, 1995) are needed to improve their flexibility. An alternative method to prepare zein films involves plasticization of zein by forming an emulsion with oleic acid followed by precipitation of the protein-lipid mixture to form a soft moldable resin (Lai et al., 1997). The plastic resin then can be stretched over rigid frames to obtain thin membranes that set into flexible films.

Films formed upon solvent evaporation contain hydrophobic, hydrogen and disulfide bonds and have been characterized as tough, glossy and scuff-resistant (Pomes, 1971). Cross-linking agents such as aldehydes will improve moisture barrier and tensile properties of zein films (Szyperski and Gibbons, 1963). Epoxy resins also increase tensile strength of zein films by crosslinking between epoxy groups and phenolic, and aliphatic hydroxyl protein groups (Howland, 1961; Howland and Reiners, 1962; Yamada et al., 1995).

The oxygen permeability of zein films is quite low when compared to synthetic films such as low density polyethylene (LDPE) and polyester film; however, oxygen permeability of zein film is greater than that of collagen (Hanlon, 1992; Krochta, 1997). Higher plasticizer levels will increase the oxygen permeability of zein films. Films prepared from zein have lower or similar WVP compared to methylcellulose, ethyl cellulose, hydroxypropylmethyl cellulose, and hydroxypropyl cellulose under similar conditions (Table 1.1).

Zein films produced using different solvents possess distinct properties. For example, Yamada et al. (1995) found that zein films prepared by dissolution in 30% acetone film showed lower WVP than when dissolved in 20% ethanol. They also reported that film strength increased linearly as film thickness increased except for films prepared from the aqueous acetone solution with 1, 2- Epoxy-3-chloropropane. At similar plasticizer levels, zein film appears to have tensile strength (TS) and percent elongation (% E) similar to collagen film (Table 1.2).

The concentration of plasticizer greatly affects the mechanical and water vapor barrier properties of zein protein films (Aydt et al., 1991; Butler and Vergano, 1994).

Table 1.2. Tensile strength (TS) and percent elongation (% E) of edible films (23°C, 50% RH).

Film	TS (Mpa)	% E	References
Coll:Sor:Gly (3.4:0.8:1)	8.1	25	Hood (1987)
Coll:Sor:Gly(8.8:0.8:1)	9.1	38	Hood (1987)
Corn Zein:Gly (1:0.5)	2.7-15.7	43-198	Gennadios et al. (1993), Aydt et al. (1991)
WG:Gly (2.5:1)	4.4	194.7	Gennadios et al.(1993)
SPI:Gly (2:1)	4.3	78	Brandenburg et al. (1993)
WPI:Gly (5.7:1)	29.1	4.1	Krochta (1994)
WPI:Gly (2.3:1)	13.9	30.8	Krochta (1994)
WPI:Gly (1.5:1)	18.2	5.0	Krochta (1994)
Casein: Gly (1.4:1)	4.5	223	Chick and Ustunol (1998)
Alginate:Gly (2:0.7)	2.5	7.9	Parris et al. (1995)
Pectin: Gly (3:0.5)	2.3	5.0	Parris et al. (1995)
Chitosan	6.3 – 31.8	14-70	Caner et al. (1998)
MC	12.5	20	Debeaufort and Voilley (1997)
Starch:ethylene-acrylic acid (2:0.8)	23.9	260	Otey et al. (1977)
Pectin:Starch: Gly (1:1:0.5)	27-34	1.8-13	Coffin and Fishman (1994)

Coll: Collagen, Gly: Glycerin, MC: Methylcellulose, WG: Wheat gluten, WPI: Whey protein isolate, SPI: Soy protein isolate, Sor: Sorbitol

When Park et al. (1994) evaluated the impact of two plasticizers, glycerin (0.36 ml/g protein) and polyethylene glycol (PEG) (0.39 ml/g protein), on the properties of zein film, % E of corn zein films containing glycerin or PEG was 3 and 94%, respectively. TS of zein films reportedly increased from 13.4 to 25.8 MPa during 20 days of storage; however, percent elongation decreased from 76 to 12%. WVP of these zein films also decreased from 0.59 to 0.41 ng.m/m^2.s Pa as the ratio of glycerin to PEG decreased.

1.1.3.3. Casein

1.1.3.3.1. Composition and Structure

Casein, the major protein in milk, represents 80% of total milk protein (Dalgleish, 1989). Casein consists of three principal components, α, β, and κ-casein, and a minor component, γ-casein, which together form colloidal micelles in milk containing large numbers of casein molecules that are stabilized by calcium-phosphate bridging (Kinsella, 1984). Caseins are phosphoproteins that precipitate at pH 4.6 and 20°C.

The α-caseins have an approximate molecular weight of 23,500 and an isoelectric point of pH 5.1 (Xiong, 1997). They are more phosphorylated than other caseins, more calcium sensitive than β-casein and contain more charged residues and fever hydrophobic residues than β- casein (Dalgleish, 1989).

β-casein, which comprises up 30-35% of the total casein content in milk, has a molecular weight of 24,000 and an isoelectric point of pH 5.3 (Xiong, 1997). The last 50 residues of the terminus are charged, whereas the rest of the molecule is highly hydrophobic (Dalgleish, 1989). β-casein shows temperature, concentration, and pH

dependent association-dissociation. At neutral pH and high temperatures, β-casein associates into threadlike polymers (Dalghleish, 1989).

κ-casein, which comprises approximately 15% of the total casein fraction, has a molecular weight of 19,000 and an isoelectric point of pH 3.7 to 4.2 (Xiong, 1997). Being calcium sensitive, κ-casein associates with α_1- and β-caseins in the presence of calcium to form thermodynamically stable micelles. Apolar residues in κ-casein are concentrated at the N-terminus with the charged portion being near the C-terminus. The hydrophobicity of κ-casein is between that of α- and β-caseins (Dalgleish, 1989).

The negatively charged κ-casein is cleaved by the enzyme rennin used to coagulate milk in cheese production. When κ- casein is cleaved, the micelle structure is stabilized and the casein precipitates.

Casein contains low levels of cysteine, and thus few disulfide cross-linkages which produces an open random structure. Caseinates function as good emulsifiers and foaming agents because of even hydrophilic and hydrophobic amino acid distribution.

1.1.3.3.2. Film Formation

Casein films are formed from aqueous solutions of casein. No further treatment is necessary because of their random coil structure and propensity for extensive hydrogen bonding. Hydrophobic, ionic, and hydrogen bonding involve interactions in the film matrix with the surfactant nature of casein making it very suitable for producing emulsion films that are transparent, flavorless and flexible.

1.1.3.3.2. Film properties

The inherently poor water barrier properties of caseinate films can be improved by treatment with lactic or tannic acid (Guilbert, 1986). Films can also be prepared from sodium or calcium caseinate without addition of plasticizer. Addition of plasticizers (glycerol or sorbitol) to casein film solutions at ratios of 0.6:1, 1:1, and 1.4:1 decreased tensile strength, but increased percent elongation, with glycerol being more effective than sorbitol (Chick and Ustunol, 1998). Calcium caseinate films have lower WVP compared to sodium caseinate films. However, when treated with calcium chloride sodium caseinate films have even lower WVP, likely because of more effective calcium cross-linking (Avena-Bustillos and Krochta, 1993). Incorporating various lipids, waxes, and fatty acids into the caseinate film solution also has been examined for reduction of water vapor permeability, with beeswax being more effective than paraffin or carnauba (Krochta et al., 1990; Ho, 1992). Caseinate film formation at the isoelectric point of casein insolubilizes the film and also reduces water vapor permeability by about one-half compared to films at higher pH levels (Krochta et al., 1988). Overall, caseinate films have moisture barriers that are similar to wheat gluten and soy protein films and somewhat greater moisture barriers than corn zein films (Table 1.1).

Additional modification techniques, including UV light and γ-irradiation, also can be used to improve the properties of casein films. The formation of bityrosine with γ-irradiation (16-64 kGy) or UV light reportedly improved tensile strength and WVP properties of calcium caseinate film (Brault et al., 1997; Ressounany et al., 1998; Mezhgheni et al., 1998). Furthermore, addition of $CaCl_2$ increased the fluorescence signal

of bityrosine by shortening molecular distances between polypeptides, thus aiding the formation of bityrosine.

1.1.3.4. Wheat Gluten

1.1.3.4.1. Composition and Structure

Wheat kernels contain 8-15% protein based on dry weight (Kasarda et al., 1976). Gliadin and glutenin are the main wheat endosperm storage proteins, comprising 85% of wheat flour protein, the remaining 15% consisting of various albumins, globulins and smaller peptide structures (Holme, 1966). While gliadin is soluble in 70% ethanol, glutenin is not. By hydration gliadin and glutenin form a colloidal complex known as gluten (Pomeranz, 1988). These properties are used to produce films from gluten protein.

The amino acid composition of wheat gluten is characterized by high levels of glutamic acid and low levels of lysine and other basic amino acids (Kasarda et al., 1976; Lasztity, 1986). The amide group of glutamine promotes hydrogen bonding between gluten chains. The relatively high amounts of nonpolar amino acids, such as proline and leucine cause wheat gluten insolubility in water at neutral pH (Krull and Inglet, 1971). Both gliadin and glutenin contain disulfate bonds. Disulfate bonds link glutenin chains together to produce polymers of high molecular weight. The gliadin-glutenin complex involves both covalent and noncavolent bonding in the film matrix.

1.1.3.4.2. Film Formation

Wheat gluten (WG) films are produced by deposition and drying of wheat gluten dispersions. Aqueous ethanol is the most common solvent used in film-forming solution.

Mechanical mixing and heating under acidic or alkaline conditions are used to form homogeneous gluten dispersions (Gontard et al., 1992; Gennadios et al., 1993a). Covalent disulfide bonds are also important in wheat gluten film formation. Disulfide bonds in gluten are reduced to sulfhydryl groups in wheat gluten dispersed under alkaline conditions (Okamoto, 1978). During casting of film-forming solutions, disulfide bonds are reformed by reoxidation in air and sulfhydryl-disulfide interchange reactions. Reformed disulfide bonds link polypeptide chains, to produce the film structure. Hlynka, (1949), Beckwith et al. (1965), and Wall et al. (1968) describe the reduction and reoxidation of disulfide bonds while Steward and Mauritzen (1966) and McDermott et al. (1969) describe sulfhydryl-disulfide interchange reactions in wheat gluten.

1.1.3.4.3. Film Properties

Mechanical and barrier properties of cast WG films have been reported by several investigators (Krull and Inglett, 1971; Aydt et al., 1991; Gontard et al., 1992; Gennadios et al., 1993b; Park et al., 1994; Rayas and Ng, 1997; Rayas et al., 1998). Extensive intermolecular interactions in WG result in quite brittle films, which require plasticizers. Plasticizer molecules mediate between polypeptide chains, which decreases the rigidity of the film structure (Wall and Beckwith, 1969). However, increasing film flexibility by raising the plasticizer content (glycerin) reduces film strength and WVP (Gontard et al., 1993).

Oxygen permeability of wheat gluten is similar to commercial nylon (Rayas et al. 1998). Unlike WVP, at comparable plasticizer content and test conditions, the oxygen permeability of gluten films prepared from alkaline solutions is an order of magnitude

lower than oxygen permeability of zein films. The effects of subjecting wheat gluten films to several treatments, including lactic acid, calcium chloride have been investigated by Gennadios et al. (1993c). Soaking the film in calcium chloride and in buffer solution at the isoelectric point of wheat gluten reportedly increased tensile strength (47 and 9%, respectively) and decreased WVP (14 and 13%, respectively).

Effects of replacing a portion of the wheat gluten with keratin, soy protein, corn zein, or soy protein with cysteine on film properties was also studied (Gennadios et al. 1993a, b; Were et al., 1999). The addition of hydrolyzed keratin into a wheat-gluten based film solution reportedly lowered oxygen permeability by 83%, WVP by 23%, and TS by 35%, but increased % E by 32%. Replacing 30% of the wheat gluten with SPI decreased oxygen permeability, % E, and WVP by 40, 9, and 16%, respectively, while increasing TS by 69%. Corn zein addition had no effect on OP, but decreased WVP and % E by 23 and 37%, while increasing TS by 58%. Adding cysteine (1%) to WG: SPI (4:1) increased the disulfate content of the film forming solution and thus increased TS, but had no affect on WVP and oxygen permeability (Were et al., 1999).

Properties of wheat gluten films are affected by the pH of the film-forming solution (Gontard et al., 1992; Gennadios et al., 1993a). Gontard et al. (1992) showed that wheat gluten films produced at acidic conditions had significantly lower WVP than wheat gluten films produced at alkaline conditions (Table 1.1). However, Gennadios et al. (1993a) reported that films prepared under alkaline conditions had greater TS than films prepared under acidic conditions with %E and WVP not affected by the pH difference.

1.1.3.5. Soy Protein

1.1.3.5.1. Composition and Structure

Soybeans contain 38-44% protein, most of which is insoluble in water but soluble in dilute neutral salt solutions. Soy protein is a globular protein and is separable into four different fractions: 2S, 7S, 11S and 15S (Wolf and Smith, 1961) with the 7S and 11S fractions comprising about 37% and 31% of the total protein, respectively (Wolf et al., 1962). Soy protein is rich in asparagine and glutamine residues. Both 7S and 11S fractions are tightly folded proteins, with the former containing two or three cystine groups and the later containing 20 intermolecular disulfate bonds (Gennadios et al., 1993c).

Protein in the form of meal is one of the typical end products from industrial soybean processing. Protein meal can be further concentrated for the production of soy protein concentrates and soy protein isolates, which contain at least 70% and 90% protein on a dry basis, respectively.

1.1.3.5.2. Film Formation

Edible films from soybeans have been traditionally produced in the Orient on the surface of heated soymilk. In these films, lipids and carbohydrates are incorporated with protein. Films obtained from soymilk are known as "yuba" in Japan, "tou-fu-pi" in China, "kong kook" in Korea, and "fu chock" in Malaysia (Snyder and Kwon, 1987).

The mechanism for forming these films involves polymerization of heat-denatured protein followed by surface dehydration of the soymilk emulsion (Farnum et al. 1976). Soy protein films contain a protein matrix formed by heat-catalyzed protein-

protein interactions that result in disulfate, hydrogen and hydrophobic bonds. Fukushima and Van Buren (1970) showed that the mechanism of polymerization involves intermolecular disulfate and hydrophobic bonds. Film formation is favored in more alkaline soymilk solutions.

Soymilk used in making these films is prepared from soybeans. In one soymilk manufacturing procedure, (Wu and Bates, 1972) soybeans that were presoaked in water for 1 hour at 65°C were by drained, ground with water, passed through a screw expeller and finally followed by a clarification to produce soymilk with a pH of about 6.7.

Guilbert (1986, 1988) produced films by casting aqueous solutions containing 10% soy protein isolate and 5% glycerin as a plasticizer. Films were characterized as flexible, smooth and transparent. Production of films and coatings from soymilk and soy protein has been reviewed by Gennadios and Weller (1991). Heating and alkaline conditions favor soy protein polymerization by unfolding the protein secondary structure and exposing sulfhydryl and hydrophobic groups. Sian and Ishak (1990) and Gennadios et al. (1993) reported that film formation was possible only within pH 1-2 and pH 6-12. The optimum pH range for producing soymilk films is between 7.0 and 8.0, with darkening of the film occurring above pH 8.0 (Wu and Bates, 1972). Brandenberg et al. (1993) produced film by heating alkaline solutions of soy protein isolate (5%) and glycerin (3%) at 60°C for 10 min. These films had a smoother appearance and fewer insoluble particles, as evidenced by scanning electron microscopy. Heat treatment of SPI film-forming solutions at 85°C promoted intermolecular cross-linking and produced SPI films that were smoother and more transparent, and possessed lower WVP and increased % E, compared to those produced from unheated solutions (Stuchell and Krochta, 1994).

The moisture barrier properties of SPI films are similar to wheat gluten films prepared from alkaline solutions but somewhat inferior to corn zein films (Table 1.1). SPI films having similar TS to zein and alkaline wheat gluten films have lower E (Table 1.2). The oxygen permeability of SPI films has been measured, but only at 0% (Brandenburg et al., 1993, Gennadios et al., 1994). Like wheat gluten films, the oxygen permeability of SPI films appears to be an order of magnitude lower than the oxygen permeability of zein films at similar plasticizer levels (Table 1.2).

Soy protein films are transparent and flexible when plasticizer is added, but have poor water barrier properties (Guilbert, 1986). When the plasticizer content was increased from 20 to 40% the % E of soy protein films increased from 1.5 to 106%; however, tensile strength decreased from 15.8 to 2.6 MPa (Cunningham et al., 2000).

Ionizing radiation, such as γ-irradiation, cross-links soy protein films, thus improving their functional properties. Exposing soy protein film to a γ-irradiation dosage of 5-30 kGg in the presence of poly(ethylene oxide) or poly(vinyl alcohol) increased tensile strength (Grorpade et al., 1995; Sabato et al., 2001). Gennadios et al.(1998) also reported that UV irradiation (103.7 J/m^2) improved tensile strength of soy protein films from 3.7 to 6.1 MPa; however, percent elongation decreased from 124 to 85% with increasing UV irradiation. Modification of SPI film properties by heat curing at 80 and 95°C was also studied by Gennadios et al. (1994), with this treatment reportedly reducing WVP, % E, moisture content, and water solubility and increasing TS. Changes in film properties were attributed to heat-induced cross-linking and lower moisture content within the film, with the greatest effect observed at 95°C.

Cross-linking interactions of calcium chloride or calcium sulfate with SPI can also improve tensile strength (Park et al., 2001). In the same study, glucono-delta-lactone (GDL) incorporation into soy protein increased elongation. In addition, both calcium salts and GDL-treated SPI films had lower WVP than the SPI control film.

1.1.3.6. Collagen

1.1.3.6.1. Composition and Structure

Collagen is a fibrous protein generally isolated from hides, tendon, cartilage, bone and connective tissue (Ballian and Bowes, 1977). This protein is arranged in fibrils which are packed together to form bundles of parallel fibers having cross-sectional diameters of 20-40 microns (Ballian and Bowes, 1977). Collagen is unique in that every third amino acid residue throughout most of the structure is glycine, while proline and hydroxyproline account for about one-fourth of the residues (Harrington and Von Hippel, 1961, Ramachanran and Ramakrishnan, 1976). Collagen fibers swell with immersion in alkali, acid or neutral salt solutions. They are resistant to proteolytic enzymes but are easy targets for collagenase. When heated, collagen fibers shrink and are converted to gelatin (Harrington, 1966).

1.1.3.6.2. Film Formation

Collagen films can be produced from animal hides using a dry or wet process. The dry process involves alkaline treatment of hide corium and acidification to pH 3.0 (Hood, 1987) followed by shredding of acid-swollen coriums to preserve maximum fiber structure, mixing of acid-swollen fibers to produce a high-solids dough (>12%), addition

of plasticizing and cross-linking agents, high pressure pumping and extrusion of dough to form tubular casings, drying, conditioning, neutralizing and/or providing additional cross-linking. The wet process consists of acid or alkaline dehairing of hides, deacidification of hide coriums, grinding mixing of ground collagenous material with acid to produce a swollen slurry (4-5% solids), slurry homogenization, extrusion into tubular casings, washing to remove salts, treatment with plasticizing and cross-linking agents and drying.

The tensile strength of collagen is less than that of methyl cellulose, corn zein, WPI, and chitosan-based films (Table 1.2). Several techniques have been developed to improve the properties of collagen films and casings. The strength of collagen casings can be improved by drying to a moisture content of 15 to 40% in the presence of a polyhydric alcohol, such as poylethylene glycol, or a salt, preferably sodium chloride (Kidney, 1970). Treatment with glyceraldehyde was found to promote cross-linking which increased the strength and thermal resistance of collagen casings (Jones and Whitmore, 1972). Alkyl diols also improved the mechanical properties of collagen casings by reducing internal hydrogen bonding while increasing intermolecular spacing (Lieberman and Gilbert, 1973; Boni, 1988). Furthermore, exposing edible tubular collagen casings to ultraviolet irradiation (180-420 nm) increased casing strength (Miller and Marder, 1998).

Exposure to proteolytic enzymes such as papain, bromelain, ficin, trypsin, chymotrypsin, pepsin and fungal proteose can be used to partially stabilize collagen, which will improve uniformity in diameter and wall thickness of collagen casings (Fujii, 1967; Tsuzuki and Lieberman, 1972; Miller, 1983).

Water vapor pemeability of collagen films can also be altered. Lieberman and Gilbert (1973) found that formaldehyde cross-linking and chrome tanning significantly reduced the gas permeability of collagen films. Formaldehyde reacted with collagen by combining with free amino groups of basic amino acids, whereas chrome tanning promoted reactions between carboxyl groups of amino acids.

1.1.3.7. Gelatin Films

1.1.3.7.1. Composition and Structure

Gelatin, a protein produced from partial hydrolysis of collagen, ranges in molecular weight from 3000 up to 200,000 depending on production methods. Gelatin lacks internal order with the polypeptides configured randomly in aqueous solutions (Xiong, 1997). The process for hydrolysis of collagen to gelatin includes thermal denaturation (40°C), which cleaves hydrogen bonds and electrostatic bonds, and hydrolytic breakdown of covalent bonds (Eastoe and Leach, 1977). Thermally reversible gels are produced by forming cross-links between amino and carboxyl components of amino acid residual side groups (Glicksman, 1982).

Raw materials commercially used for gelatin manufacture include pork skin as well as bovine hide, skin, and bones. A pretreatment or curing step is applied before gelatin extraction of the raw material. Curing removes impurities and initiates collagen hydrolysis. Two different curing methods, acid or alkali, produce Types A and B gelatin, respectively. Type A gelatin with an isoelectric point between pH 7 and 9 is mostly made from pork skin, while Type B gelatin with an isoelectric point between pH 4.6 and 5.2 is made from bones and bovine hides (Xiong, 1997).

2.1.3.7.2. Film Formation

Gelatin films are prepared by casting aqueous film-forming solutions of 20% gelatin and 0-10% glycerin (Guilbert, 1986, 1988). Such films are clear, flexible, strong and oxygen impermeable when the aqueous solution contains plasticizer such as glycerol or sorbitol.

Gelatin gels dry to produce films with poor moisture barrier and tensile strength properties. Treatment with lactic or tannic acid improves water-barrier properties although these films are less flexible and less transparent (Guilbert, 1986). Tensile strength of gelatin films can be improved by drying 5% solutions at 20°C rather than 60°C, due to a higher degree of crystallization in films dried at the lower temperature (Bradbury and Martin 1952). In addition, gelatin also reportedly has a configuration that promotes interchain hydrogen bonds at temperatures less than 35°C (Robinson, 1953).

1.1.4. Polysaccharide-based edible films

Several carbohydrate-based edible films were developed and tested. This review will address some common carbohydrate films including cellulose-, chitosan-, alginate-, starch-, pectin-, and dextrin-based edible films.

1.1.4.1. Cellulose Films

Cellulose, composed of linear chains of β-D-glucopyranosyl units joined by glycosidic bonds (1 → 4), is the most abundant organic compound on earth because it is the principal component of plant cells. Native cellulose is insoluble in water because of the high level of intermolecular hydrogen bonding, but can be converted into cold water-

soluble gums by esterification. In this process, cellulose is reacted with aqueous caustic, then with methyl chloride, propylene oxide or sodium monochloroacetate to yield methylcellulose (MC), hydroxypropyl methylcellulose (HPMC) and sodium carboxymetylcellulose (CMC), respectively. MC, HPMC and hydroxypropyl cellulose (HPC) are available commercially in powder or granular form in varying molecular weight. Their relative hydrophilities increase in the order of HPC<MC<HPMC<CMC (Krumel and Lindsay, 1976).

MC, HPMC and HPC are water-soluble ethers with good film-forming properties. Dissolution of these compounds is a two-step process involving dispersion and hydration. The powder must be added slowly to agitated water to separate and sufficiently wet their surfaces. Another procedure is by dispersing the cellulose ethers in a water miscible nonsolvent such as glycerin, ethanol or propylene glycol and then adding the slurry to water.

Films prepared from MC, HPMC and HPC are tough, flexible and transparent with linear structure (Krumel and Lindsay 1976). The first cellulose film, cellephane, was developed by Brandenberg in 1908 (Paist, 1958), and some modified cellulose films have been used as edible films and coating since the 1980s (Kester and Fennema, 1986). The addition of plasticizers (polyethylene glycol 400, glycerin, propylene glycol) necessary to improve elasticity of cellulose films (Torres, 1994) does not affect the visual appearance at concentrations lower than 30% (Donhowe and Fennema, 1993; Park et al., 1993; Debeaufort and Voilley, 1995a). However, addition of plasticizers during preparation increases oxygen, water vapor and aroma transfer and sorption within the films and decreases TS (Debeaufrt and Voiley, 1995).

Films based on MC, HPMC and HPC are soluble in water and insoluble in lipids, and thus have high water vapor permeability (Table 1.1). They are very good oxygen and hydrocarbon barriers, and their water vapor properties can be improved by adding lipids or fatty acids (Biquet and Labuza, 1988; Kamper and Fenema, 1984a, b; Park and Chinnan, 1990; Ayranci and Tunc, 2001). Donhowe and Fennema (1992) showed that MC and CMC prepared with beeswax or acetylated monoglycerides exhibited increased WVP with increasing RH from 0 to 80% due to hydration and swelling across the entire film thickness, thus facilitating water movement through the film. The addition of fatty acids also was shown to improve water vapor barrier properties. For example, Ayranci and Tunc (2001) reported that incorporation of stearic, palmitic or lauric acids (5-40 g/100 g MC) in MC-based edible films decreased WVP.

The ability of hydrophobic substances to retard moisture transfer through cellulose-based films depends on the homogeneity of their final re-partition in the matrix and/or on the surface. Polo et al. (1992) studied the effect of paraffin oil and paraffin wax on the moisture barrier properties of three cellulose derivative-based films with different polarity and porosity prepared using several techniques: emulsion (MC), emulsion plus coating (MC), and dipping (porous filter paper and nonporous cellophane). Cellophane films prepared with paraffin oil were more permeable than those prepared with wax. Films prepared by emulsion and dipping had highly heterogeneous systems with the least effect on movement of water. The most efficient system, in terms of water vapor permeability, was obtained using cellophane films with homogeneous re-partition of paraffin wax independent of thickness and RH.

1.1.4.2. Alginate Films

Commercial algin is a sodium salt of alginic acid, a linear polyuronic acid obtained from brown seaweed. Alginic acid contains two monomeric units, β-D-mannopyranosyluronic acid and α-L-gulopyranosyluronic acid. Segments containing only β-D-mannopyranosyluronic units are called M blocks whereas those containing only α-L-gulopyranosyluronic units are termed G blocks (Whistler and Daniel, 1990; Cottrell and Kovacs, 1980; McDowell, 1973). These blocks determine the properties of the polymer. For example, algins containing many G-block units produce high strength, more brittle and less elastic film. Solutions of sodium alginates are highly viscous. The calcium alginates are insoluble.

Alginates react with several cations including calcium and sodium to form gels Calcium ions, the most effective gelling agent (Allen et al., 1963), pull alginate chains together by ion interactions and interchain hydrogen bonding to produce a three-dimensional gel network (Grant et al., 1973; King, 1983). Calcium-induced gelation of alginate is irreversible unless treated with strong chelating agents at an alkaline pH (Klock et al., 1994).

1.1.4.2.1. Film Formation

Alginates possess good film-forming properties, with glycerol often added as a plasticizer to reduce brittleness (Glicksman, 1984). Films and coatings made from sodium alginate solutions by rapid reaction with calcium in the cold contain intermolecular associations in G-block regions. Film strength and permeability can be altered by the concentration of calcium, the rate of its addition, pH and temperature

(Kester and Fennema, 1986). Homogenous release of calcium ions within an alginate solution is necessary for uniform gelation. Alteration in pH, temperature and employment of calcium ions with low solubility can be used to control release of calcium ions in the alginate suspension (Deasy, 1984). Increasing the acidity of the gel solution with the hydrolysis of glucono-δ-lactone will slow the calcium ion dissociation rate from insoluble calcium salt. Alginate films prepared under these conditions will be homogenous, transparent and smooth (Draget et al., 1989) with the pH of the alginate solution slowly decreasing from 5.6 to 3.8 in 6 h.

Alternatively, soluble calcium salt can be released into an alginate solution at high temperature with gelation occurring upon cooling (Sime, 1990; Papageorgiou, 1994, Dominic et al., 1996). Such films are homogenous, but opaque with a rough uneven surface. Film properties depend on the gel cooling rate. Gelation occurs too rapidly (2 h) to allow the alginate chains to align for proper cross-linking. Such films have a weak gel structure because of irregular distribution of calcium crosslinks throughout the film matrix (Dominic et al., 1996).

Many calcium salts can be used to form alginate gels including chloride, acetate, lactate, tartarate, gluconate, sulfate, citrate and tri-calcium phosphate (Kester and Fennema, 1986). Calcium chloride yields the strongest gel coatings (Allen et al., 1963).

Films made by casting an aqueous solution of alginic acid are flexible and transparent, but water-soluble. Immersing alginate gels in aqueous solutions of multivalent cations is an alternate method to improve film strength (Kaletunc et al., 1990). Immersing the film in solutions containing 5 or 10% calcium and zinc for 15

minutes produced water insoluble films with tensile strength increasing from 5.6 to 31.0 and 51.67 MPa, respectively (Pavlath e al., 1999).

2.1.4.3. Pectin Films

Pectin, a complex group of structural polysaccharides, is found in plant cells (Aspinall, 1970). Pectins are composed of D-galacturonic acid with various degrees of methyl esterification. Low-methoxyl pectins dissolved in aqueous media are capable of forming gels in the presence of calcium ions (Miers et al., 1953) which react with free carboxyl groups on adjacent polymer molecules. Hydrogen bonding also strengthens this association.

Low methoxyl pectin films were first developed and studied in the 1940's (Coffin and Fishman, 1994) with calcium or other cations used as cross-linking agents. These films exhibited good mechanical properties, but had poor folding durability. When Schultz et al. (1949) evaluated dried low-methoxyl pectinate films, the water vapor permeability rate was 300 g H_2O mil/m^2.day.mmHg at 25°C with 50% relative humidity.

Incorporating high amylose pectin into high methoxyl pectin films modified the mechanical properties of the pectin films (Coffin and Fishman, 1993, 1994). Films made from high methoxyl lime pectin and high starch had very good mechanical properties. Adding a high proportion of high amylose starch resulted in only a modest decrease in dynamic mechanical properties (storage modulus and loss modulus) of the films, and had a beneficial effect on surface properties.

A two-step technique was used to apply pectinate gel coatings directly to food surfaces. This procedure is the same as that used in development of alginate coatings. An

aqueous pectin solution was applied to the surface, followed by treatment with a calcium solution to promote gelation.

1.1.4.4. Chitosan

Chitosan is derived from chitin, the second most abundant polysaccharide after cellulose (Lezica and Quesada-Allue, 1990). The polysaccharide chitin, which consists of repeating units of 1,4 linked 2-deoxy-2-acetamido-β-D-glucose, occurs naturally in the crystalline state (Minke and Blackwell, 1978). In chitin, the amino group is acetylated, thus chitin is an amide of acetic acid. Chitosan containing a free diacetylated amino group is soluble in aqueous acidic solutions (Muzzarelli, 1977; Rinaudo and Domard, 1989). Such acidic solutions containing positively charged chitosan are used in agriculture as carriers for pesticides (Anon, 1987), in the food industry as adsorbents for waste and water treatment (Knorr, 1984), and in the area of nutrition as lipid binders (Furda, 1990).

1.1.4.4.1. Film Formation

Chitosan films can be prepared by dissolving 1% chitosan with glycerol (1 or 2.5%) in acidified aqueous solutions (1% acetic or formic acid) at room temperature (Wong et al., 1992; Hoagland, 1996; Caner et al., 1998). After casting in petri dishes, the solution was dried to produce freestanding chitosan films. Films prepared using HCl or HNO_3 solution are opaque and shrink due to chitosan crystallization. Adding of pectin or starch to the film solution will produce clear and strong chitosan HCl or HNO_3 films that

are stable for at least 6 months (Hoagland, 1996). Films made from completely diacetylated chitosan HCl will not shrink and are opaque (Samuels, 1981)

Chitosan, like many carbohydrate polymer films, tends to be a good oxygen and carbon dioxide barrier but lacks resistance to water transmission. Incorporation of fatty acids or lipids such as lauric or butyric acid provides hydrophobicity and decreased water permeability (Dominic et al., 1992). Chitosan films are unsuitable foundation films because of significant swelling. Chen et al. (1996) reported that various chitosan films could absorb 40 to 80% of their weight in water.

Use of different acids (acetic, formic, lactic, propionic) in chitosan film formulations distinctly affects the film properties. Caner et al. (1998) showed that acetic acid produced the lowest WVP, followed by propionic, formic and lactic acid whereas lowest OP was observed when lactic acid was used. Films formed with lactic acid and formic acid solutions had uniquely high values for elongation at break and tensile strength, respectively. Jong-Whan et al. (1998) also evaluated the effects of acetic, citric, formic, lactic, malic, propionic, succinic, tartaric, phosphoric and hydrochloric acids on properties of chitosan films. Films made with organic acids such as formic, acetic, malic and propionic acids were more resistant to solubilization in water than those made with citric, lactic, succinic and tartaric acids.

1.1.4.5. Starch Films

Starch consists of approximately 25% amylose and 75% amylopectin. Amylose is a linear chain of D-glucose residues linked by 1,4 glycosidic bonds. Amylopectin is a branched molecule consisting of glucose units connected by 1,4 and 1,6 linkages.

Starches can be derived from tubers (potato, tapioca, arrowroot and sweet potato), stems (sago), and cereals (corn, waxy maize, wheat and rice). Although starch is insoluble in cold water due to hydrogen bonding of polymer chains, heating will break these hydrogen bonds allowing the starch granules to absorb water and swell.

Amylose is required for film forming and preparation of strong gels. Starches containing 55 or 70% amylose content are commercially available, with 70% amylose starch producing stronger, tougher and more flexible films (Jokay et al., 1967). Wolff et al. (1951) were first to produce self-supporting films by casting aqueous solutions of gelatinized amylose, followed by solvent evaporation. Such starch films are transparent and have very low permeability to oxygen at low RH (Rankin et al., 1958, Coffin and Fishman, 1993, 1994).

Addition of plasticizers and absorption of water molecules by hydrophilic polymers can increase polymer chain mobility, which generally leads to increased gas permeability (Banker, 1966; Gaudin et al., 1999). Starch and plasticizers such as glycerol contain many polar chemical functions, mainly hydroxyl groups. These characteristics can provide susceptibility to antiplasticization materials (Jackson and Caldwell, 1967). Glycerol and water play the role of internal lubricants, preventing the rigidification of non-crystallized macromolecular starch chains at ambient temperature (Shogren, 1993). Dried raisins coated with an aqueous solution of corn amylose plasticizer poured freely, and did not clump or stick together (Moore and Robinson, 1968).

Garcia et al (2000a, b) developed starch-based edible films from cold gelatinized cornstarch and high-amylose cornstarch (amylomaize) suspensions in NaOH. Glycerol or sorbitol used as a plasticizer decreased the crystalline/amorphous ratio and permeability

to CO_2, O_2 and water vapor, with films containing sorbitol showing lower permeability than those containing glycerol. They also showed that amylomaize films with a higher crystalline/amorphous ratio were less permeable to CO_2, O_2, or water vapor than the corresponding cornstarch films. Addition of 2 g/L sunflower oil to the film formulation reportedly decreases both the WVP and the amorphous/crystalline ratio (Garcia et al. 2000b).

1.1.4.6. Dextrins

Dextrin-starch hydrolysates of low dextrose equivalent also have been suggested for use as protective coatings. Allen et al. (1963) tested the barrier properties of these edible film materials as coatings using a cellulase acetate support. Water vapor transport through starch films was very high, while films comprised of low dextrose equivalent dextrin and corn syrup had WVP values that were 2- and 3-fold lower, respectively.

Dextrins are often used as film-formers and edible adhesives to replace natural gums. Dextrin-based coatings exhibit some resistance to oxygen transmission as has been shown by the reduction in browning of sliced apples (Murray and Luft, 1973). A gluten-dextrin coating was also used to coat dry roasted peanuts before application of salt (Noznick and Bundus, 1967).

1.1.5. Lipid Films

Lipid compounds include neutral lipids of glycerides which are esters of glycerol and fatty acids, and waxes which are esters of long-chain monohydric alcohols and fatty acids. From this group, acetylated monocglycerides, natural waxes, and surfactants are commonly used in edible films and coatings (Kester and Fennema, 1986).

1.1.5.1.Waxes

Solidification of the melted wax or lipid by cooling is a common technique for preparing lipid films. Wax films are also formed by casting molten wax on a dried film of methylcellulose and then dissolving away the methylcellulose film (Donhowe and Fennema, 1993). The rate of cooling plays an important role in the predominant polymorphic state, as well as degree of recrystallization in the solidified film. Several waxes have been studied for film or coating preparation: paraffin, carnauba, beeswax, candellila and polyethylene wax.

Paraffin wax is derived from the distillate fraction of crude petroleum. It contains hydrocarbon fractions ranging from eighteen to thirty-two units (Bennett, 1975). Paraffin waxes are permitted for use as protective coatings for raw fruit and vegetables and for cheeses. They can also be used in chewing gum as a defoamer and as a component for microencapsulation of flavors.

Carnauba wax is extruded from palm tree leaves of the Tree of Life. Refined carnauba wax, composed of saturated acid esters twenty-four hydrocarbons in length and saturated long chain alcohols (Bennett, 1975), has a high melting point and is often added to other waxes to increase hardness.

The surface appearance of carnuaba wax films is somewhat "hilly" with small pores disrupting the otherwise smooth contours (Donhowe and Fennema, 1993). Carnauba wax, which has low oxygen permeability probably because of its hardnesses, is permitted for use as a coating for fresh fruits and vegetables and in both chewing gums and sauces.

Beeswax is secreted by honeybees for comb building. Beeswax obtained by melting comb wax, contains 71% long-chain fatty acid esters and alcohols, 15% long-chain hydrocarbons and 8% free fatty acids (Tulloch, 1970). This wax is soft at room temperature with a melting point of 61-65°C, but becomes brittle at colder temperatures. Beeswax is partially crystalline in nature. Its crystal size is quite small, allowing for tight packing of individual crystallites. This type of crystalline morphology accounts for beeswax being a particularly effective barrier to water vapor transmission (Kester and Fennema, 1989). However, beeswax has higher water vapor permeability than candellila, carnauba, and microcrystalline wax (Donhowe and Fennema, 1993) due to higher concentrations of fatty acids, fatty alcohols and esters. Beeswax also contains low levels of unsaturated hydrocarbons which are responsible for its flexibility (Dafler, 1977) and high oxygen permeability. Unlike carnauba wax, the microscopic surface of beeswax appears smooth and devoid of distinct morphological features (Greener and Fennema, 1989; Kester and Fennema, 1989; Donhowe and Fennema, 1993).

Candelilla wax is extracted from the candelilla plant, a reed-like plant that grows in Mexico and southern Texas. Candellila wax contains 57% hydrocarbons and 29% wax esters, with the remainder consisting mainly of fatty alcohols and fatty acids (Tolluch, 1970). Its melting point is 65-68.8°C.

Polyethylene wax is the oxidation product of polyethylene, a petroleum by-product. Polyethylene waxes are available in several grades to provide desired emulsion properties. These waxes which differ in molecular weight, density, and melting point (Eastman Chemical Inc., 1990) can be used to make emulsion coatings.

Acetylglycerides are produced by acetylation of glycerol monoestearate with acetic anhydrate. Acetyglycerides exist in the α polymorphic form (Jackson and Lutton, 1952; Vicknair et al., 1954) and can be stretched up to eight times their original length. Acetyglyceride films posses a relatively smooth surface without any well-defined morphological characteristics (Kester and Fennema, 1989) because heterogeneous acetyglycerides are stable in the α form (Feuge, 1955). WVP of wax-based films is lower than most other edible films; however, oxygen permeability of beeswax is similar to some of the protein-based films such as collagen, casein, soy protein films (Table 1.1). Acetylated monoglycerides based-films have higher WVP values compared to other wax-based films.

1.1.6. Evaluating of the properties of edible films

1.1.6.1. Barrier properties

Barrier properties of the aforementioned films directly impact product shelf-life with transfer of gases (e.g. oxygen, carbon dioxide), water vapor and volatile compounds occurring from the environment to the food product and vice versa. Edible films possessing polymer structures can be used as barriers to these gases and water vapor. Depending on the products, edible films or coatings may be used to prevent moisture loss, moisture absorption, exposure to oxygen, or diffusion of CO_2. Control of the moisture and oxygen content limits the growth of aerobic microorganisms, which provids good microbiological characteristics as well as physicochemical and organoleptic characteristics to intermediate moisture or dry foods.

2.1.6.1.1. Water Vapor Permeability

One of the most important properties of an edible film is its water vapor permeability. Water vapor permeability (WVP) is defined as the rate of water vapor transmission per unit area of a flat material of unit thickness.

Several techniques for measuring WVP have been described and are based on either infrared sensors such as the Permatran-W series or the WVP tester L80-4000 series which is a colometric method. These techniques are well-suited for high barrier efficiency polymers such as plastics or wax-based edible films, but far less so for hydrophilic polymers (Krochta, 1992, McHugh and Krochta, 1994). The most commonly employed method is the "cup method" which is based on gravimetric analysis (Kester and Fennema, 1983; Kamper and Fennema, 1985). True permeability is a process of solubilization and diffusion, where the vapor dissolves on one side of the film and diffuses through to the other side.

Water vapor permeability (WVP) can be expressed as follows:

$$WVP = \frac{g \times L}{A \times t \times \Delta p}$$

Where

g = Amount of water vapor

L = Thickness

A = Film area

t = Time

Δp = Differential partial pressure of water vapor

The water vapor transmission rate, determined by the amount of water weight lost or gained, depends on the material placed in the cup water salt solution or desiccant. In the Desiccant Method, the film to be tested is sealed over the mouth of a cup containing desiccant, and is placed in a chamber under controlled temperature and relative humidity. In the water method, the test dish contains distilled water or a saturated salt solution. In both cases, the rate of water transmission is determined from periodic weighings which must be taken after a steady state rate of moisture transmission has been obtained (ASTM, 1980).

1.1.6.1.2. Oxygen Permeability

Measurement of oxygen permeability (OP) typically involves the use of colorimetric (e.g. Oxatran-Mocon, Modern Controls Inc., Minneapolis, MN, USA), or manometric sensors (Lyssy L100 series, Lyssy, Zurich, Switzerland). Oxygen transmission rates (OTR) are obtained by monitoring the permeant in the upper portion of a diffusion cell at specific time intervals, while passing a known amount of oxygen through the lower portion until the steady state is reached. To obtain the OP, the thickness of the film and the partial pressure difference must be considered as shown in the following equation:

$$OP = (OTR \times L)/ \Delta p$$

Where

L = thickness of film

Δp = partial pressure difference of oxygen

1.1.6.2. Mechanical Properties

Tensile strength and percent elongation are the most commonly tested mechanical properties. Tensile strength, the maximum tensile stress that a material can sustain before the onset of permanent deformation or failure, is determined by measuring the force per unit while the film is being stretched. Percent elongation refers to the degree of toughness and flexibility of the film, and measures the length of displacement per original length while the film is being stretched. The established method to determine tensile strength and elongation is ASTM standard D-882-83 which most often utilizes the Instron machine (ASTM, 1991).

1.1.7. Factors Affecting Film Properties

Some factors such as the chemical and structural nature of the polymer and permeant can affect the barrier properties of edible films. Chemical composition plays a major role in the barrier properties of edible films. For example, polar polymers such as many proteins and polysaccharides show low gas permeability values at low RHs but have poor moisture barrier properties. In contrast, non-polar hydrocarbon-based materials such as lipids are excellent moisture barriers and less effective gas barriers. When added to polymer films, low molecular weight additives can improve or reduce the barrier properties of edible films depending on their chemical structure. Most edible film plasticizers increase water vapor permeability by disrupting polymer chain hydrogen bonding.

The nature of the permeant also affects its transfer through films, with smaller molecules generally diffusing faster than larger molecules, and polar molecules diffusing

faster than non-polar molecules, particularly in polar films. The diffusion of gases and water vapor though film polymers is termed permeation. During permeation, adsorption of these gases and water vapors on the polymer surface and their desorption through the opposite surface is also seen (Sperling, 1992). These films also can have different barrier properties depending on their composition and method of production.

1.1.8. Application of Edible Films

Edible film barriers can serve many functions in food products. Such edible films or coatings can provide additional nutrients, enhance sensory characteristics and serve as a carrier for many food additives including flavoring/coloring agents, vitamins, antioxidants and antimicrobials, the last of which is the focus of this review.

Edible films can function as oxygen and moisture barriers to retain crispiness and inhibit oxidative and hydrolytic rancidity in fat-containing products such as nutmeats. Although coating of hydroxypropylated starch, hydroxypropyl cellulose (Ganz, 1969, Ramos et al., 1996), and WPI (Mate and Kroctha, 1996; Mate et al., 1996a, b; Mate and Krochta 1997a, b) can delay rancidity in nuts, coatings based on corn zein (Andres, 1984) or low-methoxyl pectins (Swenson et al., 1953) required the addition of an antioxidant to be effective.

Edible coatings can be used to improve the quality of fresh, frozen and processed meat, poultry and seafood by minimizing microbial spoilage, moisture loss, lipid oxidation and discoloration. Application of wax, mineral oil, or corn oil to whole poultry, meats and fish before freezing will reduce moisture loss (Gennadios et al., 1997). Many types of coatings have tested in attempt to maintain quality in frozen fish. According to

Anderson (1961), long chain saturated fatty alcohols and fatty acid coatings could control moisture loss and freezer burn in frozen meats. Stuchell and Krochta (1995) studied the effects of acetylated monoglyceride (Myvacet 9-08 and Myvacet 9-45) and acetylated monoglyceride/whey protein isolate coatings on moisture loss and lipid oxidation in frozen King salmon pieces stored at – 23°C, with application of these coatings reducing moisture loss by 42-65% during the first weeks of frozen storage. Onset of lipid oxidation was also delayed and peroxide values were lowered. In another study, Silver salmon fillets that were dipped in Myvacet 5-07 acetylated monoglyceride (60°C) and placed infrozen storage at –10°C retained more moisture and had lower peroxide values than uncoated control samples (Hirasa, 1991).

Alginate coatings have been applied to various meats such as beef and pork cuts, poultry parts and whole lamb carcasses using a two-step procedure to reduce dehydration (Mountney and Winter, 1961; Allen et al., 1963; Earl, 1968; Earl and McKee, 1976; Lazarus et al., 1976; Williams et al., 1978). Meat is typically immersed in or sprayed with an aqueous sodium alginate solution with gelation induced by application of a calcium chloride solution. These alginate coatings reduced dehydration of meat tissue compared to that of uncoated controls. Reduced dehydration is mainly due to the high moisture content of alginate gels with moisture in the gel evaporating before any desiccation of the enrobed food (Kester and Fennema, 1986). Flavor-tex®, a commercial calcium alginate coating (D.H. McKee, Inc., 1989), is used to reduce shrinkage, oxidative rancidity, moisture migration and oil absorption in various foods including meat, poultry, seafood, baked goods, vegetables, extruded foods and cheese. Alginate coatings protect against lipid oxidation in pre-cooked, ground pork patties (Wanstedt et al., 1981) with better

texture and absence of a warmed-over flavor (an indication of rancidity) observed in coated compared to uncoated samples. Wu et al. (2001) evaluated edible films of starch-alginate (SA), starch-alginate-stearic acid (SAS), SA-tocopherol, SAS-tocopherol, tocopherol-coated SA, and tocopherol-coated SAS for their effectiveness in maintaining quality of precooked beef patties during storage at 4°C. Patty weight loss, moisture loss, 2-thiobarbututric acid values and hexanal, pentane and total volatile content differed with film composition. SAS-based films controlled moisture loss more effectively than lipid oxidation, with tocopherol-treated films more effective than non-tocopherol films in inhibiting lipid oxidation.

Application of alginate coatings can also reduce the total-surface microbial count and some pathogenic bacteria on meat surfaces. Lazarus et al. (1976) reported that alginate-coated lamb carcasses had lower microbial counts than uncoated samples after 7 days of refrigerated storage. Williams et al. (1978) also observed lower microbial counts on the surface of alginate-coated beef cuts than on uncoated controls. In addition, they showed that coating with alginate protected the red color of beef muscle. Similarly, Siraguas and Dickson (1992, 1993) demonstrated that calcium alginate coatings containing organic acids (lactic acid or acetic acid) reduced the levels of *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7 on beef tissue.

Chitosan coatings which possess several important biological properties including antifungal activity (Allan and Hadwiger, 1979; Hirano and Nagao, 1989) and phytoalexin (Hadwiger and Beckman, 1980; Walker-Simons et al., 1983), have been applied to fruits and vegetables to reduce water loss and extend shelf life (El Ghaouth et al., 1991). These coatings also can retard ripening of tomatoes and extend storage life without adversely

affecting later ripening characteristics (El Ghaouth et al., 1990). Coating cucumbers and bell peppers with chitosan also reportedly reduced respiration, color loss, wilting and fungal infection (El Ghaouth et al., 1991). A similar delay in spoilage was also observed in chitosan-coated strawberries and was attributed to the fungistatic properties of chitosan (El Ghaouth et al., 1990).

Waxes and oils such as paraffin wax or oil, beeswax, carnauba wax, candelilla wax, mineral oil, vegetable oil, acetylated monoglycerides, sucrose esters of fatty acids and resins are generally good moisture barriers and have been used extensively on whole fruits and vegetables, (Hagenmaier and Shaw, 1990, 1992). Protective coatings comprised of waxes and sucrose esters of fatty acids have been developed and commercialized for fruits and vegetables. Various waxes have been traditionaly applied to cheese as peelable coatings to prevent mold growth. Kamper and Fennema (1984) prepared bilayer films with HMC and beeswax, paraffin, hydrogenated palm oil, stearic acid, or stearic-palmitic acid, with these films inhibiting water transfer from high- to low-moisture foods.

Use of protein-based films and coatings on fresh produce has been restricted because of high water vapor permeability. However, addition of hydrophobic materials to protein films presents many opportunities for coating fresh fruit and vegetables. Park (1991) investigated the effect of corn zein coatings on tomatoes and found that the shelf-life could be extended six days by delaying color change, loss of firmness, and weight loss. Other investigators (Krochta et al., 1989; Avena-Bustillus et al., 1993, 1997) have designed and applied caseinate-based coatings to minimally processed fruits and vegetables. Using such coatings on peeled carrots, white blush formation was eliminated

with decreased dehydration also observed at 2.5°C/70% RH (Avena-Bustillus et al., 1993). Coating uncut celery sticks with a caseinate/acetylated monoglyceride solution also resulted in a 75% reduction in moisture loss under the same conditions. *Penicillum spp.* were not found on apple slices coated with caseinate/acetylated monoglyceride solutions that contained included potassium sorbate (1%) (Avena-Bustillus et al., 1997). Lerdthanangkul and Krochta (1996) coated green bell peppers with a mineral oil- or cellulose-based coating, whey protein isolate, sodium caseinate, or a sodium caseinate beeswax emulsion to assess the impact on respiration, internal gases, color, firmness, and water loss during 20 days of storage at 10°C. Only the mineral oil-based coating significantly reduced moisture loss, thus maintaining fruit firmness and freshness. Cellulose and sodium caseinate coatings were the most effective O_2 and CO_2 barriers. However, none of the coatings influenced respiration or color change. In another study, soy protein film coatings reportedly extended the shelf-life of "Fuji" and "Golden 95 delicious" apples over 40 days at 24°C (Park, 2000) with these films retarding changes in apple firmness, color and acidity compared to uncoated controls.

Polysaccharide-based coating also has been developed for processed fruit and nut products. When tested on fruits, starch coatings extended the shelf-life of pitted prunes due to O_2 barrier properties (Jokay et al., 1967). Starch films are reported to be semipermeable to CO_2, but highly resistant to O_2 transmission (Rankin et al., 1958). Choi et al. (2000) showed that low pH (2.7) chitosan coatings dissolved the wax layer on apple skin, which led to increased weight loss and a lower respiration rate.

Edible film coatings have been shown to reduce oil uptake by foods and oil degradation during deep fat frying. Extension of frying-oil life may be accomplished

through appropriate selection and application of edible films to pre-fried products. Hollownia et al. (2000, 2001) evaluated the application of MC and HPMC edible films to marinated whole chicken strips before and after breading. When applied before breading, oil absorption by the chicken strips decreased during frying, with oil degradation also reduced by 50%, due to a reduction in moisture and acetic acid migration which are responsible for reduced free fatty acid generation in oils during frying. The potential for gellan gum-, HC- and MC- based films to reduce absorption of fat in a fried pastry mix was also investigated by Williams and Mittal (1999a). All coated samples had lower levels of fat after frying than uncoated samples, with fat absorption reduced by 50-91%. Williams and Mittal (1999b) also developed a mathematical model for heat, moisture and fat transfer in the film and the foods when edible films were used as coating in fried foods (potatoes and pastry). Film diffusivities were determined for gellan gum films at a thickness of 2.0 mm during frying. Moisture diffusivities of the food and film were 0.33 x 10^{-7} and 0.25 x 10^{-7} m^2/sec., respectively. Fat diffusivities were 0.103 x 10^{-8} m^2/sec. for the food and 0.604 x 10^{-9} m^2/sec. for the film. Thermal diffusivities were 0.102 x 10^{-6} m^2/sec. for the food and 0.156 x 10^{-6} m^2sec. for the film.

1.2. ANTIMICROBIALS

As mentioned at the outset, edible films can serve as carriers for a wide range of food additives including various antimicrobials, which can extend product shelf-life and reduce the risk of pathogen growth on the surface of food products. Antimicrobials including benzoates, propionates, sorbates, parabens, acidifying agents (e.g. acetic and lactic acids), curing agents (e.g. sodium chloride and sodium nitrite), bacteriocins and

natural preservatives (e.g. essential oils, lysozyme, liquid smoke) will now be addressed individually in terms of their possible use in edible films.

1.2.1. Benzoic acid or sodium benzoate

The preservative action of benzoic acid was first described in 1875 by H. Fleck. Sodium benzoate was the first chemical preservative permitted in foods by the FDA. In the United States, benzoic acid and sodium benzoate are generally regarded as safe (GRAS) preservatives (Code of Federal Regulations, 1977, Title 21, Sections 184.1021 and 184.1733) at levels up to 0.1%. In most other countries, maximum permissible levels range between 0.15 and 0.25%.

Sodium benzoate is one of the most commonly used antimicrobials in edible films, since it is soluble in most film solutions and remains active after film production. The antimicrobial activity of benzoate is related to pH. Like many other food antimicrobials, benzoate (pK = 4.20) is most effective in its undissociated form with 60% of the compound undissociated at pH 4.0. Therefore, methycellulose, chitosan, and collagen films, all of which have a relatively low pH, are good candidates for this antimicrobial. This restriction limits the use of edible films containing benzoic acid and its sodium salts to high-acid products such as cheese and fermented meat products (Lueck, 1980).

Although benzoic acid acts essentially as a mold and yeast inhibitor (Balatsouras et al., 1963), other studies have shown that sodium benzoate or benzoic acid can also inhibit the growth of both pathogenic and psychotropic bacteria (Thomas et al., 1993, Kasrazaden et al., 1994, El-Shenawy and Marth, 1988).

Use of sodium benzoate alone or in combination with various organic acids has been proven effective for decreasing microbial populations on raw chicken. In one study conducted by Hwang and Beuchat (1995), raw chicken wings were inoculated with *Salmonella*, *Campylobacter jejuni*, *Listeria monocyctogenes*, *Staphylococcus aureus* or *Escherichia coli* O157:H7 and immersed in water or a solution of 0.5% lactic acid/0.05% sodium benzoate (LB) (pH 2.64) for 30 min. Lower populations of pathogenic and naturally occurring psychrotrophic bacteria were detected on wings immediately after treatment with LB compared to the untreated controls. During refrigerated storage, populations of *Salmonella*, *C. jejuni*, *L. monocytogenes*, and *E. coli* O157:H7 decreased significantly on lactic acid-treated wings. In another study, benzoate was more effective than lactic acid against *Staphylococcus aureus* and *E. coli* at 35°C, especially when used at pH <6.0 (Thomas et al., 1993). Sodium benzoate can also be used to inhibit *Salmonella* in certain cheeses. Kasrazaden et al. (1999) found that addition of sodium benzoate (0.3%) to cheese (pH 6.6) made from milk, which was acidified to pH 5.9 with propionic acid, prevented growth of *Salmonella* in the final product during storage.

1.2.2. Sorbic Acid and sorbates

Sorbic acid, first isolated from the pressed unripened berry of the rowan or mountain ash tree in 1859 by the German chemist A. W. Hoffmann (Anonymous, 1978), is a straight chain α, β- unsaturated monocarboxylic acid and has the structure CH_3CH = CHCH = CHCOOH (Sofos, 1989). The carboxyl group reacts to form calcium, sodium or potassium salts and esters. Potassium sorbate, the commonly used salt of sorbic acid, is highly soluble in water (58.2% at 20°C). Like sodium benzoate, sorbic acid (pK = 4.80)

is most effective in acidic foods (< pH 6.5) (Cowles, 1941). At pH 4.0, 86% of sorbic acid is undissociated (Sofos et al., 1980) with the undissociated form again most able to penetrate the bacterial cytoplasmic membrane (Chichester and Tanner, 1972). Increased antimicrobial activity of sorbate at low pH values has been reported for a wide range of microorganisms (Table 1.3) (Gould, 1964; Park and Marth, 1972; Sofos et al., 1980; Lahellec et al., 1981; Blocher et al., 1982; Elliot et al., 1982; Nose et al., 1982; Roberts et al., 1982; Seward et al., 1982; Tsai et al., 1984; Tuncan and Martin, 1985; Lund et al., 1987). Therefore, edible films containing sorbate only exhibit antimicrobial activity at pH values less than 6.0.

Sorbic acid salts are the most studied antimicrobial agents in carbohydrate or protein-based edible films such as methylcellulose, WPI and chitosan since the sorbates are soluble in various film solutions and remain chemically active in the film matrix. The carboxyl group, which is the active site in sorbates, forms hydrogen bonds with carbohydrate or protein chains in films. Edible films containing sorbates have been tested against a wide variety of microorganisms (i.e. spoilage bacteria, pathogenic bacteria, yeast and molds) in laboratory media using diffusion-type assay (Torres et al., 1985; Torres and Karel, 1985; Chen et al., 1996; Cagri et al., 2001).

Typical use levels range from 0.02% to 0.3% in commercial foods (Table 1.3). These concentrations are added to bakery, dairy, fruit and meat products to inhibit a wide range of yeasts (Lueck, 1980; Monsanto, 1987), molds (*Trichosporon*, *Alterneria*, *Aspergillus*, *Mucor*, *Fusarium*) (Sofas and Busta, 1993, Chichester and Tanner, 1972; Luck, 1976), and bacteria (*E. coli* O157:H7, *Clostridium botulinum*, *Staphylococcus aureus*, *Saccharomyces italicus*, pseudomonads, and coliforms) (Bradley et al., 1962;

Table 1.3. Antimicrobials

Antimic.	pKa	Solubility	pH	Mode of action	Food Application	Film Application	Microorganisms affected	References
Benzoate	4.76	Water soluble	<6.0	Alters cell membrane function; interferes with uptake of substrates, electron transport, and the proton-motive force involved in transport functions; binds sulfyhdryl groups on various enzymes leading to disruption of vital processes involved in transport functions	Dairy, bakery, vegetable, fruit, meat, and fish products, beverages	Cellulose, chitosan	*L. monocytogenes*, *E. coli*, *S.* Typhimurium, yeast and mold	Thomas et al. (1999), Kasrazaden et al. (1994), El-Shenawy and Marth (1988), Lueck (1980)
Sorbate	4.19	Water soluble	<6.0		Baked goods, dairy products, fruits (dried fruits, fruit drinks, jams, jellies wine), vegetable products and other foods (fish, fermented sausage, mayonnaise, margarine, salad dressing)	Zein, WPI, cellulose, chitosan	*Bacillus spp.*, *E. coli*, *L. monocytogenes*, *Pseudomonas*, *Salmonella*, *Clostridium botulinum*, *Staphylococcus aureus*, yeast and mold	Park and Marth (1972), Luck (1980), Sofos et al. (1980), Lahellec et al. (1981), Blocher et al. (1982), Elliot et al. (1982), Seward et al. (1982), Tsai et al. (1984), Tuncan and Martin (1985), Lund et al. (1987), Chen et al. (1996), Cagri et al. (2001)
Propionate	4.87	Water soluble	<6.0		Cheese, bakery products	WPI	*E. coli*, *S. aureus*, *Sarcina lutea*, *Salmonella* spp., *Proteus vulgaris*, *Lactobacillus spp.*, *L. monocytogenes*, *B. subtilis*, *Aspergillus*, *Candida* spp. and *Saccharomyces cerevisiae*	Chung and Goepfert (1970), Eklund (1985), El-Shenawy and Marth, (1989), Cherrington et al. (1990), Cherrington et al. (1991)

Table 3. Antimicrobials (cont'd)

Antimic.	pKa	Solubility	pH	Mode of action	Food Application	Film Application	Microorganisms aftected	References
Parabens	3.0-8.0	Water soluble	2-10	Physical damage to the cytoplasmic membrane of microorganis mscausing the release of cytoplasmic components	Butter, margarine, ices, soy sauce, maple syrup, meats	WPI (PABA)	*L. monocytogenes, S. aureus, Clostridium botulinium, Bacillus cereus, B. megaterium, Lactobacillus lactis, Streptococcus faecalis, Sarcina lutea, E. coli, Salmonella* Typhimurium, *S. typhosa, Yersinia enterocolitica, Vibrio parahaemolyticus, Pseudomonas spp., Canadida albicans, Fusarium oxysporum, Penicillum citrinum, P. Chrysogenum, Saccharomyces cerevisae, Torula utilis, Zygosaccharomycess spp.*	Aalto et al.(1953), Jurd et al. (1971), Lee (1973), Kato and Shibasaki (1975), Robach and Pierson, (1977), Dymicky and Huhtanen (1979), Eklund (1980), Lueck (1980), Eklund (1981), Reddy and Pierson (1982), Reddy et al. (1982), Marwan and Nagel (1986), Bargiota et al. (1987), Jermini and Schmidt-Lorenz (1987), Payne et al.(1989), Thompson, (1991), Juneja and Davidson (1992), Moir and Eyles (1992), Davidson (1993)
Fatty Acids	-	Water soluble	-	impairing cell permeability and the transport of nutrients	-	Chitosan, HPMC, WPI	*L. monocytogenes, E. coli, Salmonella spp.*,yeast and mold	Kabara (1978), Greenway and Dyke (1979), Notermans and Dufrenne (1981), Baker et al. (1985), Knapp and Melly (1986)

Table 1.3. Antimicrobials (Cont'd)

Antimic.	pKa	Solubility	pH	Mode of action	Food Application	Film Application	Microorganisms affected	References
Acetic acid	4.77	Water soluble	-	Uncouples substrate transport and oxidative phosphorylation from the electron transport system	Bakery products, mustard, salad dressing, mayonnaise, pickled, sausages, cheeses, dairy product analogs, fats and oils, gravies and sauces, meats, breakfast cereals, cheeses, gelatin, candy, jams and jellies, and soup mixes	Chitosan, WPI	*L. monocytogenes, Bacillus spp., E. coli* O157:H7, *Salmonella spp., Staphylococcus spp.*	Bell et al. (1986), Okrend et al. (1986), Lillard et al. (1987), Anderson et al. (1988), Arizcun et al. (1998), Dorsa et al. (1998)
Lactic acid	3.08	Water soluble	-		Jam, jellies, sherbet, beverages, pickles, olives, apple slices, fruit and bakery products	Collagen, WPI, chistosan	*Salmonella, L. monocytogenes, E. coli, S. aureus, Clostridium spp.,*	Branen and Davidson (1983), Cherigton et al. (1991), Dickson and Anderson (1992)

Table 1.3. Antimicrobials (Con't)

Antimic.	pKa	Solubility	pH	Mode of action	Food Application	Film Application	Microorganisms affected	References
Nisin	-	Water soluble	<6.0	Disrupts membrane activity by pore formation and effects on electron transfer chain components	Cheese, fermented meat products	WPI, zein, cellulose	*Listeria monocytogenes*, *Bacillus spp.*, *Clostridium spp.*, *Corynebacterium spp.*, *Lactobacillus spp.*, *Leuconostoc spp.*, *Micrococcus spp.*, *Pediococcus spp.*, *Streptococcus spp.*	Mattick and Hirsch (1947), Rayman et al. (1981), Collins-Thompson et al. (1985), Ogden and Tubb (1985), Bell and DeLack (1986)
Pediocin	-		3-9		Fermented meat products	Cellulose	*L. monocytogenes*, *Bacillus cereus*, *S. aureus*, *Lactobacillus. brevis*, *Lb. plantarum*, *Lactoccucus lactis*, *Clostridium botulinum*, *C. perfringens*, *C. sporogenes*, *Micrococcus luteus*, *Enterococcus faecalis*,	Luchansky et al. (1992), Yousef et al. (1999)
Lyzosyme	11	Water soluble	2-11	Cell wall degradation and lysis	Seafood, kimchi, Chinese noodles, potato salad, custard (Japan), hard cheese (Europe)	Zein, cellulose	*L. monocytogenes*	Yousef et al. (1999)

Table 1.3. Antimicrobials (Cont'd)

Antimic.	pKa	Solubility	pH	Mode of action	Food Applications	Film Applications	Microorganisms Affected	References
Essential oils	-	Water soluble	-	Disrupt both electron transport and nutrient uptake	Meat and vegetable products	-	*E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Lactobacillus viridescens*	Davidson (1993), Juven et al. (1994), Del Campo et al. (2000), Elgavyar et al. (2000), Tabanca et al. (2001)
Lactoferrin	9.5	Water soluble	-	Chelation of iron as well as calcium and magnesium, alter the permeability of the membrane because of its cationic feature	-	-	*Bacillus subtilis*, *B. stearothermophilus*, *L. monocytogenes*, *Micrococcus* spp., *Klebsiella* spp., *Salmonella* Typhimurium, *Pseudomonas fluorescens* *E. coli* O157:H7, *Shigella*, *Candida* spp., *Yersinia enterocolitica*, *Camplylobacter jejuni*, *Streptococcus mutants*, *Corynebacterium diphtheria*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Clostridium perfringens*	Oram and Reiter (1968), Reiter (1978), Payne et al. (1989), Payne et al. (1990), Bellamy et al. (1992), Wakayabayashi et al. (1992), Conner (1993), Jones et al. (1994), Payne et al. (1994)

Table 1.3. Antimicrobials (Cont'd)

Antimic.	pKa	Solubility	pH	Mode of action	Food Applications	Film Applications	Microorganisms Affected	References
NaCl	-	Water soluble	-	Plasmolytic effect, limits oxygen solubility, alters pH, and is toxic for microbial cells due to sodium and chloride ions	Meat products, vegetables	-	Gram-negative bacteria, Gram-positive bacteria, yeast and molds	Banward, 1979, Sperber (1983), Corry (1987), Doi and Kitabastake, 1997
Nitrite	-	Water soluble	<7.0	Blocking sulfhydryl sites within the bacterial cells, inhibits active trasnport, oxygen uptake, and oxidative phosphorylation	Meat products	-	*Clostridium* spp. , *Staphyloccocus aureus, E. coli, Achromobacter* spp., *Enterobacter* spp., *Flavobacterium* spp., *Micrococcus* spp., *Pseudomonas* spp., *Listeria monoctogenes*	Woods and Woods (1982), Yang, (1985), Gibson and Roberts (1986a, a,c), Carpenter et al. (1987), Roberts et al. (1991), Pelroy et al. (1994)

Gandhi et al., 1973; Kasrazadeh and Genigeorgis, 1995; Briozzo et al, 1985) and to prevent aflatoxin and enterotoxin production (Lueck, 1980).

Antimicrobial activity of sorbate is generally enhanced at low temperature. Investigators showed that *L. monocytogenes*, *Y. enterocolitica*, *A. hydrophila*, and *Pseudomonas putida* were more sensitive to potassium sorbate at refrigeration temperature than at higher temperature (El-Shenawy and Marth, 1988; Moir and Eyles, 1992). Increased microbial inhibition by sorbate in the presence of higher solute concentrations also has been reported in both laboratory media and foods. Sodium chloride reportedly acts synergistically with potassium sorbate to inhibit *S. aureus* (Robach and Stateler, 1980) and outgrowth of *C. sporagenes* (PA 3679) spores (Robach and Statelen 1980). Razavi-Rohami and Griffiths (1999) also found that the inhibitory activity of sorbic acid increased with increasing NaCl concentration from 3 to 20% (w/v) and lower pH (3.5) for *Candida*, *Sporothrix*, *Fusarium*, *Penicillium*, *Paecilomyces* and *Aspergillus* spp. The inhibitory mechanism of 3% NaCl is not know but may be related to the effects of added solutes on membrane activities as suggested by Walter et al. (1987).

1.2.3. Propionic Acids

Propionic acid, a monocarboxylic acid (pKa = 4.87), is produced by *Propionabacterium freudenreichi* subsp. *shermani*. Swiss cheese contains up to 1% propionic acid from the growth of propionibacteria, which gives it a characteristic flavor and prevents mold growth (Davidson and Branen, 1993).

Antimicrobial activity of the propionates is again pH dependent, with the undissociated form being 45 times more inhibitory than the dissociated form (Eklund,

1985). Therefore, it is only effective in low pH edible films such as collagen and chitosan.

Propionic acid or its salts are commonly used food preservatives due to their wide spectrum of activity. While primarily active against molds, some yeasts and bacteria are also inhibited (Table 1.3) (Chung and Goepfert, 1970; Eklund, 1985; El-Shenawy and Marth, 1989; Cherrington et al., 1990; Cherrington et al., 1991). *Bacillus cereus* which causes rope formation in bread dough, can be inhibited by the addition of propionic acid at pH 5.6 to 6.0 (Woolford, 1975; O'Leary and Kralovec, 1986). Propionates can be added directly to bread dough because they have no effect on the activity of baker's yeast. Amounts of propionate used in foods are generally less than 0.4% (Robach, 1980).

Propionic acid was most effective among the organic acids at inhibiting aflatoxin production by *Aspergillus flavus* on betel nuts after 2 (62%) and 4 (85%) weeks (Raisuddin and Misra, 1991). Kwon et al. (1998) reported that when grown in tryptic soy broth (TSB) containing buffered propionic acid (BPA), the growth rate of *S.* Typhimurium gradually decreased as the level of BPA increased and the broth pH decreased. No growth was detected at BPA concentrations above 3% (v/v), with growth also markedly suppressed under anaerobic conditions and at pH 5.0 compared to 7.0.

Propionic acid and the propionates (8-12%) were also effective in controlling the growth of mold on the surface of cheese and butter (Deane and Downs, 1951). When used at pH 4.5 at a level of 0.2%, propionic acid completely inhibited growth and aflatoxin formation (Ghosh and Haggblom, 1985).

1.2.4. Mode of action of benzoate, sorbate, and propionic acid

Benzoic, sorbic and propionic acid act by inducing changes in the morphology and appearance of microbial cells. Incorporating these acids into specific cell structures may also inhibit specific biosynthetic pathways in the cell (Sofos et al., 1986). As one example, sorbate prevented amino acid (L-serine and L-histidine) uptake by *S.* Typhimurium at low pH (Tuncan and Martin, 1985).

These acids also have been shown to reduce the intracellular pH (pHi) in *E. coli* cells (Salmond et al., 1984) and vesicles (Eklund, 1985), suggesting that this common preservative may act as a protonophore. Ronning and Frank (1987) reported that undissociated sorbic acid reduced the pHi in vegetative *C. sporogenes* PA 3679 cells, and decreased the protonmotive force which energizes cellular activities such as amino acid transport. They also proposed that the loss in energy and reduced uptake of essential amino acids induced a stringent-type response that inhibited but did not kill the cells.

Past evidence has suggested that the inhibitory action of these antimicrobials on yeasts is due to reduction of pH. However, using a novel method to measure pHi in growing cells, little correlation was found between reduced growth rate on exposure to sorbic acid and reduction of pH. In fact, growth inhibition correlated with an increase in the intracellular ADP/ATP ratio due to increased ATP consumption by the cells. This was partly attributed to the activation of protective mechanisms, such as increased proton pumping by the membrane H+ -ATPase, which ensured that pH did not decline when the cells were exposed to sorbic acid (Bracey et al., 1998). Thus, the inhibitory action of sorbic acid was likely due to the induction of an energetically expensive protective

mechanism that compensated for any disruption of pHi homeostasis but resulted in less available energy for normal growth.

Benzoic, sorbic and propionic acid also alter cell membrane function by producing pores (Freese and Levin, 1978) that interfere with uptake of substrates, electron transport and the proton-motive force involved in transport functions. In addition, sorbic acid can bind sulfhydryl groups on various enzymes, thus leading to disruption of vital processes involved in transport functions, cell metabolism and growth. Sorbate also reportedly inhibits microbial growth by combining with coenzyme A (Nose et al., 1982) with Harada et al. (1968) theorizing that sorbate may act competitively with acetate at the site of acetyl coenzyme A.

1.2.5. Parabens

Esterification of the carboxyl group of benzoic acid produces parabens. Having the ability to remain undissociated at pH values up to 8.5, most parabens are active at pH 3.0 to 8.0. The methyl, propyl and heptyl parabens can be used as food preservatives in most countries, while the ethyl and buthyl esters are more restricted. Parabens can be used effectively in a wide range of foods (Table 1.3).

Parabens with a longer alkyl chain posses more antimicrobial activity than those having a shorter alkyl chain (Aalto et al., 1953; Shibasaki, 1969). Parabens are more inhibitory on Gram-positive than Gram-negative bacteria due to their decreased in polarity. Methyl, ethyl, propyl and butyl parabens completely inhibit the growth of Gram-positive bacteria and Gram-negative bacteria at concentration levels of 40-2000 and 50-4000 μg/ml, respectively (Table 1.3) (Aalto et al., 1953; Jurd et al., 1971; Lee, 1973;

Kato and Shibasaki, 1975; Robach and Pierson, 1977; Dymicky and Huhtanen, 1979; Eklund, 1980; Lueck, 1980; Eklund, 1981; Reddy and Pierson, 1982; Reddy et al., 1982; Payne et al., 1989; Juneja and Davidson, 1992; Moir and Eyles, 1992; Davidson, 1993). However, parabens are generally more active against molds and yeasts than bacteria. Using esters of *p*-hydroxybenzoic acid, concentrations of 32 to 1000 μg/ml are normally needed for complete inhibition of bacteria and fungi (Table 1.3) (Aalto et al., 1953; Kato and Shibasaki, 1975; Marwan and Nagel, 1986; Jermini and Schmidt-Lorenz, 1987; Juneja and Davidson, 1992; Thompson, 1991)

The antimicrobial mechanism for paraben is very similar to that of phenols and related phenolic compounds, since parabens are phenolic derivatives. Vas (1953) and Judis (1963) both proposed that phenol physically damages the cytoplasmic membrane of microorganisms, which causes the release of cytoplasmic compounds. Furr and Russel (1972) detected similar leakage of intracellular RNA by *Serratia marcescens* in the presence of parabens, with the amount of leakage proportional to the alkyl chain length of the paraben. Additional studies have shown that the parabens inhibit nutrient uptake through the cytoplasmic membrane, inhibiting both membrane transport and the electron transport system (Freese et al., 1973; Eklund, 1980). According to Freese et al. (1973) serine uptake by cytoplasmic membrane of *B. subtilis* is inhibited by parabens with Eklund (1980) showing inhibition of alanine, serine, phenylalanine, and glucose uptake by vesicles in *E. coli*, *B. subtilis*, and *P. auruginosa*.

1.2.6. Free Fatty Acids and Their Esters

Low concentrations of long-chain fatty acids are inhibitory to microorganisms, especially Gram-positive bacteria and yeasts (Kabara, 1978). Saturated fatty acids having chain lengths of C_{12} to C_{16} and C_{10} to C_{12} possess the most antimicrobially activity against bacteria and yeasts, respectively (Kabara, 1993). Decreasing effectiveness of longer chain fatty acids may be related to increased hydrophobicity and decreased solubility (Wang and Johnson, 1991). Fatty acids are also more active at low pH (<5.0). Fatty acid structure and function has been reviewed by Kabara (1982, 1993). The inhibitory effects of unsaturated fatty acids increase as the number of double bonds increases. For example, linoleic acid was far more inhibitory than oleic acid (Fuller and Moore, 1967). Fatty acids and monoglycerides are inhibitory to bacterial species (Table 1.3) (Notermans and Dufrenne, 1981; Baker et al., 1985; Knapp and Melly, 1986). According to Sprong et al. (1999), increased intake of bovine milk fat enhanced gastrointestinal killing of *L. monocytogenes,* but had little effect on infection with *Salmonella enteritidis* in rats. Free fatty acids $C_{10:0}$, $C_{12:0}$ and $C_{12:0}$ and the monoglycerides of $C_{12:0}$, $C_{12:0}$, and $C_{12:0}$ likely play a pivotal role in this enhanced listeriacidal activity. In contrast, infection with *Salmonella* was not affected by milk fat consumption.

Monoesters of glycerols and the esters of sucrose are more antimicrobial than their corresponding free acids. Monolaurin (lauricin), the most effective of the glycerol monoesters, is inhibitory to various Gram-positive bacteria and some fungi at 5-100 ppm (Andrews and Grodner, 1997). Monolaurin is most effective at pH 5.0-8.0. Antimicrobial activity of monolaurin has been demonstrated in various foods including mechanically

deboned chicken meat, minced fish, chicken sausage (Baker et al., 1982), soy sauce (Kato, 1981), meat slurries (Notermans and Dufrenne, 1981), cottage cheese, pork homogenate (Robach et al., 1981), skim milk (Wang and Johnson, 1992), and crawfish tail (Oh and Marshal, 1994). However, use of monolaurin as a food preservative is limited due to off-flavors and the loss of activity from the interaction with lipophilic proteins, fat globules and starch. Fatty acids and poylglycerides are added to edible films and coatings to decrease WVP properties. Long-chain alcohols (e.g. stearyl alcohol) and fatty acids (e.g. stearic, palmitic) are commonly used as additives in edible coatings due to their high melting point and hydrophobic chracteristic (Hagenmaier and Shaw, 1990). Vojdani and Tores (1990) developed composite films with MC and fatty acids of different chain lengths to decrease migration of preservatives like potassium sorbate from the surface of foods such as cheese. The cellular membrane has been suggested as a primary target for antimicrobial activity (Freese et al., 1973) with fatty acids impairing cell permeability and the transport of nutrients (Greenway and Dyke, 1979).

1.2.6. Organic Acids

1.2.6.1. Acetic Acid

Acetic acid (CH_3COOH), the primary component of vinegar, is produced by *Acetobacter* species. Acetic acid and its salts are commonly used in variety of different foods (Table 1.3). Acetic acid, like other organic acids, can be used to acidify edible films prepared from chitosan, alginate, collagen and WPI. Addition of acetic acid also increases the activity of other antimicrobial agents such as sorbic acid and benzoic acid that can be incorporated into edible films.

Application of acetic acid and its salts in the meat industry has met with variable success. Adding acetic acid to chiller water (pH 2.5) in poultry processing reportedly extended the shelf-life of poultry (Mountney and O'Malley, 1965). Processing poultry in scald tank water containing 0.1% acetic acid decreased the heat resistance of *Salmonella newport*, *S.* Typhimurium, and *Campylobacter jejuni* (Okrend et al., 1986). However, in another study, incorporating 0.5% acetic acid in scald water had no effect on *Salmonella*, total aerobic bacteria, or members of the family *Enterobactericea* on poultry carcasses (Lillard et al. 1987). Acetic acid solution dips (1 to 3%) can reduce the number of pathogenic and spoilage microorganisms on beef and lamb tissue (Bell et al., 1986; Anderson et al., 1988). Growth of *Listeria innocua*, *S. Typhimurium*, *E. coli* O157:H7, *Clostridium sporogenes*, aerobic bacteria, lactic acid bacteria, and Pseudomonads was suppressed or eliminated in ground beef prepared from carcasses which were washed with 2% lactic acid, 2% acetic acid, and 12% trisodium phosphate solution as compared to the untreated control (Dorsa et al., 1998). The combination of 100 mM NaOH (pH 10.5) and 76.7 mM acetic acid (pH 5.4) applied sequentially at 55°C for 5 min was very effective at eliminating *L. monocytogenes* from biofilms (~ 4.5- to 5.0 logs CFU/cm^2 decrease) (Arizcun et al., 1998)

Acetic acid is variably effective as an antimicrobial when used as a spray on meat carcasses. Washing with water (35°C) followed by a 2% acetic acid spray (55°C) treatment was more effective at reducing the numbers of *E. coli* O157:H7 and S. Typhimurium than trimming or washing alone (Hardin et al., 1995). Similarly, a 5% acetic acid spray decreased populations of *E. coli* O157:H7 on beef tissue by 1 log/cm^2 as compared to the untreated control (Delzari et al., 1998). When used alone or in

combination with a pulsed electric current, another acetic acid treatment reportedly decreased *Salmonella* populations 1 log on carcasses (Tinney et al., 1997).

Acetic acid caused greater inactivation of *L. monocytogenes* than lactic and citric acid with inhibition increasing at lower incubation temperatures. Lactic acid concentrations > 0.3% inhibited *L. monocytogenes* at 13 and 35°C (Ahamad and Marth, 1989, 1990). Addition of 0.1% acetic to TPB minimized the inhibitory effect of 100 and 200 CFU/ml SML during the first 32 h of incubation. *Staphylococcus aureus* was inhibited to a lesser extent when cultured in TSB supplemented with SML alone compared to SML with organic acids (Monk et al., 1996).

A 2% acetic acid wash reportedly killed *L. monocytogenes* in a model system of fresh meat fluids within 24 h while the pathogen increased 1.0 to 2.0 logs using a nonacid water wash (Samelis et al., 2001). Sodium diacetate is also inhibitory to *L. monocytogenes*, *E. coli*, *Pseudomonas fluorescens*, *Salmonella enteritidis*, and *Shewanella putrefaciens* (Shelef and Addala, 1994). Sodium acetate at 1% reportedly increased the shelf life of catfish fillets by 6 days when stored at 4°C compared to the control (Kim et al., 1995). Sodium acetate will also inhibit the rope-forming bacteria and various molds at pH 3.5 to 4.5 in baked goods (Table 1.3) (Glabe and Maryanski, 1981) and prevented mold growth in cheese spreads (Doores, 1993).

Vinegar vapor (4-6% acetic acid) has been used to inactivate conidia of fungal pathogens on various fruits. Vapors of several common vinegars containing 4.2% to 6.0% acetic acid effectively prevented conidia of brown rot (*Monilinia fructicola*), gray mold (*Botrytis cinerea*), and blue mold (*Penicillium expansum*) from germinating and causing decay of stone fruit, strawberries, and apples, respectively. Vapor from 1.0 ml of red

wine vinegar (6.0% acetic acid) eliminated decay on apricot caused by *M. fructicola.* Similarly, vapors from 1.0 ml of white vinegar (5.0% acetic acid) reduced decay on strawberries by *B. cinerea* from 50% to 1.4%. Eight different vinegars, ranging from 4.2% to 6.0% acetic acid, of which 0.5 ml of each vinegar was heat-vaporized, reduced decay by *P. expansum* to 1.0 % or less on apples (Sholberg et al., 2000).

1.2.6.2. Lactic Acid

Lactic acid ($CH_3CHOHCOOH$), produced naturally by lactic acid bacteria during food fermentation, is primarily used for improving and controlling the quality and microbial stability of foods. Lactic acid sprays (1 to 3% solutions) often have been used to sanitize meat surfaces as discussed in several reviews (Smulders, 1986; Cherrigton et al., 1991; Dickson and Anderson, 1992) with psychrotrophic Gram-negative bacteria generally being more sensitive than Gram-positive organisms to this treatment.

Lactates are generally used as humectants and flavor enhancers in meat and poultry products (Duxbury, 1988). Levels of 2% are used in hot dogs, frankfurters and similar products. Evans et al (1991) reported that injecting a 4% lactic acid solution into beef roast before cooking increased the cooking yield (15%) and lowered the aerobic plate count during refrigerated storage.

The antimicrobial activity of lactic acid depends on the food application and the target microorganism. Lactic acid is more effective than malic, citric, propionic or acetic acid in inhibiting growth of *Bacillus coagulans* in tomato juice (Rice and Pederson, 1954). Lactic acid is also inhibitory to spore forming bacteria as well as *S. aureus* and *Y. enterocolitica* (Minor and Marth, 1970; Woolford, 1975; Bracket, 1987).

Like other organic acids, lactic acid can be used in edible film formulations such as chitosan and collagen for acidification purposes. As mentioned earlier, lactic acid also can be used modify both the tensile strength and antimicrobial properties of collagen casings.

1.2.6.3. Mode of action of organic acids

Organic acids inhibit oxygen uptake and resultant ATP production in whole cells of *B. subtilus* (Sheu and Freese, 1972; Freese et al., 1973). However, they do not inhibit NADH oxidation by isolated membranes. Glycerol phosphate- or NADH-energized uptake of serine transport in membrane vesicles yields similar results. Hence, they appear to inhibit growth by uncoupling substrate transport and oxidative phosphorylation from the electron transport system, which in turn inhibits uptake of metabolites by the cell. Sheu et al. (1975), later determined that acetic acid interfered with PME, with these organic acids also acting on cellular enzymes to reduce intracellular pH (Huang et al., 1986).

1.2.7. Bacteriocins

Bacteriocins are antimicrobial substances that have a peptide or protein component essential for their activity. Although most bacteriocins have a narrow spectrum of inhibition and only inhibit closely related species, nisin and pediocin exhibit a broader spectrum of activity and have consequently some attention as antimicrobial additives to edible film.

1.2.7.1. Nisin

Nisin, the first bacteriocin to be used in the food industry, was recognized as a safe biological food preservative by a joint FAO/WHO commission on food additives in 1968 (FAO/WHO 1969). Twenty years later, nisin was accepted by the United States Food and Drug Administration (FDA, 1988). Nisin has been shown to be very effective in cheese products, including processed cheeses and cold-pack cheese spreads (Delves-Broughton, 1990). The legal precedent for use of nisin in U.S. foods was set with pasteurized cheese spreads (900 IU/mg) (Food and Drug Administration, FDA, 1988).

Nisin, a protein of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis*, (Jung, 1991) possesses amphiphilic characteristics with clusters of hydrophobic and hydrophilic residues at the N and C-terminus, respectively. Gross and Morell (1967) identified didehydroalanyllysine and isoleucine at the C- and N-terminus of nisin, respectively (Gross and Morell, 1970). The thioether cross-linkages and highly reactive double bonds are likely responsible for important functional properties of nisin, including acid tolerance, thermostability and bactericidal activity.

One of the mostly investigated bacteriocins for antimicrobial edible film studies, nisin can be incorporated into film solution or applied directly to the film surface after casting. Various nisin-containing protein-based films (e.g. whey protein, corn zein-, wheat protein-, and soy protein) have been assessed for antimicrobial activity against Gram-positive bacteria such as *L. monocytogenes* and lactic acid bacteria. Since nisin is more active in hydrophilic environments, WPI films which contain higher numbers of hydrophilic residues than zein or wheat protein films reportedly produce larger inhibition zones against *L. monocytogenes*.

The solubility, stability and biological activity of nisin are highly pH dependent. Biological activity and stability decrease sharply at higher pH values (Barranova et al., 1976; Liu and Hansen, 1990). Nisin is insoluble at neutral and alkaline conditions (Hirsch, 1951; Hurst, 1981; Liu and Hansen, 1990) and more stable to heat at low rather than high pH (Tramer, 1964). Stability of nisin to heat and storage depends on pH, chemical composition of the solution and the temperature.

Nisin inhibits the majority of Gram-positive bacteria (Table 1.3) (Mattick and Hirsch, 1947; Ogden and Tubb, 1985). The effectiveness of nisin against various bacteria in meat and meat products has been investigated. Nisin can reportedly inhibit the growth of *Bacillus licheniformis* (Bell and DeLack, 1986), *Clostridium sporogenes* (Rayman et al., 1981) and lactic acid bacteria in cured and fermented meat products (Collins-Thompson et al., 1985).

When combined with chelating agents, several reports suggest that nisin may be effective against Gram-negative bacteria in food (Shelef et al., 1995; Mahmoud and El-Baradei, 1998; Wells et al., 1998; Boziaris and Adams, 1999). While the outer membrane of Gram-negative bacteria prevents penetration of nisin into the cytoplasmic membrane (Stevens et al., 1991; Schved et al. 1994), chelating agents such as EDTA as well as sublethal heating, low pH, or freezing disrupt the permeability barrier thus increasing the sensitivity of Gram-negative organisms such as *Salmonella enterica*, *S.* Typhimurium, *E. coli*, and *Shigella* to nisin (Stevens et al., 1991; Kalchayanand et al., 1992; Ganzle et al., 1999). EDTA (0.3mM) and nisin (50μg), in combination with heating (0.3 min) at 37°C, reduced the numbers of *Erwinia carotovora*, *E. chrysanthemi*, *Pseudomonas fluorescens*, and *P. viridiflava* in trypticase soy broth by 2 logs, and at 49°C by 3 logs compared to the

unheated control at 25°C. Nisin activity against some Gram-negative bacteria can be enhanced by trisodium phosphate (TSP). Following a 10 minute exposure to sublethal concentrations of TSP (0.5 to 5 mM), Carneiro de Melo et al. (1998) reported that cell suspensions of *Campylobacter jejuni*, *Escherichia coli*, *Pseudomonas fluorescens* and *Salmonella enteritidis* showed greatly enhanced susceptibility to nisin (1μM). Under optimal conditions at 37°C, viable counts decreased up to 6 logs after 30 minutes. Pulsed-electric field treatment, which induced sublethal injury, also enhanced the bactericidal action of nisin against *Bacillus cereus* and *Escherichia coli* (Pol et al., 2000; Terebiznik et al., 2000).

Activity of nisin is strongly influenced by various environmental factors including pH, temperature and NaCl. Benkerroum and Sandini (1988) showed that *L. monocytogenes* was more sensitive to nisin in tryptose soy broth at pH < 5.94 than at pH 7.0. Increased antimicrobial activity of nisin against various pathogens at low pH was also reported by Harris et al.(1991) and Tatini (1992) with low pH increasing the sensitivity of *E. coli* and *S. enterica* to nisin (Ganzle et al., 1999). Tolerance of vegetative *B. cereus* cells to nisin increased as the pH of the broth increased from 5.53 to 6.01 and 6.57. Nisin activity is also directly related to incubation temperature. According to Tatini (1992), the minimum concentration of nisin needed to inhibit growth of *L. monocytogenes* was two to three times higher at 35 than at 4°C. Similarly, 5 and 50 μg/ml were needed to inhibit outgrowth of *B. cereus* spores at 8 and 15°C, respectively. Harris et al and Ganzle et al. (1999) also reported that addition of 2 to 3% sodium chloride enhanced the activity of nisin against *L. monocytogenes*, *S.* Typhimurium, and *E. coli* (< 10 μg /ml) in laboratory media.

1.2.7.2. Pediocin

Pediocin is produced by *Pediococcus acidilactici*. Among the pediocins isolated from different strains, only pediocin PA1 (*Pediococcus acidilactici* PAC 1.0) and pediocin AcH (*P. acidilactici* LB42-923) have been well characterized. Pediocin AcH, having a molecular mass of about 2700 Da (Bhunia et al., 1987), contains 62 amino acids and two disulfide bonds.

The pediocins are another commonly studied group of bacteriocins for edible film use, due to their wide spectrum of antimicrobial activity and their effectiveness over a wide range of pH and temperature. Antimicrobial activity of pediocin is retained at 100°C, reduced at 121°C and most evident at pH 4-7 with substantial losses at pH <3 or > 9. Pediocin remains active following treatment with lipase, phospholipase C, lysozyme, DNase or RNase, but its activity is destroyed by protease, papain and α-chymotrypsin (Gonzalez and Konka, 1987). While pediocin activity was unaffected during 6 months of frozen storage, over 50% of the activity was lost after 12 weeks at ambient temperature (Ray, 1996).

Pediocin is inhibitory to a broad range of bacteria (Table 1.3). Inhibition of *L. monocytogenes* with pediocin AcH in various foods has been well documented. *L. monocytogenes* numbers decreased by 0.74 log within 2 h in beef wieners that were inoculated with a pediocin-producing strain of *P. acidilactici* (Yousef et al., 1999). Numbers of *L. monocytogenes* increased initially and then markedly decreased with pediocin production by *P. acidilactici* with pediocin activity also detected in the wiener exudate during the last stage of *P. acidilactici* growth. Degnan et al. (1992) investigated

survival of *L. monocytogenes* in temperature-abused, vacuum-packed wieners which contained pediocin- and non-pediocin-producing *P. acidilactici*. *Listeria* populations increased 3.2 logs after 8 days of storage at 25°C in the absence of any *Pediococcus* strain, remained unchanged in the presence of the non-pediocin-producing strain, and decreased by 2.7 logs using the pediocin-producing strain.

Inhibition of *L. monocytogenes* by pediocin-producing starter cultures was reported during fermentation of dry and semi-dry sausage (Berry et al., 1991). Using a pediocin-producing strain of *Pedioccocus* and a nonbacteriocinogenic starter, *L. monocytogenes* populations decreased ~2 logs and <1 log respectively, during fermentation of semi-dry sausage. Antilisterial activity from a pediocin-producing strain also was demonstrated during manufacture of turkey summer sausage (Luchansky et al., 1992) with pediocin and non-pediocin-producing strains decreasing populations of *L. monocytogenes* by 3.4 and 0.9 log CFU/g, respectively, after 12 h of fermentation.

Several investigators observed potential benefits of adding pediocin to dairy products. Pucci et al. (1988) showed that incorporating a crude extract of pediocin PA1 into cottage cheese, cheese sauce and half-and-half decreased the numbers of *L. monocytogenes* in all inoculated samples after the first day of refrigerated storage. The effectiveness of pediocin AcH in controlling *L. monocytogenes* in dry milk, ice cream, and cottage cheese was also studied by Motlagh et al. (1992), who found that pediocin decreased numbers of *L. monocytogenes* by ~1 log in sterile ground beef, sausage mix and cottage cheese after 1 h of storage at 4°C. Pediocin (1350 AU/ml) also reduced *Listeria* populations 2 to 4 logs in dry milk after 1 day of storage at 4°C.

In addition to meat and dairy products, other foods may benefit from biopreservation with pediocin and pediocin-producing strains. Choi and Beuchat (1994) added a bacteriocin extract from *P. acidilactici* M to kimchi during fermentation. This treatment immediately reduced numbers of *L. monocytogenes* in the inoculated product and inhibited growth of the organism during 16 days of fermentation.

1.2.7.3. Mode of Action of Bacteriocins

Bacteriocins disrupt membrane activity by pore formation and may have additional effects on electron transfer chain components. Binding of bacteriocin to the cell membrane results in the formation of peptide pre-aggregates that induce conformational changes and pore formation (Sahl, 1991; Benz et al., 1991; Freund et al., 1991) which in turn decreases the membrane potential and proton gradient across the membrane. For example, Bruno et al (1992) showed that the addition of 2.5 μg of nisin/ml completely dissipated both components of the proton motive force in *L. monocytogenes.* Okereke and Montville (1992) also found that nisin inhibited the growth *C. sporogenes* PA 3679 by dissipating the proton motive force and possibly inhibiting ATPase activity, thereby exhausting intracellular ATP.

Like other bacteriocins, pediocin's bactericidal mode of action against Gram-positive bacteria involves adsorption to lipoteichoic acid receptors on the cell surface followed by penetration through the wall and contact with the cytoplasmic membrane (Gonzales and Kunka, 1987; Bhunia et al., 1991). Gram-negative bacteria do not possess lipoteichoic acid and are therefore unable to adsorb pediocin. Pediocin produces a

conformational change in the cell wall of Gram-positive bacteria which interferes with proper barrier function (Jack et al., 1995)

Sublethally stressed Gram-negative bacteria can become sensitive to bacteriocins even though the molecule is not adsorbed by the cell. In such cells, the cell-surface barrier function is impaired, thus allowing bacteriocins to pass through the impaired wall, come in contact with the cytoplasmic membrane, and destabilize its function leading to cell death. Pediocin is also active against some *Bacillus* and *Clostridium* species (Kalchayanand, 1990), preventing outgrowth of germinated spores.

1.2.8. Natural Antimicrobials

1.2.8.1. Lysozyme

Lysozyme, another popular choice for the production of antimicrobial films, is an enzyme comprised of 129 amino acids, crosslinked by four disulfide bonds. Dried egg white, the commercial source for lysozyme, contains about 3.5% lysozyme. However, lysozyme is also present in mammalian milk, tears and other secretions, insects and fish. It is heat stable (100°C) at pH <5.3, but is inactivated at lower temperatures when the pH is increased (Smolelis and Hartsell, 1952; Matsuka et al., 1966). This remarkable stability is attributed to the four disulfide bonds present in this small protein. Plasticizers used in edible film such as glycerol and sorbitol help stabilize lysozyme against heat through hydrophobic interactions that reduce the complete transfer of hydrophobic groups from an aqueous to a non-polar environment (Yashitake and Shininichiro, 1977; Back et al., 1979). Therefore, lysozyme is highly suited for heat processed films such as heat-pressed corn zein-based films (Dawson et al., 1997; Padget et al., 1998).

Lysozyme is most active against Gram-positive bacteria, most likely because peptidoglycan of the cell wall is more exposed. Penetration through the outer membrane can be accomplished by using either chelating agents or osmotic shock. The outer membrane of Gram-negative organisms contains divalent cations that stabilize lipopolysaccharide within the membrane. Chelating agents remove cations and thus increase cell permeability of Gram-negative bacteria to lysozyme (Hancock, 1984; Samuelson et al., 1985; Hughey et al., 1989; Payne et al., 1994; Razavi- Rohani and Griffits, 1996).

Vedmina et al. (1979) tested the sensitivity of lysozyme against 476 strains of Gram-negative bacteria and found high resistance to lysozyme in *Vibrio cholera* El Tor and *Pseudomonas*. Organisms exhibiting varying sensitivity include *Aeromonas* and enteropathogenic *Escherichia coli*.

1.2.8.1.1. Mode of Action

Lysozyme catalyzes the hydrolysis of β-1,4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan in bacterial cell walls, which leads to cell wall degradation and lysis in hypotonic solutions. Depending on the enzyme source, chitin and certain esters are also susceptible to lysozyme.

1.2.8.2. Spices, Herbs, and Essential Oils

Essential oils are responsible for the odor, aroma, and flavor of spices and herbs. These compounds can be added to edible film to modify flavor, aroma and odor as well as antimicrobial properties. Films containing these ethanol-soluble compounds will also

show activity against both Gram-negative and Gram-positive bacteria, with Gram-positive organisms being more sensitive (Beuchat and Golden, 1989). Ting and Deibel (1992) reported that addition of cloves, oregano, sage, rosemary and nutmeg to TSB inhibited growth of *L. monocytogenes* at 24°C. According to Bahk et al.(1981), addition of 5% cinnamon also inhibited growth of *L. monocytogenes* in TB at 4°C; however, 0.5% ginger, garlic, onion and mustard as well as ginseng, saponin and mulberry extract were noninhibitory.

Activity of garlic powder and cloves was also investigated against *S.* Typhimurium and *S. aureus* by Teeraporn (1995). Results showed that 12% garlic powder and 0.4% clove oil led to a > 5-log reduction of *S.* Typhimurium, whereas 0.4% clove oil and a much higher concentration of garlic powder (58%) were needed for similar inhibition to *S. aureus*.

Antimicrobial activities have been known among various plant essential oils for centuries. However, their use as food additives is limited by their strong flavor. These extracts contain mostly phenolic compounds such as abietane diterpenes (Moujir et al, 1993), carnosol and ursolic acid (Collins and Charles, 1987) which are presumably responsible for their antimicrobial action. Antimicrobial activity and composition of the essential oils from *Micromeria cristata subsp. phrygia* was investigated against Gram-negative and Gram-positive bacteria by Tabanca et al. (2001). The essential oils showed inhibitory activity against *E. coli*, *S.* Typhimurium, *S. aureus*, *P. aeruginosa*, *E. aerogenes*, *P. vulgarus*, and *C. albicans* with the major oil compenent being borneol (39%).

Ethanol extracts of rosemary (Oxy'less) (100 mg/ml) effectively killed *L. monocytogenes, Leuconostoc mesenteroides, S. aureus, Streptococcus mutanss* and *Bacillus cereus*, whereas no activity was observed with Gram-negative bacteria (e.g. *E. coli, Salmonella enteriditis, Erwinia carotovora*) or yeasts (e.g. *Rhodotorula glut, Cryptococcus laurentii*) (Del Campo et al., 2000).

Elgavyar et al. (2000) evaluated the antimicrobial activity of essential oils from parsley, anise, angelica, carrot, cardamom, coriander, dill weed, fennel, oregano and rosemary using a disc diffusion assay. Essential oils from cardoman, coriander, oregano, basil, celery and parsley inhibited growth of *S.* Typhimurium, *Y. enterocolitica, E. coli*, S. *aureus, L. monocytogenes* and *Aspergillus niger*. However, essential oils from dill weed, fennel, carrot, rosemary and anise were noninhibitory to *L. monocytogenes* on standard methods agar (SMA). Inhibition zones were observed for all essential oils except those from carrots against *S.* Typhimurium, *E. coli* and *S. aureus* on SMA.

The effectiveness of some essential oils is enhanced at lower temperatures and lower pH values. Ting and Deibel (1992) showed that refrigeration temperatures increased the inhibitory effect of sage, but not that of cloves or oregano against *L. monocytogenes*. In addition, essential oils from thyme and oregano were ten-fold more effective against *B. cereus* at 8 compared to 30°C (Ultee et al., 1998).

Surface-active agents in foods such as dairy cream and albumin also were shown to reduce the inhibitory effect of essential oils (Davidson, 1993; Tassou and Nychas, 1994; Juven et al., 1994; Del Campo et al., 2000). For example, Juven et al. (1994) showed that serum albumin (9 mg/ml) eliminated the antimicrobial activity of thymol against *S.* Typhimurium.

Essential oils alter the fatty acid composition and phospholipid contact of the cell membrane, which results in the release of cellular constituents that interfere with metabolism, disrupting both electron transport and nutrient uptake. These essential oils also have the ability to disrupt various enzyme systems, including those involved in cellular energy and structural synthesis

1.2.8.3. Lactoferrin

Lactoferrin (lactotransferrin), an iron binding glycoprotein, is present in bovine milk and can bind two iron atoms per molecule (Ashton and Busta, 1968; Reiter, 1983). This protein was shown to effectively inhibit the growth of several bacteria including *Bacillus subtilis*, *B. stearothermophilus*, *L. monocytogenes*, *E. coli*, *Micrococcus* spp, and *Klebsiella* spp. (Oram and Reiter, 1968; Reiter, 1978; Payne et al., 1989; Payne et al., 1990; Korhonen, 1978). Payne et al (1990) showed that lacroferrin had a bacteristatic effect against *L. monocytogenes*. At 30 mg/ml Apo-lactoferrin (iron-free lactoferrin) reduced *Listeria* population 10 fold. At 2.5 mg/ml, lactoferrin had no activity against *S.* Typhimurium or *P. fluorescens* with only minimal activity against *E. coli* O157:H7 and *L. monocytogenes* (Payne et al., 1994). Some Gram-negative bacteria may be lactoferrin resistant due to the presence of siderophores that aid in adaption to low-iron environments (Crichton and Chaloteux-Wauters, 1987). In addition, bacteria with low iron requirements such as lactic acid bacteria would not be adversely impacted by lactoferrin (Reiter and Oram, 1986).

Ashton and Busta (1968) reported that the inhibitory activity of lactoferrin was likely due to chelation of iron as well as calcium and magnesium ions. Inhibition of *L.*

monocytogenes by lactoferrin is directly related to iron availability in the medium, with *L. monocytogenes* surviving best in iron-rich media (Payne et al. 1989). However, Arnold et al. (1982) showed that lactoferrin inhibited many bacteria in an iron-rich environment. Lactoferrin caused the release of anionic polysaccharides from the outer membrane of *E. coli* by chelation of cations that stabilize lipopolysaccharides. Thus, lactoferrin may increase outer membrane permeability to hydrophobic compounds.

Lactoferricin B, the active region of lactoferrin, was isolated by acid-pepsin hydrolysis from the N-terminal region of the molecule (Bellamy et al., 1992). Lactoferricin contains 25 amino acid residues. Bellamy et al. (1992) and Jones et al. (1994) determined that lactoferricin was inhibitory to bacteria at concentrations of 0.3 to 150 μg/ml (Table 1.3). *Pseudomonos fluorescens*, *Enterococcus faecalis* and *Bifidobacterium biffidum* strains were highly resistant to this peptide. These results confirm and expand on earlier inhibition studies with lactoferricin B (Tomita et al., 1992;Wakabayashi et al., 1992). According to Kumar et al. (1999), lactoferricin B (100 μg/g) also reduced *E. coli* O157:H7 populations by 0.8 log CFU/g in ground beef. While the mode of action of lactoferricin has not been fully elucidated, it is thought to alter the permeability of the membrane because of its cationic feature (Conner, 1993; Jones et al., 1994).

1.2.8.3. Liquid Smoke

Liquid smoke is a solution of natural wood smoke flavors prepared by burning a wood (e.g. hickory, maple) and capturing the natural smoke flavors in water.

Alternatively, liquid smoke can be derived from the destructive distillation of a wood - i.e the breakdown or cracking of the wood fibers into chemical constituents which are distilled out of the wood residue. Most liquid smokes are very acidic, although some partially neutralized liquid smokes are also available. While some commercially available smoke can be used at full strength, others are normally diluted in water or another appropriate diluent. Commercial liquid smoke products used in processed meats, sausages and cheeses contain phenols and acetic acid, which are bactericidal at relatively low concentrations. Liquid smoke can inactivate common food-borne pathogens including *E. coli*, *Salmonella*, *S. aureus*, and *L. monocytogenes*.

Several investigators examined the potential of commercial liquid smokes to inactivate *L. monocytogenes* in culture media and meat products. Wendorff (1989) found that liquid smoke compounds (0.5%) were listericidal in both phosphate buffer and processed meats. Faith et al. (1992) also reported that *L. monocytogenes* was inactivated by addition of 0.2 and 0.6% liquid smoke to wiener exudate with D-values of 36 and 4.5 h, respectively; however, numbers of *Listeria* in the untreated exudate increased to 8 logs after 3 days at 25°C. Among 11 individual phenols tested, only isoeugenol showed antilisterial activity in tryptose broth (TB) during incubation at 37°C. In the presence of isoeugenol (100 ppm), greater inhibition of the pathogen was observed in TB acidified with acetic acid to pH 5.8 compared to pH 7.0.

In addition to meat products, antimicrobial activity of liquid smoke was also evaluated against molds on Cheddar cheese (Wendorf et al., 1993). Liquid smoke reportedly inhibited the growth of *Aspergillus oryzae* and increased the lag phase of *Penicillium camemberti* and *P. roqueforti*. Among 8 phenolic compounds tested, only

isoeugenol retarded the growth of all three molds. *P. camemberti* was slightly inhibited by m-cresol and p-cresol, while *A. oryzae* was inhibited by guaiacol, 4-methylguaiacol, m-cresol and p-cresol.

Based on these findings, liquid smokes which possess antimicrobial, antioxidant, color, and flavor properties, have the potential to become attractive edible film additives. Thus a, incorporation of liquid smoke has only been studied for edible collagen casings. Liquid smoke was introduced into the acid-swollen collagen mass before extrusion as a casing or film (Miller, 1975). Since liquid smoke is generally very acidic (pH 2.5 or less), it is compatible with the gel system and, in fact, can replace a portion of the acid normally added to induce swelling. The resultant edible collagen casings with uniformly dispersed liquid smoke reportedly had increased tensile strength and improved film clarity.

1.2.9. Curing Agents

1.2.9.1. Sodium Chloride

Sodium chloride (NaCl), recognized as a food preservative since ancient times, can be used alone or in combination with other preservation techniques such as pasteurization or fermentation. Bacterial food-borne pathogens are generally susceptible to NaCl, except *S. aureus*, which can grow at low water activities (0.83-0.86) (McLean et al., 1968). Another salt-tolerant pathogen is *L. monocytogenes*, which can grow at concentrations up to 10% NaCl and survive for long periods of time in saturated brine solutions. Yeasts and molds are also more tolerant to low water activity than bacteria. with xerotolerant fungi growing at a water activity value as low as 0.61 (Corry, 1987).

Sodium chloride inhibits microbial growth by its plasmolytic effect. The antimicrobial activity of sodium chloride is related to its ability to reduce water activity in the food. Microbial cells lose water when the water activity of the external environment is reduced, which results in growth inhibition or possible death (Sperber, 1983). In addition to this osmotic effect, NaCl limits oxygen solubility, alters pH, and is itself toxic for microbial cells (Banward, 1979).

Incorporation of NaCl into protein-based films as an antimicrobial agent is of limited use since physical properties of protein films decrease with increasing ionic strength of the film solution. At high ionic strength, proteins aggregate to form turbid opaque gels possessing a high-water holding capacity (Doi and Kitabastake, 1997).

1.2.9.2. Nitrite

Sodium nitrite ($NaNO_2$) and potassium nitrite (KNO_2) are primarily used to inhibit *C. botulinium* growth and toxin production in cured meats. Nitrite inhibits bacterial sporeformers by preventing outgrowth of the germinated spores (Cook and Pierson, 1983; Duncan and Foster, 1968). Nitrite effectiveness also depends on other environmental factors. For example, nitrite is more active at low pH and anaerobic conditions (Buchanan and Solberg, 1972; Woods et al., 1989). When used as reducing agents, ascorbate and isoascorbate enhance the antibotulininal action of nitrite (Tompkin et al., 1978; Roberts et al., 1991). Nitrite is also inhibitory to other bacteria including *C. perfringens*, *E. coli*, *Acromobacter*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, and *Pseudemonas* spp. at 200 μg/g (Gibson and Roberts, 1986b). Growth of *L. monocytogenes* was inhibited for 40 days at 5°C by treating smoked salmon with 200

ppm sodium nitrite (Pelroy et al., 1994). However, Gibson and Roberts (1986a, 1986b) found that enteropathogenic *E. coli*, *Salmonella* spp., fecal streptococci, *Lactobacillus* (Castellani and Niven, 1955) and *Bacillus* (Grever, 1974) were resistant to 400 μg/g nitrite when used with 6% salt.

Incorporation of nitrite into edible films has not yet been studied, even though nitrite appears to be a suitable antimicrobial agent for antimicrobial edible film production. In this regard, application of the films containing nitrite on ready-to-eat-meat products may provide a possible solution for preventing growth of *L. monocytogenes* and spoilage bacteria that may contaminate such products after processing, with the potential benefit of also improving surface color.

Nitrite works by inactivating various enzymes or enzyme systems. Using vegetative cells of *C. sporogenes*, Woods et al. (1981) showed that pyruvate-ferrodoxin oxidoreductose and ferrodoxin were susceptible to nitrite. Inhibition of these enzymes causes a reduction in intracellular ATP and excretion of pyruvate. The phosphoroclastic system of *C. botulinium* and *C. pasteurianum* is also inhibited by nitrite (Woods and Wood, 1982; Carpenter et al., 1987). Rowe et al. (1979) showed that the other antimicrobial mechanism for nitrite was inhibition of active transport, oxygen uptake, and oxidative phosphorylation by oxidizing ferrous iron from an electron carrier (Rowe et al., 1979, Yang, 1985)

1.3. ANTIMICROBIAL EDIBLE FILMS

Various antimicrobial edible films have been developed to control the growth of spoilage and pathogenic microorganisms that may contaminate the surface of foods after processing. In most solid foods, contamination and microbial growth occurs on the food surface, which leads to a reduction in product shelf-life. Edible films containing various antimicrobials such as benzoic acid, sorbic acid, propionic acid, lactic acid, nisin, and lysozyme have been investigated to retard the growth of bacteria, yeasts and molds on different product surfaces.

The primary advantage of antimicrobial edible films is that the antimicrobial agents in these films can be specifically targeted to post-processing contaminants on the food surface, with the diffusion rate of the antimicrobial into the product partially controlled by levels incorporated into the film. Guilbert and his research group (Giannakopoulos and Guilbert, 1986; Guilbert et al., 1985; Guilbert et al., 1988) evaluated diffusivity of sorbic acid from casein films in different model systems. Using a cell membrane separated by casein film, their data showed that low temperatures (10°C) decreased the diffusivity of sorbic acid; however, lower water activity had no effect. They theorized that at higher levels, increased networking within the gel acted to restrict the movement of sorbic acid. Vojdani and Torres (1989a, b, 1990) also evaluated the permeability of several polysaccharide-based films prepared both with and without various combinations of lipids and potassium sorbate. Using permeability cells methylcellulose-palmitic acid films appeared to be most promising, with permeability of the film to sorbic acid decreasing from 10^{-8} to 10^{-10} mg/sec.cm^2 as pH increased from 3 to 7 and a_w decreased from 0.8 to 0.65 (Rico-Pena and Torres, 1991).

Chitosan, like other polysaccharides, forms a strong film that can carry a high amount of antimicrobials. Chitosan also has been reported to be a good choice for antimicrobial films due to its good film-forming properties, ability to adsorb nutrients used by bacteria (Darmadji and Izumimoto, 1994), capacity to bind water and inhibit various bacterial enzyme systems (Young et al., 1982). However, neutralized chitosan alone had no effect on bacterial growth when it was applied to the surface of meat products. Antimicrobial chitosan films were subsequently prepared by dissolving chitosan in hydrochloric, formic, acetic, lactic and citric acid solutions (Begin and Calstren, 1999). Films made from hydrochloric, formic and acetic acid were hard and brittle, whereas those containing lactic or citric acid were soft and could be stretched. The same research group designed antimicrobial chitosan films containing acetic acid or propionic acid, with or without the addition of lauric acid or cinnemaldehyde, to improve refrigerated shelf-life of vacuum-packaged processed meats (Ouatarra et al., 2000a). They indicated that film application delayed or completely inhibited enteric bacteria, *Lactobacillus sakei and Serratia liquefaciens,* on meat products. Films containing propionic acid were more effective than films containing acetic acid for reducing growth of *L. sakei* with the opposite observed for *S. liquefaciens*. Diffusion of acetic acid from the film matrix was limited by addition of lauric acid, with 2-22% of acetic acid remaining in the chitosan after 168 h of storage at 4°C. However, propionic acid almost totally diffused from the film after 48 h of storage. Release of organic acids from chitosan film is dependent on many factors, including electrostatic interactions between the acid and polymer chains (Demarger-Andre and Domad, 1994), ionic osmosis and structural changes induced by presence of the acid (Narisawa et al, 1996). Ouattara et al. (2000b)

also tested the impact of temperature (4 to 24°C) and pH (5.7 to 7.0) on diffusion of acetic and propionic acid from chitosan films immersed in water. Whereas diffusion was unaffected by pH, a decrease in temperature from 24 to 4°C decreased the diffusion coefficients for acetic and propionic acid from 2.59 x 10^{-12} m^2/sec to 1.19 x 10^{-12} m^2/sec and 1.87 x 10^{-12} m^2/sec to 0.91 x 10^{-12} m^2/sec, respectively. The dependency of diffusion on temperature is explained by effects on solubility of the diffusing molecule, the nature of adhesive forces at interfaces, and molecular mobility (Vojdani and Torres, 1990; Myint et al., 1996). Ouattara et al. (2000b) stated that addition of lauric acid (1%) or essential oils (0.5%) (cinnemaldehyde or eugenol) decreased the diffusion of propionic acid, since these additives increased film hydrophobicity and modified the pore construction/blind porosity of the film, thereby impairing water uptake and molecular transformation.

Besides propionic acid, fatty acids, and essential oils, sorbate and benzoate also have been tested in methylcellulose and chitosan films. For example, Chen et al. (1996) developed antimicrobial methylcellulose, chitosan and methylcellulose:chitosan films (3:2) containing 2, 4, or 5% sodium benzoate or potassium sorbate. Methylcellulose films containing 2% sorbate or benzoate yielded clear inhibition zones for *Rhotorula rubra* and *Penicillium notatum* on potato dextrose agar. Chitosan films containing 2% sorbate or benzoate yielded no zones of inhibition, since the high affinity between chitosan and the preservatives prevented diffusion of the antimicrobials. Incorporation of both potassium sorbate and sodium benzoate into methylcellulose/chitosan films did not change the tensile strength or percent elongation. In a glycerol-water model system (a_w=0.8), 40% and 50-60% of both antimicrobial agents were released from the films after 6 h at 4 and

25°C, respectively. Another research group from Taiwan evaluated the antimicrobial activity of methylcellulose coatings containing benzoic and palmitic or stearic acid against two osmophilic yeasts (*Zygosaccharomyces rouxii and Zygosaccharomyces mellis*) on Taiwanese-style fruit preserves made from plums (Chen et al. 1999). Coatings containing 50-100 μg/g benzoic acid inhibited *Z. rouxii* and *Z. mellis* at room temperature, with sensory characteristics of preserves such as flavor, texture, appearance and overall acceptability not affected by the coating.

Protein-based edible films are also very good carriers of food additives, including antimicrobial and flavor agents, due to their favorable encapsulated nature. Zein films have been used in conjunction with potassium sorbate to control surface microbial growth. The diffusion barrier properties of zein films were confirmed in microbial tests using a model food system and *S. aureus* as the challenge organism. A reduced preservative diffusion rate due to barrier properties of zein films was identified as the mechanism for product shelf-life enhancement (Torres et al., 1985; Torres and Karel, 1985). The diffusion of sorbic acid from various wheat gluten films into a model food was also measured and modeled. When *Penicillium notatum* was used as the test organism, simple gluten-based films had no fungicidal effect. However, the gluten/lipid-based films showed strong sorbic acid retention and marked fungicidal activity at 30 and 40°C, delaying *P. notatum* growth for more than 21 days.

Similarly, antimicrobial soy and corn protein-based films were developed by Dawson and his lab group at Clemson University. When prepared to contain nisin and lysozyme, these films were inhibitory to Gram-positive bacteria both on solid and in liquid media (Orr et al., 1996a, b; Dawson et al., 1995; Dawson et al., 1997; Padget et al.,

1995; Padget et al., 1998). Addition of EDTA to these films also led to inhibition of Gram-negative organisms (Orr et al., 1996a). Modifying the water permeability by incorporating short chain fatty acids (lauric acid) reduced the effectiveness of nisin on solid media, whereas films with lauric acid were as effective as nisin against Gram-positive bacteria in liquid media (Dawson et al., 1997). Padget et al. (1998) incorporated nisin and lysozyme into soy protein and corn zein films using the heat press and casting methods. Both antimicrobial films containing lysozyme (10 to 133 mg/g film) or nisin (0.1 to 6.0 mg/g film) inhibited *Lactobacillus plantarum* on MRS media. Orr et al. (1998) found that corn zein films containing 150 mg of nisin reduced *L. monocytogenes* populations 1.3 to 2.2 logs in milk after 72 h at 4°C, with no inhibition observed with nisin free-films. Use of zein film coatings containing nisin (1000 IU/g) also reportedly reduced *L. monocytogenes* populations 1 to 3 logs on ready-to-eat chicken during 30 days of refrigerated storage (Janes et al., 1999).

Rodrigues and Han (2000) reported that when incorporated into WPI films, lysozyme and nisin effectively inhibited *Brochotrix thermosphacta* but not *L. monocytogenes*. Addition of EDTA increased the inhibitory effect of these films against *E. coli* and *L. monocytogenes* on trypticase soy agar. Other antimicrobial WPI films containing sorbic acid (SA) or p-aminobenzoic acid (PABA) were developed by Cagri et al. (2001). Both of these films reportedly inhibited the growth of *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium on TSAYE agar. Subsequently, these films were tested between beef bologna and summer sausage slices that were surface inoculated with the same pathogens at a level of 10^6 CFU/g (Cagri et al., 2002a). WPI films containing SA or PABA decreased *Listeria*, *E. coli* and *S.* Typhimurium populations 3.4-4.1, 3.1-

3.6, and 3.1-4.1 logs on bologna and sausage after 21 days of aerobic storage at 4°C, respectively. Growth of mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB) and mold/yeast on slices was also inhibited with WPI films containing SA or PABA compared to antimicrobial-free control films. In the same study, film tensile strength decreased while % elongation remained unchanged following 72 h of product contact. Subsequently, heat-sealed WPI casings containing SA, PABA, or SA:PABA (1:1) were developed for hot dog manufacture, with these casings compared to commercial collagen and natural casings (Cagri et al., 2002b). WPI casings containing PABA inhibited *L. monocytogenes* growth on hot dogs during 42 days of refrigerated storage; however, films with SA or SA:PABA were less effective. Sensory (texture, flavor, juiciness, overall acceptability), chemical (TBA, pH, moisture, fat, protein), physical (purge, color), and mechanical (shear force) characteristics of hot dogs with WPI casings containing PABA were comparable to hot dogs prepared with collagen and natural casings. Consequently, WPI casings containing PABA may eventually prove useful in minimizing risk of *Listeria* growth on hot dogs.

Ko et al. (2001) also tested the antilisterial activity of nisin (200 – 8000 IU/g film) when incorporated into WPI, soy protein isolate, egg albumin, and wheat gluten films. All of these films inhibited *Listeria,* with greatest activity observed at low pH (2.0 or 3.0). WPI films containing nisin were most effective against *L. monocytogenes* due to their increased hydrophobicity, with their mechanical properties also remaining unchanged by the addition of nisin.

Various edible antimicrobial films have also found use in vegetables to prevent the growth of spoilage and pathogenic bacteria. Zhuang et al. (1996) investigated use of

antimicrobial cellulose-based edible films containing citric acid, acetic acid, or sorbic acid (0.2 to 0.6%) on tomatoes inoculated with *Salmonella montevideo*. Although coating with a hydroxypropyl methylcellulose (HPMC) solution reduced *Salmonella* populations by 4.5 logs on the surface of tomatoes; a reduction of only 2.0 logs was achieved in core tissue. *S. montevideo* cells penetrating into the core tissue when tomatoes were dipped in the 30°C bacterial suspension were likely protected from inactivation during coating (Zhuang et al.,1995). Among the antimicrobials tested in HPMC films, only 0.4% sorbic acid enhanced the inactivation of *S. montevideo* (~1.0 log) on the surface of tomatoes. However, tomatoes coated in HPMC containing 0.4% sorbic acid appeared chalky, less firm and exhibited color changes that may limit possible commercial applications.

Most recently, Cha et al. (2001) reported that nisin-containing κ-carrageenan, MC and HPMC films prepared by either a heat press or casting method were inhibitory to *Micrococcus luteus* in agar well diffusion assay. Nisin reportedly diffused faster from MC than from κ-carrageenan or HPMC films. Not surprisingly, the heat-pressed films had lower antimicrobial activity than the cast films.

In conclusion, the use of edible films or coatings on various food products continues to expand. The numerous benefits of edible films as carriers of antimicrobial as well as flavors, antioxidants and color agents justifies further research in this field. Edible films containing antimicrobial agents have been shown to effectively inhibit both pathogenic and spoilage organisms on a wide variety of ready-to-eat food surfaces. These films have the ability to control the diffusion rate of the antimicrobial agent and also serve as a good barrier against oxygen and water vapor transmission. The antimicrobial edible films appeared to be a possible solution to reduce the incidence of pathogens

especially *L. monocytogenes* on food surfaces. However, only some of these antimicrobial edible films received commercial acceptance. Further research is needed for development of feasible application methods for the industry such as extrusion of film solution into tubular shaped film in various sizes for processed meat products.

CHAPTER 2.

ANTIMICROBIAL, MECHANICAL, AND MOISTURE BARRIER PROPERTIES OF LOW PH WHEY PROTEIN- BASED EDIBLE FILMS CONTAINING P-AMINOBENZOIC OR SORBIC ACIDS

Cagri, A., Ustunol, Z., Ryser, E.T.

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2.1. ABSTRACT

Low pH (5.2) whey protein isolate–based edible films containing p-aminobenzoic acid (PABA) or sorbic acid (SA) were developed and assessed for inhibition of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium DT104 in a disc diffusion assay. Water vapor permeability (WVP), tensile strength (TS), and percent elongation (% E) were also determined. Using 1.5% PABA and SA, average inhibition zone diameters were 21.8, 14.6, 13.9 and 26.7, 10.5, 9.7 mm for *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium DT104, respectively. Three strains of *S.* Typhimurium DT104 were resistant to 0.5% SA. Addition of PABA and SA increased %E, but decreased TS. WVP was not affected by 0.5 and 0.75% SA; however, PABA increased WVP.

2.2. INTRODUCTION

Microbial stability of a food surface is a major determinant of product quality and safety during storage and distribution since most Class I product recalls in the U.S. result from post-processing contamination during subsequent handling and packaging. In December 1998, new food safety concerns were raised when consumption of hot dogs was traced to over 100 cases of listeriosis, including 21 fatalities in 22 states (CDC, 1999). A nationwide recall was subsequently issued for 35 million pounds of contaminated product. This pathogen continues to threaten the processed meat industry. Sixty-three of 97 microbiologically related Class I recalls issued from January 1999 to October 2000 involved a total of more than 3.5 million pounds of cooked/ready-to-eat

meats contaminated with *Listeria monocoytogenes* (USDA-FSIS, 2000). Two additional foodborne pathogens, namely *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 are also raising considerable public health concerns. *E. coli* O157:H7 has been responsible for many widely publicized outbreaks involving ground beef (Bell et al., 1994), fermented meat products (Tilden et al., 1996) and fresh produce (Besser et al., 1993). In addition, over 30 million pounds of raw ground beef have been recalled since 1995 due to *E. coli* O157:H7 contamination (USDA-FSIS, 2000). *S.* Typhimurium DT104, a multiantibiotic resistant strain, is also emerging as a serious foodborne pathogen of public health concern with 103 of 306 (34%) *S.* Typhimurium isolates serotyped at CDC (Centers for Disease Control and Prevention) resistant to ampicilin, chloramphenical, streptomycin, sulfonomides and tetracyclines (Glynn et al. 1998).

Incorporating antimicrobial compounds into edible films or coatings provides a novel means for enhancing the safety and shelf life of ready-to-eat foods. Dawson et al. (1997) and Padget et al. (1998) used nisin and pediocin in soy protein and corn zein films to inhibit *Lactobacillus plantarum* and *E. coli* on laboratory media. Antimicrobial edible films are receiving attention as a potential pathogen intervention strategy for various muscle foods. Sirugusa and Dickson (1993) demonstrated that calcium alginate coatings containing organic acids were marginally effective on beef carcasses, reducing levels of *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7 by 1.80, 2.11, and 0.74 logs, respectively. According to Ming et al. (1997), pediocin-coated cellulosic casings inhibited *L. monocytogenes* on ham, turkey breast meat, and beef. In addition, McDade et al. (1999) reported that dipping frankfurters in an aqueous whey protein solution (pH 5.2)

containing propionic/sorbic acid prevented growth of *L. monocytogenes* on the product during the first 2 to 3 weeks of storage at 4 °C.

Sorbic acid, p-aminobenzoic acid, lactic acid and acetic acid have a long history as GRAS food preservatives. The World Health Organization has set the acceptable daily intake for sorbic and p-aminobenzoic acid at 25 and 30 ppm, respectively (Kabara et al., 1991). When used in combination with lactic and/or acetic acid, sorbic acid can inhibit the growth of *L. monocytogenes*, *S.* Typhimurium, and *E. coli* O157:H7 in many low acid foods including cold-pack cheese (Ryser and Marth, 1988), bologna (Wederquist et al., 1994), beaker sausage (Hu and Shelef, 1996) and apple cider (Zhao et al., 1993; Uljas and Ingham, 1999). p-Aminobenzoic acid reportedly exhibited greater inhibitory activity against *L. monocytogenes*, *E. coli* and *Salmonella enteritidis* than formic, propionic, acetic, lactic or citric acids (Richards et al., 1995).

Use of whey protein antimicrobial-containing films as a casing for frankfurters appears to be a promising means of retarding surface microbial growth, thereby enhancing product safety and extending shelf life. However, if used as a sausage casing, the mechanical properties of such an edible film (that is tensile strength, percentage elongation and water vapor permeability) are of equal importance if the film is to function properly and provide adequate physical protection for the product during production and storage. Consequently, our objectives were to (1) develop an edible film (pH 5.2) from whey protein isolate (WPI) containing p-aminobenzoic acid (PABA) or sorbic acid (SA) that is inhibitory to *L. monocytogenes, E. coli* O157:H7 and *S.* Typhimurium DT104 and (2) assess the film for water vapor permeability (WVP), tensile strength (TS) and percentage elongation (%E) at break.

2.3. MATERIALS AND METHODS

2.3.1. Film Preparation

Whey protein isolate (WPI, Alacen 895) (New Zealand Milk Products, North America, Inc., Santa Rosa, Ca., USA) (5% w/v) and glycerol (Sigma Chemical Co., St. Louis, Mo.) (2% w/v) were dissolved in distilled water containing 0.04% $CaCl_2$ (w/v) (Sigma). After mixing and adjusting the pH to 8.0 with 1.0 N NaOH, the solution was heated at 90 °C for 30 min in a shaking water bath (170 Marcel Drive water bath, Precision Scientific, Winchester, VA). Following the addition of candelilla wax (Stahl Pash, Inc., New York, NY) (0.4%, w/v) during the last 5 min of heating, the solution was homogenized for 2 min in a SD-45 homogenizer (Tekmar Co., Cincinnati, Ohio, USA), filtered through cheese cloth and cooled to 23 ± 2 °C. After incorporating 0.5, 0.75, 1.0, or 1.5% (w/v) sorbic acid (SA) or p-aminobenzoic acid (PABA), the pH was adjusted to 5.2 using three different solutions of lactic acid and acetic acid at ratios of 1:0, 1:1, and 7:3 lactic acid (1.0 N): acetic acid (1.0 N). Following degassing by vacuum, the whey protein solution (40 ml / plate) was cast by pipetting the solution into sterile 17 cm-diameter Teflon plates. The solutions were dried for approximately 24 h at 23 ± 2 °C / 50 ± 5% RH, after which the films were peeled from the plates and stored at 23 ± 2 °C / 50 ± 5% RH until used.

2.3.2. Bacterial Strains

Four strains of *Listeria monocytogenes* (CWD 95 and CWD 249 from silage, CWD 201 from raw milk, and CWD 1503 from ground turkey) and three strains of

Escherichia coli O157:H7 (AR, AD 305, AD 317) were obtained from C. W. Donnelly (Dept. of Nutrition and Food Sciences, University of Vermont, Burlington, Vt., USA). Five strains of *Salmonella* Typhimurium DT104 (G01074, G11601, G10931, G10601, G10127) were obtained from B. Swaminathan (Centers for Disease Control and Prevention, Atlanta, Ga., USA). All strains were maintained at –70 °C in trypticase soy broth containing 10% (v/v) glycerol and subcultured twice in trypticase soy broth containing 0.6% (w/v) yeast extract (Difco Laboratories, Detroit, Mich., USA) at 35 °C / 18-24 h before use.

2.3.3. Diffusion-type Assay

WPI films were aseptically cut into 16-mm diameter discs using a sterile cork borer. The discs were then aseptically transferred to pour plates containing exactly 15 ml of either trypticase soy agar + 0.6% yeast extract (TSAYE) (pH ~6.5) or TSAYE acidified to pH 5.2 with 1.0 N lactic acid (Difco) which had been previously seeded with 0.1 ml of an 18-24 h culture of the test organism. After 24 h of incubation at 35°C, the diameter of the inhibition zone around the edible film disc was measured perpendicularly to the nearest millimeter. The end result was the average of two measurements.

2.3.4. Film Thickness

A model M micrometer (Testing Machine Inc., Amityville, N.Y., USA) was used to determine film thickness. Measurements were taken at five different locations and the mean value was used in further calculations for moisture barrier and mechanical properties.

2.3.5. Mechanical Properties

Films were cut into strips measuring 101.6 mm by 25.4 mm using a Precision Sample Cutter (Thawing Albert Instrument Co., Philadelphia, Pa., USA). All films were conditioned for 48 hours at 23 ± 2 °C / 50 ± 5% RH before testing. Tensile strength (TS) and percent elongation at break (%E) were determined according to standard D-882-91 (ASTM, 1992). The test was run using the Instron Universal Testing Machine Model 2401 (Canton, Mass., USA) at 23 ± 2 °C / 50 ± 5% RH with a static load cell of 1 kN and a cross head speed of 50.8 cm/min. TS was calculated in MPa from the following equation:

TS = load /(sample width × sample thickness)

% Elongation at break was determined by the following equation:

% E = (distance sample stretched / original length of sample) × 100

2.3.6. Water Vapor Permeability

Standard Method E96 - 80 (ASTM 1992) was used in which the film was sealed on top of an aluminum test cup containing desiccant (calcium sulfate) and then placed in a chamber at 37 °C / 85% RH. The area of the cup mouth was 54 cm^2 and the cup well depth was 1.1 cm. Cups were weighed at 2-h intervals during ~12 h of controlled storage. Square edible film samples having a surface area of 9 cm^2 were placed in the chamber to examine moisture absorption. WVP was calculated from the water vapor transmission rate through film, the partial vapor pressure difference between the two sides of the film, and the thickness according to McHugh et al. (1993).

2.2.7. Statistical Analysis

All experiments were replicated three times using a complete randomized design. Two- way analysis of variance (ANOVA) was performed using the SAS Statistical Analysis System (SAS Institute Inc., 1990). Means were compared using the Duncan Grouping test at p=0.05.

2.4. RESULTS AND DISCUSSION

2.4.1. Antimicrobial Properties

Increasing the concentration of PABA and SA in the film discs increased the diameter of inhibition zones for *L. monocytogenes* (4 strains), *E. coli* O157:H7 (3 strains), and *S.* Typhimurium (5 strains) on TSAYE (pH 5.2) ($p<0.05$). SA and PABA are weak acids and are most effective in the undissociated form (Luck, 1980) due to their increased ability to penetrate the cytoplasmic membrane of bacteria. (Chichester and Tanner,1972). At pH 5.2 and 6.5, 28.48 and 1.25 % and 26.18 and 1.11 % of SA (pKa = 4.75) and PABA (pKa = 4.8) is undissociated. Hence, no inhibition was observed in TSAYE adjusted to pH 6.5 (results not shown). Control films (pH 5.2) without antimicrobials were non-inhibitory. Therefore, the antimicrobial containing films developed in our study would be best suited for foods such as meats and cheeses that have pH values $\leq$ 5.2.

All *L. monocytogenes* strains were inhibited using WPI film discs (pH 5.2) containing SA or PABA at levels of 0.5, 0.75, 1.0 or 1.5% with inhibition zones ranging from 12.0 to 32.0 and 4.0 to 27.0 mm, respectively (Table 2.1, 2.2). El-Shenawy and Marth (1988) also showed that *L. monocytogenes* was inhibited when 0.2 to 0.3%

Table 2.1. Antimicrobial activities of whey protein based edible films containing p-aminobenzoic acid against 4 strains of *L. monocytogenes*

		Diameter of Inhibition Zone (mm)			
PABA. (%)(w/v)	LA:AA*	CWD 95	CWD 249	CWD 201	CWD 1503
0	1:0	0^{a}	0^{a}	0^{a}	0^{a}
	7:3	0^{a}	0^{a}	0^{a}	0^{a}
	1:1	0^{a}	0^{a}	0^{a}	0^{a}
0.50	1:0	13.3 ± 2.6^{bcd}	8.7 ± 2.3^{bc}	15.0 ± 1.7^{cd}	11.7 ± 2.6^{cd}
	7:3	9.3 ± 1.5^{bc}	8.0 ± 2.2^{bc}	8.3 ± 3.7^{def}	17.0 ± 3.2^{def}
	1:1	8.0 ± 0.8^{ab}	4.3 ± 1.2^{bc}	5.3 ± 0.1^{ab}	4.0 ± 0.2^{ab}
0.75	1:0	18.0 ± 0.6^{def}	15.0 ± 1.6^{ab}	20.0 ± 0.5^{cde}	13.0 ± 1.2^{cde}
	7:3	13.0 ± 0.4^{bcd}	12.0 ± 0.9^{cd}	17.0 ± 1.8^{cde}	14.0 ± 1.5^{cde}
	1:1	14.0 ± 3.2^{cde}	8.7 ± 0.4^{bcd}	12.3 ± 1.1^{bc}	9.0 ± 3.5^{bc}
1.00	1:0	21.0 ± 0.4^{ef}	17.0 ± 5.3^{bc}	22.3 ± 0.5^{def}	19.3 ± 2.3^{def}
	7:3	18.3 ± 2.9^{def}	19.3 ± 4.9^{de}	16.0 ± 0.6^{ef}	20.3 ± 3.5^{ef}
	1:1	18.7 ± 2.3^{def}	13.0 ± 5.7^{de}	18.3 ± 2.1^{def}	17.3 ± 3.8^{def}
1.50	1:0	24.7 ± 4.6^{f}	19.7 ± 3.3^{cd}	25.0 ± 3.8^{f}	22.3 ± 4.1^{f}
	7:3	22.8 ± 0.1^{ef}	22.8 ± 0.9^{de}	22.7 ± 0.1^{ef}	16.7 ± 3.2^{def}
	1:1	20.3 ± 2.3^{def}	18.1 ± 1.1^{e}	27.0 ± 0.7^{ef}	20.3 ± 5.8^{ef}

Geometric mean ± standard deviation (n=3). Means in same column with different superscript are significantly different ($p<0.05$).

*Ratio of lactic acid (LA) to acetic acid (AA).

Table 2.2. Antimicrobial activities of whey protein based edible films containing sorbic acid (SA) against 4 strains of *L. monocytogenes*

SA (%)(w/v)	LA:AA*	Diameter of inhibition zone (mm)			
		CWD 95	CWD 249	CWD 201	CWD 1503
0	1:0	0^{a}	0^{a}	0^{a}	0^{a}
	7:3	0^{a}	0^{a}	0^{a}	0^{a}
	1:1	0^{a}	0^{a}	0^{a}	0^{a}
0.50	1:0	19.0 ± 2.3^{b}	19.3 ± 5.7^{bc}	12.3 ± 2.1^{bc}	13.7 ± 3.8^{a}
	7:3	18.0 ± 1.3^{b}	12.3 ± 3.1^{ab}	21.3 ± 3.7^{cd}	12.7 ± 3.2^{a}
	1:1	17.0 ± 1.3^{b}	17.0 ± 1.5^{bc}	15.3 ± 0.1^{bcd}	18.0 ± 1.2^{ab}
0.75	1:0	21.0 ± 1.9^{bc}	20.0 ± 0.2^{bc}	12.0 ± 0.7^{b}	23.0 ± 0.5^{abc}
	7:3	24.7 ± 4.2^{bc}	16.7 ± 3.4^{bc}	21.7 ± 1.4^{cd}	25.7 ± 2.5^{bc}
	1:1	27.3 ± 0.6^{bc}	21.7 ± 3.0^{bc}	20.3 ± 1.1^{cd}	28.3 ± 2.5^{c}
1.00	1:0	27.0 ± 3.3^{bc}	23.3 ± 1.9^{bc}	26.3 ± 1.2^{e}	27.7 ± 3.3^{bc}
	7:3	29.7 ± 2.5^{bc}	25.7 ± 1.3^{bc}	24.0 ± 0.8^{de}	28.3 ± 1.5^{bc}
	1:1	30.0 ± 2.8^{bc}	25.7 ± 3.2^{bc}	23.3 ± 1.2^{de}	30.0 ± 3.8^{c}
1.50	1:0	31.3 ± 2.5^{c}	30.3 ± 0.4^{c}	22.3 ± 3.8^{de}	30.7 ± 3.1^{c}
	7:3	27.3 ± 3.1^{bc}	13.7 ± 0.9^{ab}	20.7 ± 1.1^{cd}	30.0 ± 2.0^{c}
	1:1	25.3 ± 4.0^{b}	30.0 ± 4.8^{c}	27.7 ± 1.8^{e}	32.0 ± 3.8^{c}

Geometric mean ± standard deviation (n=3). Means in same column with different superscript are significantly different ($p<0.05$).

*Ratio of lactic acid (LA) to acetic acid (AA).

potassium sorbate was added to trypticase soy broth at pH 5.0. Films containing SA were generally more inhibitory to *L. monocytogenes* than films containing PABA. McDade et al. (1999) reported that growth of *L. monocytogenes* was inhibited on frankfurters during 2 to 3 weeks of storage at 4 °C by coating the frankfurters with a whey protein film-forming solution that contained propionic/sorbic acid (pH 5.2). However, non-uniformity of the antimicrobial coating on frankfurters after dipping, draining and drying would likely produce a less effective antimicrobial barrier as compared to pre-casted films. In our study, all antimicrobial edible films were uniform in thickness. Consequently, these films would be better suited to inhibit post-processing surface contaminants such as *L. monocytogenes.*

Film discs containing SA or PABA also were inhibitory to *E. coli* O157:H7 with inhibition zones ranging from 0.7 to 13.3 mm and 5.3 to 21.3 mm, respectively (Table 2.3, 2.4). When used at concentrations of 0.5, 0.75, or 1.0%, PABA was more effective against *E. coli* than SA. Richards et al., (1995) and Tsai and Chou (1996) showed similar inhibition of *E. coli* O157:H7 on laboratory media using PABA and SA, respectively.

Using SA and PABA, inhibition zones for *S.* Typhimurium DT104 ranged from 0 to 12.2 mm and 3.0 to 16.3 mm, respectively (Table 2.5, 2.6). Films containing PABA inhibited all strains of *S.* Typhimurium DT104 on TSAYE, whereas film discs containing 0.5 and 0.75% SA and lactic acid:acetic acid (1:0 and 7:3) failed to inhibit three strains of *S.* Typhimurium DT104 (G10127, G10931 and G10601). While laboratory media containing 0.2 or 0.5% SA is reportedly bacteriostatic to *Salmonella* at pH 5.5 (Restaino et al., 1981; Elliot and Gray, 1981), the amount of SA released from our film discs containing 0.5 or 0.75% SA was presumably too low to inhibit these three strains.

Table 2.3. Antimicrobial activities of whey protein based edible films containing p-aminobenzoic acid (PABA) against 3 strains of *Escherichia coli* O157:H7

PABA (%)(w/v)	LA: AA*	Diameter of Inhibition zone (mm) AR (Acid Resistant)	AD 305	AD 317
0	1:0	0^{a}	0^{a}	0^{a}
	7:3	0^{a}	0^{a}	0^{a}
	1:1	0^{a}	0^{a}	0^{a}
0.50	1:0	5.7 ± 1.2^{cb}	7.7 ± 0.5^{b}	0.7 ± 0.2^{a}
	7:3	7.0 ± 0.8^{bcd}	9.7 ± 0.5^{bc}	1.7 ± 0.4^{a}
	1:1	5.3 ± 1.2^{b}	10.0 ± 1.6^{bc}	4.0 ± 0.2^{a}
0.75	1:0	7.3 ± 2.2^{bcd}	9.2 ± 1.3^{b}	3.0 ± 1.3^{a}
	7:3	7.7 ± 1.1^{bcd}	12.8 ± 1.2^{bc}	2.0 ± 0.2^{a}
	1:1	7.5 ± 0.2^{bcd}	11.8 ± 0.9^{bc}	2.7 ± 0.3^{a}
1.00	1:0	10.8 ± 0.2^{ef}	12.5 ± 0.5^{bc}	3.0 ± 0.2^{a}
	7:3	10.3 ± 2.3^{def}	12.3 ± 3.0^{bc}	5.3 ± 0.1^{ab}
	1:1	9.0 ± 0.6^{def}	12.5 ± 1.2^{bc}	3.0 ± 0.7^{a}
1.50	1:0	13.2 ± 2.2^{f}	15.8 ± 0.2^{cd}	9.5 ± 0.9^{bc}
	7:3	13.0 ± 1.0^{f}	15.8 ± 1.2^{cd}	10.3 ± 1.5^{bc}
	1:1	13.2 ± 0.4^{f}	21.3 ± 1.8^{cd}	11.0 ± 1.0^{c}

Geometric Mean ± standard deviations (n=3). Means in same column with different superscript are significantly different ($p<0.05$).
*Ratio of lactic acid (LA) to acetic acid (AA).

Table 2.4. Antimicrobial activities of whey protein based edible films containing sorbic acid (SA) against 4 strains of *E. coli* O157:H7

SA(%)(w/v)	LA: AA*	Diameter of inhibition zone (mm)		
		AR (Acid Resistant)	AD 305	AD 317
0	1:0	0^{a}	0^{a}	0^{a}
	7:3	0^{a}	0^{a}	0^{a}
	1:1	0^{a}	0^{a}	0^{a}
0.50	1:0	1.7 ± 0.2^{ab}	0.7 ± 0.3^{a}	0.7 ± 0.2^{a}
	7:3	1.7 ± 0.5^{ab}	2.3 ± 0.3^{a}	1.7 ± 0.4^{a}
	1:1	2.3 ± 0.3^{abc}	4.0 ± 0.4^{abc}	4.0 ± 0.2^{a}
0.75	1:0	2.0 ± 0.2^{abc}	2.7 ± 0.2^{ab}	3.0 ± 1.3^{a}
	7:3	4.3 ± 1.3^{abc}	4.3 ± 0.5^{abc}	2.0 ± 0.2^{a}
	1:1	2.3 ± 0.3^{abc}	1.7 ± 0.4^{a}	2.7 ± 0.3^{a}
1.00	1:0	5.0 ± 1.2^{bcd}	5.0 ± 1.1^{abc}	3.0 ± 0.2^{a}
	7:3	5.3 ± 1.2^{bcd}	4.5 ± 0.4^{abc}	5.3 ± 0.1^{ab}
	1:1	8.3 ± 0.5^{cde}	7.5 ± 1.2^{bc}	3.0 ± 0.7^{a}
1.50	1:0	9.0 ± 3.2^{cde}	8.3 ± 2.1^{cd}	9.5 ± 0.9^{bc}
	7:3	11.3 ± 1.5^{de}	10.0 ± 0.7^{d}	10.3 ± 1.5^{bc}
	1:1	13.3 ± 0.9^{e}	12.3 ± 0.3^{d}	11.0 ± 1.0^{c}

Geometric mean ± standard deviation (n=3). Means in same column with different superscript are significantly different ($p<0.05$).
*Ratio of lactic acid (LA) to acetic acid (AA).

In accordance with previously published data (Ahamad and Marth, 1989; Richards et al., 1995), acetic acid is more inhibitory to *L. monocytogenes, E. coli* O157:H7, and *Salmonella* in laboratory media at the same pH value than lactic acid. Three ratios of lactic acid: acetic acid (1:0, 7:3, or 1:1) were used to adjust the pH of our film solutions. We expected that the solution containing more acetic acid (1:1) would be most inhibitory based on the aforementioned studies. However, incorporating three different ratios of lactic acid:acetic acid in films containing PABA or SA did not synergistically alter inhibition of the three test pathogens on TSAYE at pH 5.2.

The antimicrobial findings in this study are based on the measurement of clear inhibition zones surrounding film disks where growth of the pathogen was inhibited. Diffusion of antimicrobials from the film disc depends on the size, shape, and polarity of the diffusing molecule as well as the chemical structure of the film and the degree of molecular cross-linking (Guilbert, 1986). According to Michaels et al., (1962), the shape of the diffusing molecule (linear, branched or cyclic) may impact the diffusion rate. When Chen et al., (1996) measured diffusion rates for SA and benzoic acid from chitosan film in a water-glycerol solution (a_w = 0.8), more SA (57%) was released than benzoic acid (65%). The different interactions of SA and PABA in our WPI-based edible films likely resulted in different diffusion rates leading to varying degrees of inhibition.

2.4.2. Mechanical properties

Average film thickness was 127.11μm (± 35.39) with no significant differences observed between films (Table 2.7). When SA and PABA concentrations increased from 0 to 1.5%, % E increased from 6.37% to 74.28 and 42.16%, respectively (Table 2.7). While TS of WPI films significantly decreased with increasing levels of SA ($p<0.05$)

Table 2.5. Antimicrobial activities of whey protein based edible films containing p-aminobenzoic acid (PABA) against 5 strains of *Salmonella* Typhimurium DT104

PABA (%)(v/w)	LA: AA*	Diameter of Inhibition zone (mm)				
		G10931	G10127	G01074	G10601	G11601
0	1:0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	7:3	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	1:1	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
0.50	1:0	6.8 ± 0.3^{b}	6.7 ± 0.5^{bc}	6.7 ± 1.5^{bc}	7.3 ± 1.6^{bc}	4.7 ± 0.8^{b}
	7:3	9.2 ± 0.2^{bcd}	8.3 ± 0.4^{c}	7.7 ± 2.5^{bcd}	6.8 ± 2.4^{bcd}	6.3 ± 0.1^{bc}
	1:1	7.0 ± 0.6^{bc}	3.0 ± 0.5^{ab}	4.7 ± 1.5^{b}	7.7 ± 1.2^{b}	6.2 ± 1.6^{bc}
0.75	1:0	9.7 ± 1.5^{bcd}	8.7 ± 0.2^{c}	7.7 ± 0.6^{bcd}	8.5 ± 2.0^{bcd}	4.7 ± 2.8^{b}
	7:3	11.3 ± 1.5^{cde}	7.7 ± 0.4^{c}	8.3 ± 1.5^{cd}	10.7 ± 0.5^{bc}	8.5 ± 0.5^{bc}
	1:1	8.5 ± 2.3^{bcd}	3.0 ± 0.7^{ab}	7.5 ± 2.9^{bcd}	9.2 ± 2.0^{cd}	7.8 ± 1.3^{bc}
1.00	1:0	12.0 ± 2.8^{bcd}	9.8 ± 2.3^{cd}	9.8 ± 2.3^{cd}	10.0 ± 2.1^{cd}	5.0 ± 2.5^{b}
	7:3	13.7 ± 1.2^{de}	10.2 ± 3.6^{cd}	10.2 ± 3.6^{de}	11.2 ± 1.3^{de}	10.5 ± 2.6^{c}
	1:1	11.0 ± 1.5^{bcd}	6.0 ± 0.5^{bc}	9.0 ± 1.0^{cd}	11.7 ± 1.1^{cd}	10.2 ± 0.5^{c}
1.50	1:0	16.3 ± 1.0^{e}	14.0 ± 1.8^{d}	15.3 ± 2.5^{f}	14.0 ± 3.0^{f}	14.0 ± 2.4^{d}
	7:3	14.5 ± 1.3^{bcd}	13.7 ± 3.2^{d}	13.8 ± 1.1^{f}	13.7 ± 2.1^{f}	13.7 ± 1.9^{cd}
	1:1	15.0 ± 1.8^{cde}	10.3 ± 0.6^{cd}	13.0 ± 3.2^{ef}	15.3 ± 0.6^{ef}	14.3 ± 0.1^{d}

Geometric mean ± standard deviations (n=3). Means in same column with different superscript are significantly different ($p<0.05$).

*Ratio of lactic acid (LA) to acetic acid (AA).

Table 2.6. Antimicrobial activities of whey protein based edible films containing sorbic acid (SA) against 5 strains of *Salmonella* Typhimurium DT104

SA (%)(v/w)	LA: AA*	Diameter of Inhibition Zone (mm)				
		G10931	G10127	G01074	G10601	G11601
0	1:0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	7:3	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	1:1	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
0.50	1:0	0^{a}	0^{a}	1.7 ± 0.1^{ab}	2.0 ± 0.3^{ab}	3.2 ± 0.7^{ab}
	7:3	0^{a}	0^{a}	5.3 ± 0.7^{abc}	4.0 ± 0.8^{bcd}	0^{a}
	1:1	3.3 ± 0.1^{a}	0.7 ± 0.2^{a}	1.0 ± 0.2^{ab}	4.5 ± 1.5^{bcd}	3.0 ± 0.5^{ab}
0.75	1:0	4.9 ± 0.4^{ab}	0^{a}	1.7 ± 0.5^{ab}	2.7 ± 0.9^{abc}	1.7 ± 0.3^{ab}
	7:3	6.3 ± 0.5^{bcd}	2.0 ± 0.2^{a}	7.3 ± 0.9^{abc}	7.7 ± 2.3^{def}	2.0 ± 0.8^{ab}
	1:1	5.3 ± 1.4^{bcd}	2.7 ± 0.5^{ab}	5.7 ± 1.4^{abc}	5.7 ± 0.3^{cde}	2.0 ± 0.1^{ab}
1.00	1:0	7.5 ± 1.1^{bcd}	5.0 ± 0.2^{ab}	5.3 ± 0.3^{abc}	5.3 ± 1.5^{cde}	6.2 ± 0.4^{bc}
	7:3	5.3 ± 0.9^{bcd}	4.7 ± 0.3^{ab}	6.2 ± 1.4^{bcd}	7.2 ± 2.7^{de}	6.0 ± 1.2^{bc}
	1:1	8.3 ± 1.5^{cd}	8.0 ± 1.8^{bcd}	8.0 ± 2.2^{cd}	7.5 ± 3.7^{def}	6.5 ± 2.4^{bcd}
1.50	1:0	9.3 ± 0.6^{d}	9.7 ± 0.6^{cd}	7.3 ± 2.7^{bcd}	8.3 ± 3.1^{def}	9.7 ± 1.7^{cd}
	7:3	8.0 ± 0.3^{cd}	9.3 ± 1.2^{cd}	9.7 ± 1.4^{cd}	9.7 ± 1.9^{ef}	8.0 ± 0.8^{bc}
	1:1	11.0 ± 1.5^{d}	11.3 ± 1.8^{d}	11.6 ± 1.4^{d}	12.2 ± 1.8^{f}	11.0 ± 1.8^{de}

Geometric mean ± standard deviations (n=3). Means in same column with different superscript are significantly different ($p<0.05$).

*Ratio of lactic acid (LA) to acetic acid (AA).

(Table 2.7), TS of films containing 1.5% PABA (5.7 MPa) was similar to the control (5.92 MPa). Films containing SA exhibited lower TS and higher % E as compared to films containing PABA. The reason for this phenomenon could be that the straight chain of SA can more easily penetrate into WPI chains than PABA which has a benzene ring. Consequently, SA may have allowed more mobility between protein chains, thereby producing films of lower TS and greater flexibility. The various organic acid mixtures used to adjust the pH of the film solutions did not significantly alter % E or TS.

Increasing the amount of additives other than cross-linking agents generally produced films with lower TS and greater elongation since these molecules insert between protein chains to form hydrogen bonds with amide groups of proteins (Guilbert, 1986; Kester and Fennema, 1986). Reduced interactions between these protein chains lead to increased flexibility and movement. In our study, SA, PABA, acetic acid and lactic acid might be functioning as plasticizers to increase elongation and decrease TS as was previously suggested for lactic acid (Krull and Inglett, 1971).

$CaCl_2$ was incorporated into our film solution as a cross-linking agent to improve the mechanical and water vapor permeability properties of the low pH films as previously suggested by others (Guilbert, 1986; Avena-Bustillos and Krochta, 1993). As a divalent cation, calcium cross-links between negatively charged groups on proteins, thereby increasing cohesion between protein chains, reducing protein polymer segmental mobility and improving both the mechanical properties and water vapor permeability (Krochta et al., 1990). Jeyarajah and Allen (1994) reported that $CaCl_2$ induced a change in β-lactoglobulin conformation, which facilitated polymerization during heating. Calcium

ions also increased the reactivity of SH groups at low pH. Although the SH – S-S interchange reaction is not possible at pH < 6.5, aggregation of most whey proteins can still occur in the presence of calcium (de Wit, 1981).

Whey protein films are formed by heat-catalyzed protein-protein interactions that involve disulfide, hydrogen, and hydrophobic bonds. Heating denatures the protein and exposes internal SH and hydrophobic groups (Watanabe and Klostermeyer, 1976; Shimada and Cheftel, 1998) which promote intermolecular S-S and hydrophobic bonding upon drying (McHugh and Krochta, 1994b). Film formation is favored in more alkaline film solutions since SH reactivity increases at pH > 8 (Kella and Kinsella, 1988; Banerjee and Chen, 1995). In the present study, the film solution was at pH 8.0 during heating at 90 °C, after which the pH was decreased to 5.2 using lactic and acetic acid. A low pH environment would likely prevent S-S bond formation in the protein matrix, thereby weakening the film structure. Thus, tensile strength of the low-pH film (5.92 MPa) was substantially lower than that reported for high-pH film (13.9 MPa) (McHugh and Krochta, 1994a). However, tensile strength of our low-pH film was higher than that reported for corn zein (0.4 MPa) (Aydt et al., 1991), soy protein (4.5 MPa) (Gennadios and Weller, 1991), and wheat gluten based edible films (1.9-4.4 MPa) (Gennadios et al., 1993) when tested at 23 °C / 50%RH.

2.4.3. Water Vapor Permeability

Films containing 0, 0.5, 0.75, 1.0, or 1.5% PABA exhibited average WVP values of 27.24, 53.73, 53.90, 55.34, and 54.00 g.mm/m^2.d.kPa, respectively (Table 2.7). Increasing the concentration of PABA from 0.5 to 1.5% did not significantly alter WVP

Table 2.7. Thickness, Tensile strength (TS), Percent elongation (% E), and Water Vapor Permeability (WVP) of whey protein isolate-based films containing sorbic acid (SA) and p-aminobenzoic acid (PABA).

Antimic. (%)(w/v)	LA:AA *	Thickness (μm)	%E	TS (Mpa)	WVP (g.mm/m^2.day.kPa)
Control	1:0	128.4 ± 24.4^a	6.4 ± 3.3^a	5.9 ± 1.4^a	27.2 ± 1.3^a
SA (0.50)	1:0	121.1 ± 36.6^a	20.0 ± 1.4^b	4.9 ± 2.7^b	27.3 ± 9.8^a
	7:3	126.4 ± 24.7^a	23.4 ± 1.1^b	4.5 ± 0.3^b	21.3 ± 3.2^a
	1:1	127.6 ± 40.2^a	31.6 ± 1.9^c	4.6 ± 0.7^b	31.5 ± 2.1^a
SA (0.75)	1:0	137.3 ± 30.3^a	26.6 ± 3.2^c	4.9 ± 0.5^b	28.6 ± 6.2^a
	7:3	134.6 ± 23.1^a	27.1 ± 0.9^c	4.8 ± 1.4^b	27.5 ± 1.2^a
	1:1	112.9 ± 48.2^a	24.6 ± 8.7^c	4.4 ± 1.4^b	32.9 ± 1.4^a
SA (1.00)	1:0	130.5 ± 40.4^a	67.8 ± 6.4^e	3.8 ± 0.2^c	43.5 ± 5.3^b
	7:3	132.9 ± 22.3^a	73.5 ± 1.4^e	3.8 ± 0.1^c	41.8 ± 4.2^b
	1:1	120.4 ± 19.5^a	70.7 ± 1.3^e	3.9 ± 0.3^c	41.5 ± 1.4^b
SA (1.50)	1:0	118.9 ± 43.1^a	73.0 ± 2.1^e	3.1 ± 0.5^d	43.8 ± 6.7^b
	7:3	123.7 ± 23.5^a	74.3 ± 4.5^e	2.6 ± 1.0^d	45.6 ± 1.9^b
	1:1	133.4 ± 27.3^a	73.3 ± 5.3^e	2.7 ± 0.9^d	44.1 ± 2.1^b
PABA (0.50)	1:0	123.7 ± 22.3^a	18.3 ± 5.6^b	5.4 ± 0.9^a	51.8 ± 2.2^b
	7:3	145.4 ± 45.8^a	19.9 ± 6.5^b	5.4 ± 0.7^a	55.2 ± 3.1^b
	1:1	120.5 ± 27.3^a	16.2 ± 3.9^b	4.4 ± 1.1^a	54.3 ± 1.2^b
PABA (0.75)	1:0	134.1 ± 23.5^a	20.9 ± 8.5^b	5.2 ± 2.8^a	56.4 ± 1.2^b
	7:3	119.1 ± 45.1^a	30.8 ± 3.6^c	5.2 ± 3.4^a	50.19 ± 2.4^b
	1:1	136.3 ± 56.2^a	28.5 ± 15.2^c	5.2 ± 2.4^a	55.08 ± 1.6^b
PABA (1.00)	1:0	133.2 ± 43.1^a	34.7 ± 6.3^c	4.4 ± 0.2^a	59.79 ± 3.5^b
	7:3	111.8 ± 29.3^a	33.3 ± 2.5^c	5.3 ± 2.2^a	47.08 ± 1.2^b
	1:1	120.7 ± 39.3^a	30.1 ± 8.3^c	5.8 ± 1.8^a	59.17 ± 2.6^b
PABA (1.50)	1:0	123.4 ± 57.7^a	34.9 ±10.6^d	5.3 ± 0.1^a	56.11 ± 2.1^b
	7:3	131.7± 34.9^a	38.7 ± 5.1^d	5.8 ± 1.4^a	56.95 ± 5.4^b
	1:1	130.0 ± 43.1^a	42.2 ± 10.1^d	6.0 ± 1.6^a	48.96 ± 2.2^b

Mean ± standard deviation (n=3). Means in the same column with different superscript are significantly different ($p<0.05$).

* Ratio of lactic acid (LA) to acetic acid (AA).

($p>0.05$). Average WVP values for films containing 0.5 and 0.75% SA were 26.69 and 29.70 g.mm/m^2.d.kPa, respectively, and were not significantly different from the control (27.24 g.mm/m^2.d.kPa) ($p>0.05$). However, addition of 1.0 and 1.5% SA significantly increased WVP to 42.29 and 44.46 g.mm/m^2.d.kPa, respectively ($p<0.05$).

WVP is a measure of the ease with which a material can be penetrated by water vapor. WPI edible films tend to be poor moisture barriers due to abundant hydrophilic groups in proteins. Their moisture barrier properties can be improved by adding non-polar compounds such as lipids (McHugh and Krochta, 1994b). We incorporated candelilla wax into the film solution to reduce WVP. In preliminary experiments, diffusion of SA and PABA as demonstrated by inhibition zones was similar for films prepared with and without candellila wax (results not shown). Adding SA and PABA to the film solution increased WVP because both antimicrobials are hydrophilic compounds. Addition of polar additives may increase the hydrophilic character and the solubility coefficient of the film (McHugh et al., 1994). Moreover, additives such as SA or PABA weaken chain packing in the film to produce a looser structure which increases water mobility.

2.5. CONCLUSION

Incorporating 0.5 to 1.5% of SA or PABA into WPI films (pH 5.2) led to inhibition of *L. monocytogenes, E. coli* O157:H7, and *S.* Typhimurium DT104 on TSAYE at pH 5.2. Addition of PABA and SA increased %E and WVP, but decreased TS. Given our current work involving ready-to-eat meat products which will be reported elsewhere, these films may prove useful for inactivating post-processing contaminants on ready-to-eat foods such as processed meats.

CHAPTER 3

INHIBITION OF *LISTERIA MONOCYTOGENES*, *ESCHERICHIA COLI* O157:H7, AND *SALMONELLA* TYPHIMURIUM DT104 ON BOLOGNA AND SUMMER SAUSAGE USING ANTIMICROBIALS EDIBLE FILMS

Cagri, A., Ustunol, Z., Ryser, E.T.

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3.1.ABSTRACT

Whey protein isolate (WPI) films (pH 5.2) containing 0.5 to 1.0% p-aminobenzoic acid (PABA) and/or sorbic acid (SA) were assessed for antimicrobial activity and mechanical properties while in contact with sliced bologna and summer sausage that were inoculated with *Listeria monocytogenes, Escherichia coli* O157:H7, and *Salmonella* Typhimurium DT104. WPI films containing SA or PABA decreased *Listeria*, *E. coli* and *S.* Typhimurium populations 3.4-4.1, 3.1-3.6, and 3.1-4.1 logs on bologna and sausage after 21 days at 4°C, respectively. Background flora on slices was inhibited compared to controls. Film tensile strength decreased while % elongation remained unchanged following 72 h of product contact. Consequently, these films may prove useful in extending the self-life of ready-to-eat meats.

3.2. INTRODUCTION

Post-processing contamination of ready-to-eat meat products has emerged as a serious public health concern. In 1998, 101 cases of listeriosis in 22 states, including 21 fatalities were traced to consumption of hot dogs that became contaminated after processing (CDC, 1998). A Class I recall was subsequently issued for 35 million pounds of tainted product. During the second half of 2000, another outbreak involving 29 cases in 10 states (including 7 fatalities) prompted the recall of approximately 14.5 million pounds of turkey and chicken delicatessen meat because of probable contamination with *L. monocytogenes* (CDC, 2000). *Listeria* continues to threaten the processed meat industry with over 75 Class I recalls involving more than 75 million pounds of cooked ready-to-eat meats (approximately 40 million pounds of hot dogs) issued from April 1998

to October 2001 (USDA-FSIS, 2001a). In 2000, 2,298 cases of foodborne listeriosis were reported in the United States at an estimated cost of $2.33 billion (~$ 1 million /case), making listeriosis the costliest food-borne disease (USDA-FSIS, 2001b).

Two other meat-borne pathogens also have emerged as major public health concerns. *Escherichia coli* O157:H7 has been highly publicized due to outbreaks of illness associated with ground beef (Ostroff et al., 1990; Bell et al., 1994, Mead et al., 1997, Tuttle et al., 1999) and dry-cured salami (Tilden et al.,1996). Transfer of *E. coli* O157:H7 from contaminated meat or utensils to other foods such as fresh fruits and vegetables is also a major concern (CDC, 1995). The annual cost of the 62,458 *E. coli* O157:H7 infections reported in 2000 was estimated at $659.1 million (~$10,500/case) (USDA-FSIS, 2001b). *Salmonella* Typhimurium DT104, a multiantibiotic resistant strain, is also emerging as a serious threat to public health (Glynn et al., 1998). Food-borne transmission of DT104 has been documented for several meat-related outbreaks; suspected vehicles included roast beef, ham, pork sausage, salami sticks, cooked meats, frozen sausage samples, and chicken legs (Davies et al., 1996, Anonymous, 1996). In England, 17% of 786 fresh or frozen sausage samples yielded *Salmonella* spp., including *S.* Typhimurium DT104 (Nichols and de Louvois, 1995). Sharma et al. (2001) also recovered *S.* Typhimurium DT104 from several points in pork production. These findings indicate that meat products pose a serious health risk if not cooked and handled properly.

Product slicing and packaging operations are major points at which bacterial pathogens can be introduced into cooked ready-to-eat meat products. Bologna sausage batter contains a mixed microflora, including possible pathogens, which are destroyed during cooking; however, vacuum-packed bologna is typically recontaminated during

slicing. In commercial manufacturing facilities, bacterial loads in meat reportedly increased 0.5 to 2.0 logs CFU/g during slicing (Holley, 1997b).

Several studies have demonstrated that antimicrobial edible films can reduce bacterial levels on meat products. Siragusa and Dickson (1992, 1993) showed that organic acids were more effective against *L. monocytogenes*, *S.* Typhimurium, and *E. coli* O157:H7 on beef carcass tissue when immobilized in calcium alginate than when used as a spray or dip. In another study, application of edible corn starch film containing potassium sorbate and lactic acid inhibited growth of *S.* Typhimurium and *E. coli* O157:H7 on poultry (Baron, 1993). Antimicrobial chitosan films containing acetic or propionic acid reportedly inhibited growth of *Enterobacteriaceae* and *Serratia liquefaciens* on bologna, regular cooked ham and pastrami (Ouattara et al., 2000) during 168 h of storage at 5°C. Natrajan and Sheldon (2000) also incorporated nisin and chelators into protein- and polysaccharide-based films to inhibit *Salmonella* on poultry skin. These results emphasize the potential for adding antimicrobial compounds to edible packaging materials.

In our previous study, WPI films (pH 5.2) containing 0.5 to 1.5% SA or PABA inhibited the growth of *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium DT104 on acidified (pH 5.2) trypticase soy agar containing 0.6% yeast extract (Cagri et al., 2001). In this study, WPI films containing SA or PABA were assessed for their ability to retain their desirable antimicrobial and mechanical properties while in direct contact with bologna and summer sausage slices.

3.3. MATERIALS AND METHODS

3.3.1. Products

Commercially produced all-beef bologna (dia. 9.6 cm) and fermented Thuringer summer sausage (dia. 5.4 cm) were obtained from the delicatessen counter of a local supermarket. Bologna was pre-sliced (~3 mm thick) while the summer sausage was sliced approximately 3-mm thick in the laboratory using a food slicer (Chef's Mark, Dallas, TX). All-beef bologna contained 39.3% fat, 3.6% corn syrup, 2.4% salt, 0.25% sodium nitrite, spices and sodium erythorbate as reported by the manufacturer. Similarly, Thuringer summer sausage contained 64% beef, 36% pork, 19.8% fat, 1.8% dextrose, 2.1% salt, 0.25% sodium nitrite, sodium erythorbate, a pediocin-producing starter culture, spices, and flavoring. When purchased, beef bologna and summer sausage had pH values of 6.0 and 4.6, respectively. Products used in each of three trails were from the same lot.

3.3.2. Film Preparation

Whey protein isolate (WPI, Alacen 895) (New Zealand Milk Products, North America, Inc., Santa Rosa, CA) (5% w/v) and glycerol (Sigma Chemical Co., St. Louis, MO) (2% w/v) were dissolved in distilled water containing 0.04% $CaCl_2$ (w/v) (Sigma). After mixing, the solution was adjusted to pH 8.0 with 1N NaOH (Sigma), and heated at 90°C for 30 min in a shaking water bath (Precision Scientific, Winchester, VA). Following the addition of candelilla wax (Stahl Pash, Inc., New York, NY) (0.4%, w/v) during the last 5 min of heating, the solution was homogenized for 2 min in a SD-45 homogenizer (Tekmar Co., Cincinnati, OH), filtered through cheese cloth and cooled to

23 ± 2°C. After adding 0.75 or 1.0% (w/v) sorbic acid (SA) (Sigma), p-aminobenzoic acid (PABA) (Sigma), or 0.5% SA: PABA (1.0: 1.0), the pH was adjusted to 5.2 using 1N lactic acid (Sigma). Following degassing by vacuum for 20 min, the whey protein solution was cast by pipetting 75 ml of the solution into sterile Teflon® plates (20×30 cm^2). The solutions were dried for approximately 24 h at 23 ± 2°C/50 ± 5% RH, after which the films were peeled from the plates and stored at 23 ± 2°C/50 ± 5% RH until used.

3.3.3. Bacterial Strains

Based on results from our previous WPI antimicrobial edible film work (Cagri et al., 2001), the most resistant strain of each of the three pathogens to SA and PABA was chosen. Strains used in this study included *Listeria monocytogenes* CWD 95 and *Escherichia coli* O157:H7 AR (previously obtained from C. W. Donnelly, Dept. of Nutrition and Food Sciences, University of Vermont, Burlington, VT) and *Salmonella* Typhimurium DT104 G10127 (previously obtained from B. Swaminathan, Centers for Disease Control and Prevention, Atlanta, GA). All strains were maintained at -70°C in trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol and subcultured twice in TSB containing 0.6% (w/v) yeast extract (TSBYE) (Difco) at 35°C/18-24 h before use.

3.3.4. Product inoculation and storage

Bologna and summer sausage slices were separately inoculated with 0.1 ml of an appropriately diluted culture so as to contain log 6.0 (± 0.1) *L. monocytogenes*, *E. coli*

O157:H7 or *S.* Typhimurium DT104 CFU/g. The inoculum was evenly spread on both surfaces with a sterile glass rod. The slice was then placed in a sterile 150 mm-diameter petri dish to which a piece of edible film slightly larger than the slice was previously added (Figure 3.1). Thereafter, an identically inoculated slice was placed on top of the edible film with this process repeated until a stack of eight slices, each separated by a piece of edible film, was obtained. One final piece of edible film was laid on the top slice so that both faces of all slices were in contact with the film. These petri dishes were then covered and stored aerobically to give a worst case scenario for film testing at 4°C. Two controls were used - the first consisting of bologna and summer sausage slices separated by the identical edible film prepared without antimicrobials and the second containing slices stacked together without the use of edible film.

3.3.5. Microbiological analysis

Slices of bologna and summer sausage were examined for levels of the inoculum, as well as mesophilic aerobic bacteria, lactic acid bacteria, and yeast/molds immediately after inoculation and again following 4, 7, 10, 14, and 21 days of refrigerated storage. Slices weighing 10-g each were added to 90 ml of sterile 0.1% (w/v) peptone water (PW) (Difco) and homogenized for 3 minutes in a Stomacher 400 (Tekmar Co., Cincinnati, OH). Appropriate dilutions in PW were surface plated on Modified Oxford Agar (Difco), McConkey Sorbitol Agar (Difco), McConkey Agar (Difco), Plate Count Agar (Difco), MRS Lactobaccillus Agar and Rose Bengal Agar (Difco) to quantify *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium DT104, mesophilic aerobic bacteria, lactic acid

bacteria, and yeast/molds, respectively, as outlined in the FDA Bacteriological Analytical Manual (FDA, 1998).

3.3.6. Mechanical Properties

Edible films placed between uninoculated slices of bologna and summer sausage were examined at 0, 24, 48, and 72 h for thickness, tensile strength and percent elongation after conditioning the films for 24 h at 55 ± 5 % RH / 23 ± 5° C.

A model M micrometer (Testing Machine Inc., Amityville, NY) was used to determine film thickness. Measurements were taken at five different locations and the mean value was used in further calculations for moisture barrier and mechanical properties.

Films were cut into strips measuring 101.6 mm by 25.4 mm using a Precision Sample Cutter (Thawing Albert Instrument Co., Philadelphia, PA). All films were conditioned for 48 h at 23 ± 2°C / 50 ± 5% RH before testing. Tensile strength (TS) and percent elongation at break (% E) were determined according to standard D-882-91 (ASTM, 1992). The test was run using the Instron Universal Testing Machine Model 2401 (Canton, MA) at 23 ± 2° C / 50 ± 5% RH with a static load cell of 1kN and a cross head speed of 50.8 cm/min. TS was calculated in MPa from the following equation:

TS = load /(sample width x sample thickness)

% Elongation at break was determined by the following equation:

% E = (distance sample stretched / original length of sample) x 100

3.3.7. Statistical Analysis

All experiments were replicated three times. All film thickness, TS, and %E data was analyzed by one-way analysis of variance (ANOVA) using the SAS Statistical Analysis System (SAS Institute Inc., 1990). Means were compared using the Duncan Grouping test at p=0.05.

3.4. RESULTS AND DISCUSSION

3.4.1. Antimicrobial Properties

L. monocytogenes. Using WPI films containing 0.75% PABA, 1.0% PABA, 0.75% SA, 1.0% SA, or 0.5% SA: 0.5% PABA (1:1), populations of *L. monocytogenes* decreased 1.5, 2.2, 3.0, 3.4, and 2.8 logs, respectively, on bologna slices after 21 days of storage at 4°C (Figure 3.1A) with all films remaining intact. *Listeria* counts remained relatively unchanged using films without antimicrobials; whereas in the absence of films, populations on bologna increased 2.2 logs after 21 days of refrigerated storage. Control WPI films without SA or PABA were acidified to pH 5.2 with lactic acid. Hence, lactic acid present in control films would also be expected to retard the growth of *L. monocytogenes* on bologna slices. *L. monocytogenes* was inhibited on fermented summer sausage using WPI films containing PABA or SA (Figure 3.1B). All WPI films containing antimicrobial agents reduced *L. monocytogenes* populations 3.0 to 4.1 logs on summer sausage slices after 21 days of refrigerated storage. While *Listeria* populations also decreased initially using antimicrobial-free film and slices without film, substantial regrowth occurred following 10 days of refrigerated storage.

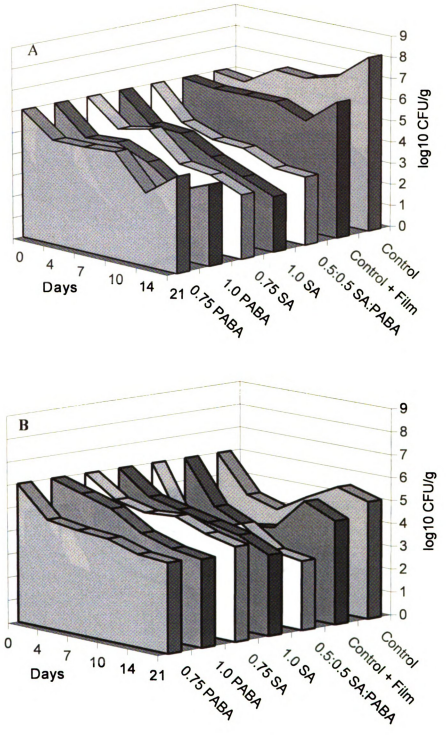


Figure 3.1. Inhibition of *L. monocytogenes* on bologna (A) and fermented sausage (B) slices with WPI edible films containing 0.75%, and 1.0% p-aminobenzoic acid (PABA); 0.75% and 1.0% sorbic acid (SA) and 0.5% SA: 0.5%PABA (1:1). Control + film (WPI edible film without SA or PABA) and control (no film used).

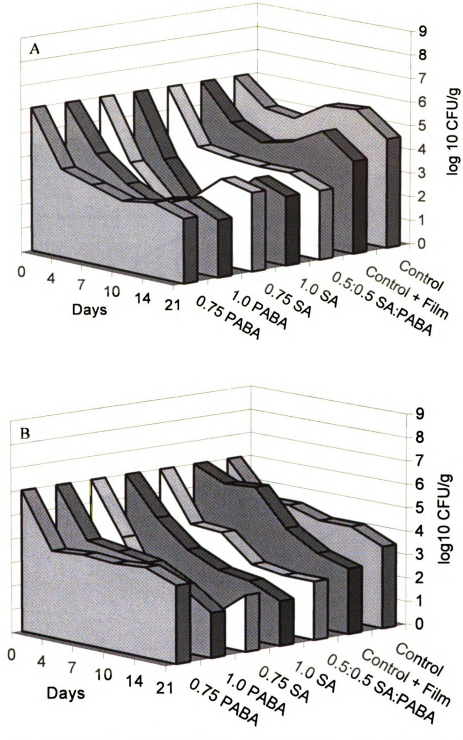


Figure 3.2. Inhibition of *E. coli O157:H7* on bologna (A) and fermented summer sausage (B) slices with WPI edible films containing 0.75%, and 1.0% p-aminobenzoic acid (PABA); 0.75% and 1.0% sorbic acid (SA) and 0.5% SA: 0.5%PABA (1:1). Control + film (WPI edible film without SA or PABA) and control (no film used).

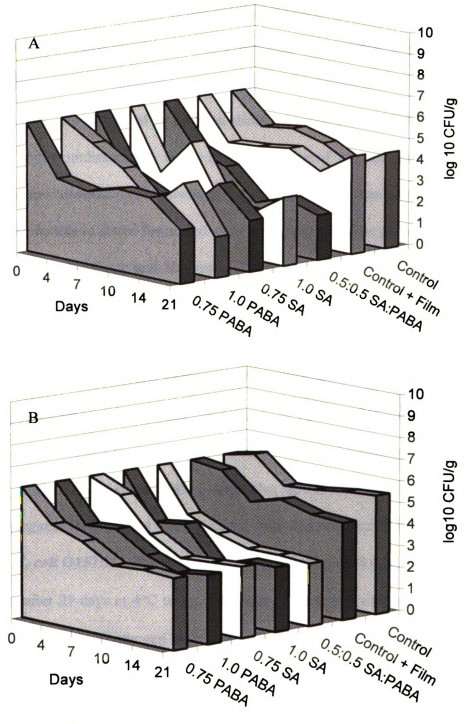


Figure 3.3. Inhibition of *S.* Typhimurium on bologna (A) and fermented summer sausage (B) slices with WPI edible films containing 0.75%, and 1.0% p-aminobenzoic acid (PABA); 0.75% and 1.0% sorbic acid (SA) and 0.5% SA: 0.5%PABA (1:1). Control + film (WPI edible film without SA or PABA) and control (no film used).

Wederquist et al. (1995) reported that potassium sorbate 0.26% (w/w) and sodium lactate 2% (w/w) were highly inhibitory to *L. monocytogenes* on bologna during 28 days of storage. In our most recent study, low pH WPI-based edible films (pH 5.2) containing 0.5 to 1.5% SA or PABA also inhibited *L. monocytogenes* on laboratory media (Cagri et al., 2001). Using antimicrobial films, greater inhibition of *Listeria* was observed on summer sausage (pH 4.6) compared to bologna slices (pH 6.0) due to higher levels of SA and PABA in the undissociated form. In the absence of antimicrobial WPI films, beef bologna also allowed more growth of *L. monocytogenes* than summer sausage. Other inhibitory factors in tested fermented summer sausage, such as pediocin produced by the starter culture (Spelhaug and Harlander, 1989), nitrite, salt, low pH and low a_w would also serve to further inhibit *L. monocytogenes*. The combined effects of nitrite and low pH (~4.6) were likely responsible for inhibiting *L. monocytogenes* on control summer sausage slices (Buchanan and Philips, 1990), with Glass and Doyle (1989) also reporting that *L. monocytogenes* was unable to multiply in vacuum-packed summer sausage (pH 4.8 - 4.9) containing 3.0 - 3.4% salt. Their results agree with those of other authors (Berry et al., 1990; Buncic et al., 1991), who also observed a decline in numbers of *L. monocytogenes* in fermented sausage during extended refrigerated storage.

***E. coli* O157:H7.** Numbers of *E. coli* O157:H7 on bologna slices decreased 2.7 to 3.6 logs after 21 days at 4°C using antimicrobial films containing SA or PABA (Figure 3.2A). However, the pathogen decreased only 2.1 and 1.4 logs, respectively, on control slices with antimicrobial-free film or without film. Growth of *E. coli* O157:H7 also was inhibited on fermented summer sausage (Figure 3.2B). Using WPI films containing 0.75 and 1.0% PABA or SA or the combination of 0.5% SA and 0.5% PABA (1:1),

populations decreased 2.3 to 2.6, 2.7 to 3.1, and 2.9 logs, respectively. Numbers of *E. coli* O157:H7 significantly decreased on both bologna and summer sausage at the end of storage using antimicrobial films ($p<0.05$) (Table 3.1). In our previous study, WPI edible films containing 0.5 to 1.5% PABA or SA inhibited *E. coli* O157:H7 in laboratory media (Cagri et al., 2001). However, numbers of *E. coli* O157:H7 on summer sausage decreased for the film-free and antimicrobial-free controls, with this inhibition likely due to the combined effects of chemical preservatives, nitrite, salt and a pH of 4.6. Several researchers (Pond et al., 2001, Riordan et al., 1998) also observed that 2.5% salt and 100 ppm sodium nitrite were inhibitory to *E. coli* O157:H7 in fermented summer sausage. However, other studies have claimed that this pathogen is very hardy (Benjamin and Datta, 1995), being able to survive more than 51 days in laboratory media at 10°C containing starter culture (10^7 CFU/ml), dextrose (0.8%), NaCl (2%), and $NaNO_2$ (200 ppm) (Glass et al., 1992, Tomicka et al., 1997). When initially present at 10^4 CFU/g, *E. coli* O157:H7 reportedly survived during manufacture and storage of fermented sausage regardless of whether a starter culture was used (Glass et al., 1992).

***S.* Typhimurium DT104.** Using WPI films containing 0.75 or 1.0% SA or PABA, populations of *S.* Typhimurium DT104 decreased 2.7 to 3.1 logs on bologna slices after 21 days of refrigerated storage (Figure 3.3A). In contrast, cell numbers on antimicrobial-free film and film-free control bologna slices decreased only 1.5 and 0.5 logs after 21 days of storage, respectively. WPI films containing 0.75 or 1.0% PABA, SA, or a combination of 0.5% PABA and 0.5% SA (1:1) decreased levels of *S.* Typhimurium DT104 3.5, 4.1, 2.8, 3.6 and 3.9 logs on fermented sausage slices after 21 days, respectively (Figure 3.4B). However, a decrease of only 1.5 logs was observed for

control slices. Cagri et al. (2001) previously concluded that WPI edible films containing 0.75 to 1.5% PABA or SA inhibited all strains of *S.* Typhimurium DT104 tested on TSAYE.

WPI films also were prepared to contain 0.5% of both SA and PABA to test their synergistic effect against growth of the three pathogens on bologna and summer sausage. WPI films containing SA or PABA were more inhibitory to the three test pathogens than WPI films containing both SA and PABA, although several studies claimed that SA and benzoic acid were more effective when used together rather than alone (Sofos and Busta, 1983, Luck, 1980)

Mesophilic Aerobic Bacteria. After 21 days of refrigerated storage, growth of mesophilic aerobic bacteria (MAB) on bologna slices was inhibited as much as 5.0 and 5.8 logs using films containing 1.0% SA or PABA as compared to the antimicrobial-free film and film-free controls, respectively (Table 3.2). However, Petaja et al.(1979) found that incorporating 0.25% potassium sorbate into cooked sausage had no effect on levels of aerobic bacteria when the product was stored at 7°C. Growth of MAB was generally inhibited on summer sausage slices by all WPI films; however, MAB populations increased 1.4 and 2.4 logs after 21 days on sausage slices with antimicrobial-free film or film-free controls, respectively (Table 3.5). Thus, WPI films can extend the shelf life of both bologna and summer sausage at 4°C.

Lactic Acid Bacteria. Using antimicrobial films, lactic acid bacteria (LAB) decreased 1.0 to 3.0 logs on bologna slices after 4 days and generally remained at these levels throughout storage; whereas no reduction in LAB populations was observed in the controls (Table 3.2). Fermented summer sausage initially contained 6.0 log CFU/g of

LAB with populations remaining relatively unchanged during 21 days of storage regardless of the type of film used (Table 3.5). However, numbers of LAB were 1.6 to 2.2 logs higher in samples stored without film. Early studies suggested that sorbate concentrations of 1.0% were markedly inhibitory to *Lactobacillus bulgaricus*, *Lactobacillus acidophilus* and *Streptococcus lactis* (Hamdan et al., 1971). LAB predominate in bologna and are often the major species present in fully ripened fermented sausage. According to Holley (1997a), bacteria present on bologna slices stored at 7°C were almost exclusively LAB, with *Lactobacillus sake* dominating. *Bacillus thermosphacta* wasabsent and coliforms were rarely detected. Fermented sausage also contained thermotolerant homofermentative lactobacilli which were quantified along with pediococci. However, growth of pediococci was limited by low temperature (<10°C) storage of the samples (Holley et al.,1988).

Yeast/Mold. As expected, mold growth on both bologna and summer sausage was inhibited with WPI films containing SA or PABA during 21 days of refrigerated storage (Tables 3.2 and 3.4). However, mold counts eventually reached 1.3-1.4 and 1.7-2.2 logs in the antimicrobial-free film and film-free controls, respectively. Using WPI films containing PABA, yeasts were not detected on bologna or summer sausage slices until day 7. Films containing 0.75 or 1.0% SA inhibited yeasts 7 to 21 days and 21 days on bologna and fermented summer sausage, respectively. The combination of 0.5% SA and 0.5% PABA (1:1) prevented growth of yeasts on bologna but not on summer sausage. In the antimicrobial-free film and film-free controls, yeast populations on bologna and summer sausage increased 3.5-3.6 and 5.0-5.6 logs, respectively, by the end of storage. Inhibition of yeasts and molds by sorbate is well documented (Liewen and

Marth; 1985, Stead, 1995). Dipping fermented sausage and raw ham in a solution of 10-20% sorbate inhibited mold growth (Leistner et al., 1975). Application of 2% gelatin, 2% liquid smoke, 0.2% corn starch, and 1% potassium sorbate on the inside of regenerated cellulose casings also prevented mold growth during sausage curing (Rose and Turbark, 1969).

Table 3.1. Population decrease (log CFU/g) of *L. monocytogenes*, *E. coli* O157:H7, and *S.*Typhimurium DT104 with WPI films containing p-aminobenzoic acid (PABA), sorbic acid (SA), or SA:PABA on bologna (B) and fermented summer sausage slices (SS) after 21 days of refrigerated storage.

		L. monocytogenes		*E. coli* O157:H7		*S.* Typhimurium DT104	
Antimicrobials	% (w/v)	B	SS	B	SS	B	SS
PABA	0.75	1.5 ± 0.3^{d}	2.1 ± 0.2^{bc}	3.3 ± 0.1^{ab}	3.3 ± 0.3^{ab}	2.7 ± 0.2^{a}	3.5 ± 0.5^{ab}
	1	2.2 ± 0.1^{c}	1.9 ± 0.3^{bc}	3.6 ± 0.3^{a}	4.1 ± 0.2^{a}	2.8 ± 0.2^{a}	4.1 ± 0.4^{a}
SA	0.75	3.0 ± 0.4^{ab}	2.2 ± 0.3^{b}	2.7 ± 0.2^{c}	3.6 ± 0.2^{ab}	2.7 ± 0.4^{a}	3.6 ± 1.0^{ab}
	1	3.4 ± 0.5^{a}	2.5 ± 0.2^{ab}	3.1 ± 0.2^{bc}	4.1 ± 1.1^{a}	3.0 ± 0.2^{a}	2.8 ± 0.4^{b}
SA: PABA	0.5: 0.5	2.8 ± 0.2^{b}	3.0 ± 0.5^{a}	3.1 ± 0.4^{bc}	3.5 ± 0.6^{ab}	3.1 ± 0.3^{a}	3.9 ± 0.1^{a}
Control + Film	0	-0.4 ± 0.1^{e}	1.5 ± 0.2^{c}	2.1 ± 0.3^{d}	3.2 ± 1.0^{ab}	1.5 ± 0.2^{b}	1.4 ± 0.2^{c}
Control - Film	0	-2.2 ± 0.2^{f}	0.9 ± 0.5^{d}	1.0 ± 0.2^{e}	2.5 ± 0.6^{b}	0.5 ± 0.3^{c}	1.5 ± 0.4^{c}

Mean ± standard deviation (n=3). Means in same column with different superscripts are significantly different ($p<0.05$).

Table 3.2. Inhibition of mesophilic aerobic bacteria (MAB) and lactic acid bacteria (LAB) on bologna slices using whey protein isolate edible films containing p-aminobenzoic acid (PABA) or sorbic acid (SA) at 4°C

		Antimicrobials (%, w/v)						
		PABA		SA		SA: PABA	Control + Film	Control - Film
	Days	0.75	1.00	0.75	1.00	0.5: 0.5	0.00	0.00
MAB	0	1.2 ± 0.5^{k}	1.3 ± 0.2^{k}	1.0 ± 0.3^{k}	1.1 ± 0.4^{k}	1.2 ± 0.2^{k}	1.4 ± 0.1^{k}	1.2 ± 0.3^{k}
	4	4.4 ± 0.4^{de}	4.8 ± 0.2^{d}	4.7 ± 0.2^{d}	4.6 ± 0.4^{d}	2.7 ± 1.2^{i}	6.8 ± 0.1^{ab}	6.7 ± 0.3^{ab}
	7	4.7 ± 0.8^{d}	4.5 ± 0.8^{de}	4.9 ± 1.5^{d}	5.0 ± 1.7^{cd}	4.1 ± 0.1^{ef}	6.2 ± 1.4^{b}	7.2 ± 1.4^{b}
	10	4.8 ± 0.5^{d}	4.0 ± 0.1^{ef}	4.1 ± 0.1^{ef}	4.2 ± 0.1^{ef}	4.7 ± 0.2^{d}	5.6 ± 0.3^{c}	6.5 ± 0.3^{ab}
	14	3.6 ± 0.2^{fg}	3.5 ± 0.2^{fg}	2.6 ± 0.3^{i}	2.1 ± 0.2^{ij}	2.9 ± 0.5^{g}	6.5 ± 1.0^{b}	5.4 ± 0.7^{c}
	21	3.6 ± 0.2^{fg}	3.4 ± 0.4^{fg}	3.1 ± 0.5^{g}	2.6 ± 0.4^{i}	2.3 ± 0.2	6.4 ± 1.5^{b}	8.4 ± 1.5^{a}
LAC	0	1.0 ± 0.2^{a}	1.3 ± 0.2^{a}	1.2 ± 0.3^{a}	1.0 ± 0.2^{a}	1.1 ± 0.3^{a}	1.2 ± 0.1^{a}	1.4 ± 0.1^{a}
	4	1.4 ± 0.4^{a}	1.2 ± 0.3^{a}	1.6 ± 0.2^{a}	1.2 ± 0.4^{a}	1.3 ± 0.3^{a}	3.8 ± 0.1^{c}	4.7 ± 0.3^{d}
	7	1.4 ± 0.2^{a}	1.3 ± 0.1^{a}	1.3 ± 0.3^{a}	1.1 ± 0.2^{a}	1.3 ± 0.1^{a}	4.2 ± 0.2^{cd}	4.8 ± 0.1^{d}
	10	1.8 ± 0.3^{ab}	1.3 ± 0.3^{a}	1.3 ± 0.1^{a}	1.2 ± 0.2^{a}	1.4 ± 0.2^{a}	4.4 ± 0.2^{d}	4.9 ± 0.1^{d}
	14	2.1 ± 0.4^{b}	1.9 ± 0.3^{ab}	1.5 ± 0.3^{a}	1.3 ± 0.4^{a}	1.5 ± 0.3^{a}	4.7 ± 0.1^{d}	4.8 ± 0.2^{d}
	21	2.5 ± 0.3^{b}	1.9 ± 0.3^{a}	2.0 ± 0.1^{b}	2.0 ± 0.2^{b}	2.3 ± 0.3^{b}	4.6 ± 0.2^{d}	4.8 ± 0.3^{d}

Unit = CFU/g ($\log_{10}$). Geometric mean ± standard deviation (n=3). Means in same column with different superscripts are significantly different ($p<0.05$).

Table 3.3. Inhibition of mold and yeasts on bologna slices using whey protein isolate edible films containing p-aminobenzoic acid (PABA) or sorbic acid (SA) at 4°C

		Antimicrobials (%, w/v)						
		PABA		SA		SA: PABA	Control + Film	Control - Film
	Days	0.75	1.00	0.75	1.00	0.5: 0.5	0.00	0.00
Mold	0	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$
	4	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$
	7	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$
	10	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$
	14	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.4^{a}$	$<1.0 \pm 0.0^{a}$	1.5 ± 0.7^{b}
	21	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.1^{a}$	1.4 ± 0.1^{b}	1.7 ± 1.0^{b}
Yeast	0	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0^{a}$
	4	$<1.0 \pm 0.0^{a}$	1.0 ± 0.0^{b}	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	1.5 ± 0.0^{b}	2.4 ± 1.3^{bc}
	7	1.5 ± 1.1^{b}	1.5 ± 1.2^{b}	$<1.0 \pm 1.1^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	1.9 ± 1.2^{bc}	2.5 ± 0.3^{bc}
	10	2.7 ± 0.7^{c}	2.4 ± 0.9^{bc}	2.2 ± 0.0^{bc}	$<1.0 \pm 0.8^{a}$	$<1.0 \pm 0.7^{a}$	3.9 ± 0.2^{d}	5.3 ± 0.2^{f}
	14	2.8 ± 0.5^{c}	1.6 ± 0.4^{b}	2.1 ± 0.0^{bc}	$<1.0 \pm 0.7^{a}$	$<1.0 \pm 0.0^{a}$	4.0 ± 0.4^{d}	4.4 ± 0.4^{de}
	21	1.3 ± 1.0^{b}	$<1.0 \pm 0.2^{a}$	3.0 ± 0.0^{c}	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	4.8 ± 0.3^{e}	4.7 ± 0.1^{e}

Unit = CFU/g ($\log_{10}$). Geometric mean ± standard deviation (n=3). Means in same column with different superscripts are significantly different ($p<0.05$).

Table 3.4. Inhibition of mesophilic aerobic bacteria (MAB) and lactic acid bacteria (LAB) on fermented summer sausage slices using whey protein isolate edible films containing p-aminobenzoic acid (PABA) or sorbic acid (SA) at 4°C

		Antimicrobials (%, w/v)						
		PABA		SA		SA: PABA	Control + Film	Control - Film
	Days	0.75	1.00	0.75	1.00	0.5: 0.5	0.00	0.00
MAB	0	5.9 ± 0.4^{c}	5.9 ± 0.6^{c}	6.0 ± 0.2^{c}	6.1 ± 0.2^{c}	5.9 ± 0.2^{c}	6.0 ± 0.7^{c}	5.8 ± 0.4^{c}
	4	6.0 ± 0.5^{c}	5.9 ± 0.6^{c}	5.9 ± 0.6^{c}	6.1 ± 0.4^{c}	5.6 ± 0.4^{cd}	5.8 ± 0.7^{c}	5.9 ± 0.5^{c}
	7	6.5 ± 0.4^{bc}	6.1 ± 0.9^{c}	5.7 ± 0.5^{cd}	5.5 ± 0.4^{cd}	5.6 ± 0.0^{cd}	6.7 ± 1.2^{bc}	8.0 ± 1.8^{a}
	10	6.5 ± 1.2^{bc}	5.4 ± 1.8^{cd}	5.0 ± 1.6^{d}	5.2 ± 1.9^{d}	5.2 ± 1.7^{d}	6.9 ± 2.1^{b}	8.2 ± 2.4^{a}
	14	6.6 ± 1.9^{bc}	5.6 ± 2.0^{cd}	5.3 ± 1.2	4.9 ± 2.7^{d}	4.7 ± 0.1^{de}	7.0 ± 2.4^{b}	8.6 ± 0.4^{a}
	21	6.8 ± 1.3^{bc}	5.9 ± 1.5^{c}	4.9 ± 0.9	5.2 ± 0.7^{d}	4.7 ± 0.2^{de}	7.4 ± 0.3^{b}	8.4 ± 0.3^{a}
LAC	0	5.8 ± 0.9^{c}	5.9 ± 0.4^{bc}	6.1 ± 0.9^{bc}	6.0 ± 0.8^{bc}	5.9 ± 0.6	6.2 ± 0.3^{b}	6.0 ± 0.0^{bc}
	4	5.9 ± 0.7^{bc}	6.0 ± 0.7^{bc}	6.1 ± 1.8^{bc}	6.1 ± 1.9^{bc}	4.9 ± 0.2	6.3 ± 2.1^{b}	4.6 ± 0.6^{d}
	7	5.6 ± 0.3^{c}	6.1 ± 0.1^{bc}	4.3 ± 0.8^{d}	5.8 ± 0.4^{c}	6.8 ± 0.6^{b}	6.5 ± 0.9^{b}	5.5 ± 2.5^{c}
	10	5.5 ± 1.8^{c}	4.8 ± 2.1^{cd}	5.2 ± 1.3^{cd}	4.8 ± 1.8^{cd}	6.5 ± 2.1^{b}	4.5 ± 1.9^{d}	5.6 ± 2.1^{c}
	14	5.5 ± 2.9^{c}	4.6 ± 2.6^{d}	5.0 ± 2.9^{d}	4.7 ± 2.5^{d}	5.5 ± 0.7^{c}	5.4 ± 3.1^{c}	6.3 ± 0.0^{bc}
	21	6.1 ± 0.2^{bc}	5.5 ± 0.8^{c}	6.0 ± 0.5^{bc}	5.8 ± 0.5^{c}	5.7 ± 0.5^{c}	6.5 ± 0.9^{b}	7.7 ± 3.2^{a}

Unit = CFU/g ($\log_{10}$). Geometric mean ± standard deviation (n=3). Means in same column with different superscripts are significantly different

Table 3.5. Inhibition of mold and yeast on fermented summer sausage slices using whey protein isolate edible films containing p-aminobenzoic acid (PABA) or sorbic acid (SA) at 4°C

		Antimicrobials (%, w/v)						
		PABA		SA		SA: PABA	Control + Film	Control - Film
	Days	0.75	1.00	0.75	1.00	0.5: 0.5	0.00	0.00
Mold	0	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.1^{a}$	$<1.0 \pm 0.0^{a}$
	4	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	2.4 ± 1.4^{c}	1.9 ± 0.0^{bc}
	7	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	1.2 ± 0.9^{b}	2.0 ± 0.0^{bc}
	10	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	1.5 ± 0.9^{b}	1.2 ± 0.0^{b}
	14	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	1.6 ± 0.0^{b}	2.4 ± 0.0^{c}
	21	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	1.3 ± 0.0^{b}	2.2 ± 0.0^{c}
Yeast	0	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.0^{a}$
	4	$<1.0 \pm 2.1^{a}$	$<1.0 \pm 1.3^{a}$	$<1.0 \pm 1.8^{a}$	$<1.0 \pm 1.9^{a}$	1.5 ± 0.5^{b}	$<1.0 \pm 1.7^{a}$	2.6 ± 1.5^{c}
	7	3.5 ± 1.7^{d}	3.8 ± 2.1^{d}	$<1.0 \pm 0.0^{a}$	$<1.0 \pm 0.9^{a}$	1.8 ± 1.3^{a}	3.7 ± 2.1^{d}	4.3 ± 2.5^{de}
	10	3.8 ± 1.5^{d}	3.1 ± 1.4^{cd}	$<1.0 \pm 1.1^{a}$	$<1.0 \pm 1.1^{a}$	2.5 ± 1.1^{c}	2.6 ± 1.3^{c}	3.4 ± 1.3^{d}
	14	4.3 ± 0.4^{de}	3.6 ± 1.2^{d}	$<1.0 \pm 0.9^{a}$	$<1.0 \pm 0.0^{a}$	2.4 ± 0.9^{c}	4.4 ± 2.4^{de}	4.4 ± 0.4^{de}
	21	4.6 ± 0.2^{e}	4.0 ± 1.4^{d}	$<1.0 \pm 0.9^{a}$	$<1.0 \pm 0.0^{a}$	2.4 ± 0.5^{c}	6.0 ± 3.4^{f}	6.6 ± 1.7^{f}

Unit = CFU/g (log_{10}). Geometric mean ± standard deviation (n=3). Means in same column with different superscripts are significantly different

3.4.2. Mechanical Properties

Films varied in thickness from 4.3 to 5.2 mil with this difference not statistically significant ($p>0.05$) (Tables 3.6 and 3.7). Percent elongation at break (% E) and tensile strength (TS) of films in contact with bologna and summer sausage slices were measured after 0, 24, 48, and 72 hours of storage to determine their eventual suitability as sausage casings. TS of all films decreased ($p<0.05$) after 24 h of contact with bologna and summer sausage slices; whereas % E increased ($p<0.05$) after contact with either meat product (Table 3.3 and 3.4). No significant increases ($p>0.05$) in % E were observed between films measured after 24 and 48 or 48 and 72 h of storage at 4°C. In addition, no significant decreases ($p>0.05$) in TS were observed beyond 24 h. Chemical stability of the films was predictably altered while in contact with bologna and summer sausage slices because of expected moisture absorption from the meat slices and release of SA, PABA, and glycerol to reach a steady state. Moisture absorption by the film likely weakened the polymer chain bonds since water molecules insert between protein chains to form hydrogen bonds with amide groups of proteins, thereby reducing tensile strength (Guilbert, 1986; Kester and Fennema, 1986). Reduced interactions between these protein chains likely led to increased flexibility and movement, as evidenced by the marked increase in % E.

Table 3.6. Percent elongation (%E), tensile strength (TS) and thickness of whey protein isolate edible film containing p-aminobenzoic acid (PABA) or sorbic acid (SA) while in contact with bologna slices at 4°C

		Antimicrobials (%)					
			SA		PABA		SA: PABA
	Hours	0	0.75	1.00	0.75	1.00	0.5:0.5
Thickness (mil)	0	4.5 ± 0.2 [a]	4.5 ± 0.3 [a]	4.8 ± 0.3 [a]	4.6 ± 0.5 [a]	5.1 ± 0.4 [a]	4.7 [a] ± 0.3
	24	5.0 ± 0.2 [a]	4.6 ± 0.2 [a]	4.5 ± 0.4 [a]	4.9 ± 0.4 [a]	5.2 ± 0.5 [a]	4.4 [a] ± 0.4
	48	4.9 ± 0.3 [a]	4.7 ± 0.4 [a]	5.0 ± 0.2 [a]	5.2 ± 0.3 [a]	4.9 ± 0.4 [a]	4.7 [a] ± 0.2
	72	4.7 ± 0.1 [a]	5.1 ± 0.3 [a]	4.7 ± 0.3 [a]	4.8 ± 0.2 [a]	4.7 ± 0.2 [a]	5.1 [a] ± 0.1
% E	0	6.4 ± 2.5 [a]	26.6 ± 9.6 [a]	67.8 ± 12.5 [a]	30.8 ± 9.7 [a]	34.7 ± 10.5 [a]	31.6 [a] ± 7.8
	24	89.2 ± 10.5 [b]	77.0 ± 19.4 [b]	84.4 ± 20.2 [b]	69.4 ± 15.5 [b]	71.7 ± 19.5 [b]	74.7 [b] ± 13.2
	48	96.5 ± 22.4 [bc]	81.4 ± 17.8 [bc]	101.8 ± 19.6 [bc]	72.3 ± 11.7 [bc]	78.0 ± 12.5 [bc]	78.2 [bc] ± 13.5
	72	104.4 ± 14.7 [c]	97.0 ± 9.7 [c]	119.0 ± 23.9 [c]	81.2 ± 16.9 [c]	82.2 ± 20.9 [c]	91.2 [c] ± 18.9
TS (MPa)	0	5.5 ± 1.5 [a]	4.9 ± 0.5 [a]	3.7 ± 0.7 [a]	5.2 ± 1.1 [a]	5.8 ± 2.2 [a]	4.6 [a] ± 0.9
	24	1.0 ± 0.3 [b]	0.9 ± 0.3 [b]	1.1 ± 0.5 [b]	0.8 ± 0.3 [b]	0.8 ± 0.1 [b]	0.8 [b] ± 0.2
	48	0.9 ± 0.2 [b]	0.9 ± 0.2 [b]	1.1 ± 0.4 [b]	0.8 ± 0.2 [b]	0.7 ± 0.1 [b]	0.8 [b] ± 0.3
	72	0.6 ± 0.1 [b]	1.0 ± 0.4 [b]	1.3 ± 0.6 [b]	0.9 ± 0.3 [b]	0.5 ± 0.2 [b]	0.6 [b] ± 0.1

Mean ± standard deviation (n=3). Means in same column with different superscripts are significantly different ($p<0.05$).

Table 3.7. Percent elongation (%E), tensile strength (TS) and thickness of whey protein isolate edible film containing p-aminobenzoic acid (PABA) or sorbic acid (SA) while in contact with summer sausage slices at 4°C

		Antimicrobials (%, w/v)					
		SA			PABA		SA: PABA
	Hours	0.00	0.75	1.00	0.75	1.00	0.5:0.5
Thickness (mil)	0	4.6 ± 0.3 [a]	4.7 ± 0.3 [a]	4.5 ± 0.3 [a]	4.5 ± 0.3 [a]	5.0 ± 0.3 [a]	4.8 ± 0.3 [a]
	24	5.1 ± 0.2 [a]	4.6 ± 0.4 [a]	4.8 ± 0.2 [a]	4.7 ± 0.5 [a]	4.9 ± 0.4 [a]	4.3 ± 0.2 [a]
	48	4.8 ± 0.2 [a]	4.8 ± 0.5 [a]	4.9 ± 0.2 [a]	4.9 ± 0.2 [a]	4.5 ± 0.3 [a]	5.2 ± 0.1 [a]
	72	4.7 ± 0.1 [a]	5.0 ± 0.1 [a]	4.6 ± 0.4 [a]	4.7 ± 0.2 [a]	4.6 ± 0.6 [a]	5.0 ± 0.5 [a]
% E	0	6.4± 1.2 [a]	26.6 ± 5.7 [a]	67.8 ± 7.3 [a]	30.8 ± 4.1 [a]	34.7 ± 6.9 [a]	31.6 ± 7.3 [a]
	24	67.8 ± 10.6 [b]	64.7 ± 10.5 [b]	78.7 ± 8.7 [b]	49.0 ± 9.6 [b]	69.9 ± 8.5 [b]	87.5 ± 12.7 [b]
	48	68.1 ± 12.7 [b]	73.3 ± 23.1 [bc]	86.5 ± 15.9 [bc]	55.5 ± 8.7 [bc]	71.1 ± 8.9 [b]	96.5 ± 10.9 [bc]
	72	80.1± 18.5 [c]	85.1 ± 16.9 [c]	112.2 ± 27.2 [c]	60.0 ± 9.2 [c]	87.6 ± 14.4 [c]	114.4 ± 22.9 [c]
TS (MPa)	0	5.5 ± 0.7 [a]	4.9 ± 2.0 [a]	3.7 ± 0.9 [a]	5.2 ± 1.3 [a]	5.8 ± 2.3 [a]	4.6 ± 2.1 [a]
	24	0.7 ± 0.3 [b]	0.5 ± 0.2 [b]	0.6 ± 0.1 [b]	0.7 ± 0.4 [b]	0.5 ± 0.3 [b]	0.5 ± 0.4 [b]
	48	0.6 ± 0.1 [b]	0.5 ± 0.2 [b]	0.6 ± 0.3 [b]	0.5 ± 0.3 [b]	0.4 ± 0.1 [b]	0.4 ± 0.1 [b]
	72	0.9 ± 0.2 [b]	0.4 ± 0.1 [b]	0.7 ± 0.3 [b]	0.4 ± 0.1 [b]	0.5 ± 0.1 [b]	0.5 ± 0.2 [b]

Mean ± standard deviation (n=3). Means in same column with different superscripts are significantly different ($p<0.05$).

3.5. CONCLUSION

WPI films containing SA or PABA clearly inhibited growth of *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium DT104 on both bologna and summer sausage slices. These films, which retained their antimicrobial activity for 21 days, also showed considerable promise in extending the shelf-life of sliced bologna and summer sausage. Percent elongation of the film increased as a result of contact with bologna and summer sausage while tensile strength sharply decreased. Eventual formulation of these antimicrobial films into sausage casings would provide the processed meat industry with another means of safeguarding cooked and ready-to-eat meats from post-processing contaminants.

CHAPTER 4

INHIBITION OF *LISTERIA MONOCYTOGENES* ON HOT DOGS USING ANTIMICROBIAL WHEY PROTEIN-BASED EDIBLE CASINGS

Cagri, A., Ustunol, Z., Ryser, E.T., Osburn, W.

4.1.ABSTRACT

Whey protein isolate (WPI) films (pH 5.2) containing 0.0 or 1.0% (w/v) p-aminobenzoic acid (PABA) were heat-sealed to form casings. A commercial-type hot dog batter was stuffed into WPI, collagen, or natural casings. After cooking, the hot dogs were surface-inoculated to contain 10^3 *Listeria monocytogenes* CFU/g, vacuum packaged and examined for numbers of *L. monocytogenes,* mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB), and yeast/mold during 42 days of storage at 4°C. Tensile strength (TS) and % elongation (% E) of casings were determined before and after cooking and after smoking using standard methods. Fat, protein, moisture, 2-thiobarbuturic acid (TBA) value, purge, shear force, color, pH, and sensory attributes of uninoculated hot dogs were also determined. *Listeria* populations on hot dogs prepared with WPI-1.0% PABA casings remained relatively unchanged; however, numbers of *Listeria* on hot dogs prepared with WPI-0.0% PABA, collagen, and natural casings increased ~2.5 logs during 42 days of refrigerated storage. Populations of MAB, LAB, and mold on WPI-1.0% PABA casings were 1-3 logs lower compared to others casings. TS and % E of WPI casings remained unchanged after cooking and smoking; however, TS and % E of collagen and natural casings decreased. TBA and pH values were similar for hot dogs with different casings. Shear force of casings prepared with WPI and collagen was lower than that for natural casings. Purge loss was higher in hot dogs prepared with WPI than collagen or natural casings. Sensory attributes (overall desirability, flavor, texture, juiciness) of hot dogs with WPI-1% PABA casings were scored higher that hot dogs with commercial collagen and natural casings. WPI casing

containing 1.0% PABA may offer another potential strategy to prevent growth of *Listeria* on hot dogs during extended refrigerated storage.

4.2. INTRODUCTION

Over the last decade, post-process contamination with *Listeria monocytogenes* has emerged as one of the largest problems faced by the processed meat industry. In 1998, more than 35 million pounds of hot dogs that had been linked to 101 listeriosis cases in 22 states, including 21 fatalities, were recalled by one Michigan manufacturer, with the product presumably contaminated at the time of packaging (CDC, 1998). Two years later, 29 listeriosis cases (7 fatalities) in 10 states prompted the recall of turkey and chicken delicatessen meat (14.5 million pounds) that likely contained *L. monocytogenes* (CDC, 2001). *Listeria* continues to threaten the processed meat industry with more than 74 Class I recalls involving approximately 100 million pounds of cooked ready-to-eat (RTE) meats issued from April 1998 to December 2001 (USDA-FSIS, 2001).

In response to the *Listeria* problem, the processed meat industry has examined post-processing thermal and high pressure pasteurization (Lucore et al., 1999, Murano et al., 1999, Yuste et al., 2000) as well as various acid washes to minimize the risk of *Listeria* on finished ready-to-eat (RTE) products. Antimicrobial edible films were investigated as another possible solution to this problem. Ming et al. (1997) reported that pediocin-coated cellulosic casings inhibited *L. monocytogenes* on ham, turkey breast meat, and beef. In addition, McDade et al. (1999) found that dipping frankfurters in an aqueous whey protein solution (pH 5.2) containing propionic/sorbic acid prevented growth of *L. monocytogenes* on the product during the first 2 to 3 weeks of refrigerated storage.

Consequently, we investigated the efficacy of antimicrobial edible films for inactivating pathogens including *L. monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104, on RTE meat products. In our initial work, whey protein isolate (WPI)-based edible films (pH 5.2) containing 0.5 to 1.5% sorbic acid (SA) or p-aminobenzoic acid (PABA) inhibited *L. monocytogenes, E. coli* O157:H7, and *S.* Typhimurium DT104 on trypticase soy agar (Cagri et al., 2001). When these WPI films containing SA or PABA were subsequently tested between inoculated slices of commercially produced bologna and summer sausage, significant reductions in numbers of *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium DT104 were observed after 21 days of storage at 4°C (Cagri et al., 2002).

The objectives of the present study were to (a) test the ability of WPI casings containing 1.0% PABA to inhibit *L. monocytogenes* on surface-inoculated hot dogs; (b) determine the ability of the WPI casings to retain their desirable mechanical properties during hot dog manufacture and storage; (c) examine the impact of different casings on storage stability of hot dogs based on numbers of total aerobic mesophilic bacteria, lactic acid bacteria, yeast and mold, 2-thiobarbutiric acid (TBA) values, purge loss, shear force, color and pH; and (d) assess sensory attributes of hot dogs prepared with WPI casings containing 1.0% PABA, WPI, collagen and natural casings.

4.3. MATERIALS AND METHODS

4.3.1. Experimental Design

In preliminary work, hot dogs were prepared using heat-sealed whey protein isolate (WPI) casings containing 0.0% and 1.0% SA, PABA, 0.5% SA:0.5% PABA and

commercial collagen casings. Hot dogs with different casings were surface inoculated with *Listeria monocytogenes* (10^3 CFU/g), vacuum-packaged and stored at 4°C for 42. *Listeria* inhibition was examined initially and after 4, 7, 10, 14, 21, 35, and 42 days of storage. WPI casings containing 1% PABA which were most inhibitory against *Listeria* were subsequently compared with commercial collagen and natural casings in the full-scale study (Figure 4.1).

In latter, hot dogs prepared with WPI casing containing 0.0% or 1.0% PABA, commercial collagen and natural casings were surface inoculated with *L. monocytogenes* (10^3 CFU/g), vacuum-packaged and stored 4°C for 42 days. Uninoculated hot dogs prepared with WPI, collagen and natural casings were tested for chemical (moisture, fat, protein, pH and TBA), microbiological (mesophilic aerobic bacteria, lactic acid bacteria, mold/yeast), mechanical (shear force, tensile strength of casings after cooking and elongation of casings after cooking), physical (purge, cook yield) and sensory (juiciness, texture, flavor, casing tenderness and overall desirability) characteristics.

4.3.2. Whey Protein Isolate Casing Preparation

WPI (Alacen 895, New Zealand Milk Products, North America, Inc., Santa Rosa, CA) (5% w/v) and glycerol (Sigma Chemical Co., St. Louis, MO) (2% w/v) were dissolved in distilled water containing 0.04% $CaCl_2$ (w/v) (Sigma). After mixing and adjusting the pH to 8.0 with 2N NaOH, the solution was heated at 90°C for 30 min in a shaking water bath (170 Marcel Drive water bath, Precision Scientific, Winchester, VA). Following the addition of candelilla wax (Stahl Pash, Inc., New York, NY) (0.4%, w/v) during the last 5 min of heating, the solution was homogenized for 2 min in a SD-45 homogenizer (Tekmar Co., Cincinnati, OH), filtered through cheese cloth and cooled to

23 ± 2°C. After incorporating 1.0% (w/v) p-aminobenzoic acid (PABA) (Sigma), sorbic acid (SA) (Sigma), or 0.5% SA: 0.5% PABA (1:1), the pH was adjusted to 5.2 with 2N lactic acid (Sigma). Following degassing by vacuum, the whey protein solution was cast by pipetting 105 ml of the solution onto sterile flat Teflon plates (35 x 23 cm^2) and drying for approximately 24 h at 23 ± 2°C / 50 ± 5% RH after which the films were peeled from the plates and heat-cured at 90°C in a vacuum oven for 12 h. Films were hand-cut with a paper cutter and heat-sealed to form tubular casings measuring 2.6 cm in diameter and 12.0 cm in length, stored at 23 ± 2°C / 50 ± 5% RH and used within 2 days.

Commercial edible collagen (clear processed type) (dia. 2.6 cm) was provided by Vista International Packaging, Inc. (Kenosha, WI). Commercial natural casings (lamb casing) (dia. 2.4-2.6 cm) were obtained from Dewied International, Inc. (San Antonio, TX).

4.3.3. Culture preparation

Listeria monocytogenes CWD 95, the most resistant strain identified from our previous work (previously obtained from C. W. Donnelly, Dept. of Nutrition and Food Sciences, University of Vermont, Burlington, VT), was maintained at –70°C in trypticase soy broth (TSB) containing 10% (v/v) glycerol and subcultured twice in trypticase soy broth containing 0.6% (w/v) yeast extract (TSBYE) (Difco Laboratories, Detroit, MI) at 35°C/18-24 h before use.

4.3.4. Hot dog processing

Beef (90% lean, 10% fat) (60%, w/w meat block) (rib lifter meat) (Food Brand America, Lansing, MI) and pork (80% lean, 20% fat) (40%, w/w meat block) (Pork picnic shoulder) (IBP, Inc., Lansing, MI) were coarse ground, placed in a vacuum bowl

chopper (Seydelmann, Frankfurt, Germany) and slowly mixed at of 80 rpm without vacuum. Half of the ice/water mix (30%, w/w ingredients) and salt (1.8% w/w ingredients) were then added after which the mixture was chopped under vacuum (50 Pa) at 4000 rpm for 2 minutes. Corn syrup solids (2.22%), potato starch (2.00%), whey protein concentrate (1.01%), spices (0.50%), beef stock (0.48%), phosphate (0.30%), sodium erythorbate (0.03%), and sodium nitrite (0.25%) were added. The mixture was chopped without vacuum at 2000 rpm for 3 min and then finally chopped at 4000 rpm under vacuum (80 Pa) for 3 min.

The meat emulsion was removed from the bowl chopper and stuffed into heat-sealed WPI casings and commercial (collagen and natural) casings using a hand stuffer (VOGT-deal, Chicago, IL) or a Vacuum Vemag Robot Cocyc 500 stuffer, respectively. After stuffing, hot dogs were hung on smoke-sticks in a smoke house (Cgi Processing Equipment, Model A 28-B0101, Cicero, IL) and processed according to a standard commercial hot dog smokehouse schedule (Table 4.1). Cellulose casings were also used to prepare cooked batter for chemical analysis with the cellulose casings peeled from the hot dogs before analysis. Two batches of hot dogs containing three replicates of each casing type were preapred. The first batch of hot dogs was used for microbiological, chemical (moisture, protein, fat content, pH, TBA), physical (purge, shear force), and mechanical (tensile strength and percent elongation) analysis. The second batch was examined for sensory properties as well as moisture and fat content.

After cooling in a chill room (0°C) for 4 h, the unpeeled hot dogs were surface-inoculated to contain *L. monocytogenes* at a level of 10^3 CFU/g for microbial analysis. Uninoculated

Table 4.1. Hot dog smokehouse schedule.

Stages	Time (min)	Internal Temp.(°C)	Dry Bulb (°C)	Wet Bulb (°C)	Smoke Source (Natural)
1	20	0	60	43	off
2	20	0	71	52	on
3	15	0	77	57	on
4	90	72	82	77	off
Shower		32	1 min on	1 min off	

hot dogs were used for chemical, physical and sensory analysis. All hot dogs were individually vacuum-packaged in Cryovac® vacuum shrink bags (5 x 9 inc^2) (Oxygen permeability- 3-6 cc./atm.day at 40°C) (Sealed Air Corporation, Duncan, SC) and stored at 4°C until analysis.

4.3.5. Chemical Analysis

Hot dog samples with casings were grounded for each casing type analyzed for moisture (AOAC 950.46B) and fat (ether extractable) (AOAC 952-47B) according to standard AOAC (1990) procedures (Appendices 1 and 2). Raw and cooked batter without casings were also ground and analyzed for moisture, fat and protein content. For moisture analysis, ~ 2.0 g-ground samples were placed in ashless wrapped filter paper and dried at 100°C in a air dry oven (Thelco model 18, GCA/Precision Scientific, Chicago, IL) overnight with weight loss after drying giving the moisture content (%). Dried samples were subjected to overnight ether extraction using soxhlet apparatus with weight loss giving the fat content. Protein content of raw and cooked batter as well as hot dogs with casings was determined using the LECO FP-2000 Protein Analyzer (LECO Corporation, St. Joseph, MI) (Appendix 3.).

4.3.6. Rancidity Measurement

The 2-thiobarbuturic acid (TBA) test (Appendix 4) (Tarladgis et al., 1960; Zipser et al, 1962) was used to assess oxidative rancidity initially and after 1, 14, 28 and 42 days of refrigerated storage. The amount of sulfanilamide added to samples for TBA analysis was based on the residual amount of nitrite in each sample according to the modification

of Shahidi and others (1985). Absorbance was read at 538 nm using a Perkin Elmer UV/VIS Spectrometer Lambda 20 (Perkin-Elmer Corporation, North American Organic Division, Norwalk, CT) with a conversion factor of 7.8 used to calculate the final TBA numbers.

4.2.7. pH test

Hot dog samples (1 g) were homogenized in 10 ml of distilled water for 1 min. The pH of the solution was determined using a pH meter (AB15, Fisher Scientific, Hanover Park, IL) equipped with a standard combination electrode (Fisher).

4.3.8. Diffusion Test

Standard Calibration Curve: Standard solutions of sorbic acid or p-aminobenzoic acid were prepared by transferring weighed samples (0.01g) to 100 ml volumetric flasks and diluting to volume with HPLC graded water. This solution was then serially diluted to prepare a series of standard solutions of known concentration for a standard calibration curve.

Sample Preparation: WPI films containing SA and PABA were peeled from the hot dogs initially and 1, 7, 14, 28, 35, and 42 days of refrigerated storage. Film samples (0.1g) were cut into small pieces and soaked in 10 ml of methanol solution (%50 v/v) for 7 days at room temperature. The sample solution was then vortexed for 1 min, diluted 1:10 in methanol-water (1:1, v/v), filtered through a 0.45 microfilter (Fisher) and analyzed for levels of SA and PABA using a Waters 717 HPLC system (Water Corporate, Milford, Massachusetts). The injection volume was 10 μl. Separations were

performed on a stainless steel column (150×5 mm I.D.) filled with 5 μm Nucleosil CN (Waters Corporate). Methanol-water (1:1, v/v) was used as the mobile-phase. The overall flow-rate was held at 1.0 ml/min. The levels of SA and PABA were obtained by substitution in following equation:

$$\%\text{SA or PABA (wt/wt)} = \frac{\text{Rs} \times \text{C.F.} \times \text{Vtotal}}{\text{Vinj} \times \text{Wtpolymer}} \times 100$$

Where: R_s = detector response value for the sample

C.F. = calibration factor (g/A.U.) (from slope of standard calibration curve)

V_{total} = total volume of solution (ml)

V_{inj} = volume of unknown solution injected into the chromatograph (ml)

Wt polymer = weight of the film

The amount of SA and PABA that diffused per unit area as a function of time for WPI edible film was plotted. Extrapolation of the steady state rate for antimicrobial diffusion was used to determine the permeation lag time. The diffusion coefficient was calculated using the following equation:

$$D = \frac{0.049 \times L^2}{t_{1/2}}$$

Where: D = Diffusion coefficient

L = Thickness of the film

$t_{1/2}$ = Time for 50% of the initial antimicrobial diffusion

4.3.9. Microbiological analysis

Hot dogs were examined for numbers of *L. monocytogenes* immediately after inoculation and again following 1, 7, 14, 21, 28, 35, and 42 days of storage at 4°C. Samples weighing 10 g each were added to 90 ml of sterile 0.1% peptone water, homogenized for 3 minutes in a Stomacher 400 (Tekmar Co., Cincinnati, OH), appropriately diluted in sterile 0.1% peptone water and quantitatively examined for *L. monocytogenes*, mesophilic aerobic bacteria, lactic acid bacteria, and yeast/mold using Modified Oxford Agar (Difco), Plate Count Agar (Difco), MRS Lactobaccillus Agar (Difco), and Rose Bengal Agar (Difco), respectively.

4.3.10. Film Thickness

A model M Micrometer (Testing Machine Inc., Amityville, NY) was used to determine casing thickness. Measurements were taken at five different locations and the mean value was used in further calculations for mechanical properties.

4.3.11. Mechanical Properties

Tensile strength (TS) and percent elongation (%E) of casings were determined before and after cooking and after smoking. After peeling, the casings were cut into strips measuring 101.6 mm by 25.4 mm using a Precision Sample Cutter (Thrawing Albert Instrument Co., Philadelphia, PA). Samples were conditioned for 48 hours at 23 ± 2°C / 50 ± 5% RH before testing. Tensile strength and percent elongation at break were determined according to standard D-882-91 (ASTM, 1992). The test was run using the Instron Universal Testing Machine Model 2401 (Canton, MA) at 23 ± 2°C / 50 ± 5% RH

with a static load cell of 1kN and a cross head speed of 50.8 cm/min. TS was calculated in MPa from the following equation:

$$TS = \frac{\text{Load}}{\text{Sample width} \times \text{Sample thickness}}$$

% E at break was determined by the following equation:

$$\%E = \frac{\text{Distance sample stretched}}{\text{Original length of sample}} \times 100$$

4.3.12. Purge loss

Purge loss of hot dogs was measured after 1, 14, 28, and 42 days of storage at 4°C. One package containing four hot dogs for each casing type was weighed and opened. After the hot dogs and residual purge in the package were dried with a paper towel, the hot dogs and package were reweighed. Purge loss was calculated as follow:

$$\%\,\text{purge loss} = \frac{\text{initial package weight} - \text{final package weight}}{\text{initial package weight}} \times 100\,\%$$

4.3.13. Shear Test

Kramer shear values were determined at room temperature using a TAHDi texture analyzer (Texture Technologies Corp., New York, NY). The sample length measured 6 cm cut from the center of the hot dog with the casing placed perpendicular to the multiblades of the Kramer shear attachment. The analyzer had a crosshead speed of 1.67 mm/s with a load cell of 10 kN. Peak force and area under the curve were calculated by the system and divided by sample weight with the results recorded as kg/g.

4.3.14. Color Measurement

Hot dogs with different casings were cut perpendicularly through the center for color measurement. L* (lightness), a* (redness), and b* (yellowness) (Hunter color system) were determined on internal and external surfaces of the hot dogs after at 1, 14, 28, and 42 days of refrigerated storage using a Minolta Chromameter (CR-300 series, Ramsey, NJ) equipped with a granular materials attachment CR-A50. The instrument was standardized using a pink ceramic tile calibrated to tristimulus values. Two hot dogs per treatment were analyzed.

4.2.15. Sensory Evaluation

An untrained, consumer panel (n=150) was used to evaluate sensory attributes of hot dogs prepared with WPI, WPI-PABA, collagen and natural casings. Following 35 days of storage at 4°C, the packaged hot dogs were heated in hot water (82°C) for 10 minutes, removed from the package, cut into 1-inch lengths, held at 60°C, and served to panelists within 15 min. Panelists consisting of students, faculty and staff, primarily from the departments of Food Science and Human Nutrition and Animal Science at Michigan State University, evaluated hot dog attributes (i.e., casing tenderness, texture, flavor, juiciness and overall desirability) by assigning a value based on a 8 point hedonic scale from 8 (extremely like) to 1 (extremely dislike). The panelists were seated in individual lighted booths and provided with cold water and unsalted crackers to allow rinsing and cleaning between samples. SIMS 2000 Computer Program (Sensory Computer System, Morristown, NJ) was used for data collection in a "survey" format. Each individual subject's sensory scores were statistically analyzed and reported using this program in an aggregate form for each product attribute tested.

4.2.16. Statistical Analysis

All experiments were replicated three times. Two-way analysis of variance (ANOVA) was performed using the SAS Statistical Analysis System (SAS Institute Inc., 1990). Means were compared using the Duncan Grouping test at p=0.05.

4.4. RESULTS AND DISCUSSIONS

4.4.1. Chemical Analysis

Chemical composition including moisture, fat and protein content of all four types of hot dogs as well as the raw and cooked batter is shown in Table 4.2. Fat content was similar for all hot dogs; whereas, moisture content was significantly lower for hot dogs prepared with either WPI casing ($p<0.05$). Hot dogs prepared with WPI casings may lose more moisture during processing and storage due to enhanced hydrophilicity of WPI casings. The higher protein content of hot dogs with WPI 1.0% PABA casings might be due to the composition of p-aminobenzoic acid (PABA) since casing protein was also measured as protein by the protein analyzer. In this study, moisture fat and protein content of hot dogs were in agreement with the composition of commercially available hot dogs as reported by Bloukas et al. (1996).

4.3.2. Thiobarbutiric acid values

TBA values, which represent the malonaldehyde concentration in hot dogs, decreased from 0.76, 0.81, 0.76, and 0.84 to 0.68, 0.70, 0.71, and 0.69 mg malonaldehyde/kg for WPI-PABA, WPI, collagen, and natural casings, respectively, after

Table 4.2. Moisture, fat and protein content of raw and cooked batter and hot dogs prepared with WPI-p-aminobenzoic acid (PABA), WPI, collagen and natural casings.

	Type of Casings					
%	WPI-1% PABA	WPI	Collagen	Natural	Raw Batter	Cooked Batter
Moisture	65.4 ± 0.7^{c}	65.4 ± 1.1^{c}	67.4 ± 1.2^{b}	66.3 ± 0.8^{cb}	73.6 ± 0.7^{a}	66.1 ± 1.4^{cb}
Fat	9.5 ± 1.3^{a}	8.6 ± 1.1^{a}	8.9 ± 1.1^{a}	8.8 ± 0.9^{a}	8.0 ± 1.4^{a}	10.2 ± 1.5^{a}
Protein	18.5 ± 0.1^{a}	17.8 ± 0.3^{ab}	17.1 ± 1.0^{ab}	17.1 ± 0.4^{ab}	13.6 ± 1.5^{c}	16.5 ± 1.3^{b}

Mean ± standard deviation (n=3). Means in same row with different superscripts are significantly different ($p<0.05$).

42 days of refrigerated storage (Table 4.3). These TBA values for hot dogs with different casings were not significantly different after 42 days ($p>0.05$). Using different casings for hot dogs did not significantly change the rancidity ($p>0.05$). A general decrease in TBA values was observed during storage. Addition of nitrite and phosphate to hot dog formulations will delay rancidity because of their action as antioxidants (Zubillaga et al., 1984). TBA values in meat are well-correlated with oxidative rancidity (Melton, 1985). However, malonaldehyde, a highly reactive secondary product of lipid oxidation, can

react with other meat components such as amino acids and amines, forming complexes that are not detected by TBA analysis (Gokalp et al., 1983). Therefore, competition for malonaldehyde in a system containing protein could result in reduced color development and incomplete quantitation of malonaldehyde. Hung and Zayas (1989) also showed that TBA values decreased from 0.79 to 0.59 mg malonaldehyde/kg in frankfurters during 45 days of storage.

4.3.3. pH

The pH of hot dogs decreased from ~6.2 to ~5.7 for all casings during refrigerated storage (Table 4.3) with none of these differences significant after 42 days ($p>0.05$). Paneras and Bloukas (1994) also found that the pH of frankfurters decreased from 6.4 to 5.8 during 5 weeks of storage 5°C with this pH decrease attributed to activity of lactobacilli.

Table 4.3. TBA values and pH of hot dogs prepared with WPI-p-aminobenzoic acid (PABA), WPI, collagen, and natural casings during 42 days of refrigerated storage.

		Type of Casings			
	Days	WPI-1.0% PABA	WPI	Collagen	Natural
TBA	1	0.76 ± 0.1^{a}	0.81 ± 0.1^{a}	0.76 ± 0.1^{a}	0.84 ± 0.0^{a}
(mg malonaldehyde /1000g)	14	0.74 ± 0.0^{a}	0.80 ± 0.1^{a}	0.82 ± 0.1^{a}	0.86 ± 0.1^{a}
	28	0.73 ± 0.0^{a}	0.72 ± 0.1^{a}	0.78 ± 0.0^{a}	0.77 ± 0.0^{a}
	42	0.68 ± 0.1^{a}	0.70 ± 0.1^{b}	0.71 ± 0.1^{b}	0.69 ± 0.1^{ab}
pH	1	6.21 ± 0.1^{a}	6.10 ± 0.1^{b}	6.20 ± 0.1^{ab}	6.21 ± 0.1^{ab}
	14	6.00 ± 0.0^{a}	6.14 ± 0.1^{b}	6.10 ± 0.0^{ab}	6.11 ± 0.0^{ab}
	28	5.80 ± 0.1^{a}	5.86 ± 0.1^{a}	5.82 ± 0.1^{a}	5.73 ± 0.1^{a}
	42	5.72 ± 0.1^{a}	5.73 ± 0.1^{a}	5.74 ± 0.1^{a}	5.73 ± 0.0^{a}

Mean ± standard deviation (n=3). Means in same column with different superscript are significantly different ($p<0.05$).

Table 4.4. Analysis of Variance on the effect of casing type on microbiological, chemical, and physical characteristics of hot dogs during 42 days of refrigerated storage (P-values for independent variables and interactions)

	Source of Variance		
	Casings	Time	Interaction (Casings x Time)
L. monocytogenes	<0.0001***	<0.0001***	0.0004**
MAB	0.019*	<0.0001***	0.31
LAB	<0.0001***	<0.0001***	0.0018**
Mold	0.64	<0.0001***	0.84
pH	0.95	<0.0001***	0.92
TBA	0.18	0.0014**	0.88
Purge	0.0015**	0.22	0.54
Color-interior			
L* (Lightness)	0.35	<0.0001***	0.39
a* (redness)	0.85	<0.0001***	0.40
b*(Yellowness)	0.89	<0.0001***	0.45
Color-exterior			
L* (Lightness)	<0.0001***	<0.0001***	0.003**
a* (redness)	0.13	0.05	0.18
b*(Yellowness)	0.0008***	0.18	0.39

*significant at p<0.01; ** significant at p<0.001, *** significant at p<0.0001.
MAB: Mesophilic aerobic bacteria, LAB: Lactic acid bacteria

4.3.4. Diffusion Coefficient

After one day of refrigerated storage, 78.7 and 75.6% of the PABA and SA diffused from the WPI film into the hot dog with only 7.7 and 6.5 % of initial PABA and SA remaining in the WPI casings after 42 days of storage, respectively. For these whey protein-based films diffusion coefficients for PABA and SA at 4°C were 1.7×10^{-13} m^2/sec and 3.2×10^{-13} m^2/sec, respectively. Chen et al. (1996) also studied diffusion of SA and benzoic acid from edible film and found that about 39% of SA and benzoic acid was released from methycellulose/chitosan films into a glycerol/water solution (a_w=0.8) within 30 minutes at 4°C. Since the storage time was only 6 hours in their study, the maximum release of antimicrobials was only 49%. Raising the temperature to 25°C enhanced the diffusion rate for both antimicrobials from these films; however, increasing the pH from 3.0 to 6.0 had no effect on diffusion.

4.4.5. Antimicrobial analysis

The numbers of *L. monocytogenes* on hot dogs with control (WPI without antimicrobials) and collagen casings increased 4.2 and 5.4 logs, respectively, after 42 days of storage at 4°C (Figure 4.1). Using casings containing 1.0% PABA, *Listeria* populations remained relatively unchanged; however, numbers of *Listeria* on hot dogs prepared with WPI casings containing 1.0% SA or 0.5% SA: 0.5% PABA (1:1) increased 2.8 and 2.1 logs after 42 days, respectively. WPI casings containing SA were less effective than WPI casings containing PABA for inhibiting *Listeria* growth on hot dogs even though SA-containing films decreased populations of *Listeria* on bologna and summer sausage slices in our previous study (Cagri et al., 2002). Acidity of WPI casings (pH 5.2) may decrease when used on hot dogs (pH 6.1) due to the diffusion of chemical

components between the hot dog batter and the casing (e.g. lactic acid). Sorbic acid (pKa 4.19) is most active at pH<6.0 since it is the undissociated form that possesses antimicrobial activity. However, PABA (pKa 4.8) still shows some effectiveness against *L. monocytogenes* at pH values as high as 6.0 (Richards et al., 1995). Overall, WPI casings containing 1.0% PABA were most inhibitory to *Listeria* and were further examined in detail.

Listeria monocytogenes: After 42 days of refrigerated storage *L. monocytogenes* populations increased about 2.5 logs on hot dogs prepared with WPI-control (antimicrobial-free), collagen, and natural casings (Figure 4.2). In contrast, WPI casings containing 1.0% PABA inhibited the growth of *L. monocytogenes* throughout 42 days of refrigerated storage. In our previous work, WPI films containing 1% PABA were more inhibitory, decreasing numbers of *L. monocytogenes* 2.8 to 3.0 logs on both bologna and summer sausage slices during 21 days of refrigerated storage (Cagri et al., 2002). Stacking these slices in sterile petri dishes may have induced greater release of PABA from the film to the slices compared to the hot dog casings, thereby allowing greater inhibition. As mentioned earlier, increasing the initial pH (5.2) of WPI casings would tend to decrease the antimicrobial activity of PABA. While phenolic compounds from the smoking process and nitrite in the hot dogs may also enhance the inhibitory effect of WPI-1% PABA casings against *Listeria* (Lou and Yousef, 1999), such effects were not observed in our study.

Previous findings (Richards and Xing, 1992; Richard, 1995) indicated that undissociated uncharged PABA diffuses through the cell membrane more freely and results in PABA exerting an increased activity as the pH decreases from 7.0 to 5.0 since the pKa value of

PABA is 4.8 (Terada, 1972). Inside the cell, the dissociation of PABA leads to the uncoupling of both substrate transport and oxidative phosphorylation from the electron transport system with growth inhibition resulting principally from the lost the cellular uptake of amino acids, phosphate and organic acids (Freese et al., 1973). However, even at pH values close to neutral (pH 6.5-7.0) PABA is still reportedly inhibitory to *L. monocytogenes* (Richards et al., 1995). This explains the inhibitory effect of WPI-PABA casings against *Listeria* on hot dogs even at above optimal pH value in our study. Incorporating a buffering agent in the WPI-PABA casing formulation may help stabilize the hot dog pH at a value that would be more optimal for inhibition of *Listeria.*

Diffusion test results showed that while only 0.12% (w/v) PABA remained the day after manufacturing, this concentration was still sufficient to prevent *Listeria* growth during 42 days of storage. However, if diffusion of PABA could be delayed by modifying the WPI casing preparation (e.g. tightening the protein chains with crosslinking agent), WPI-1.0% PABA would be even more effective against *L. monocytogenes.*

Mesophilic Aerobic Bacteria: Populations of mesophilic bacteria increased 2.6, 3.7, 4.2, and 5.1 logs on hot dogs prepared with WPI-1.0% PABA, commercial collagen, natural and WPI-control casings, respectively, during 42 days of storage at 4°C (Figure 4.3). Growth of mesophilic bacteria on hot dogs was suppressed 1.0 to 2.5 logs using WPI casings containing 1% PABA. Our previous study also showed that growth of mesophilic bacteria on bologna and summer sausage slices was retarded at 4°C using WPI films containing 0.75 and 1.0% PABA (Cagri et al., 2002). The various microbial reduction strategies used for processed meats typically select for specific groups of organisms, some of which can proliferate and cause spoilage during storage. Processed meats generally spoil due to facultative Gram-positive bacteria (lactic acid bacteria) that

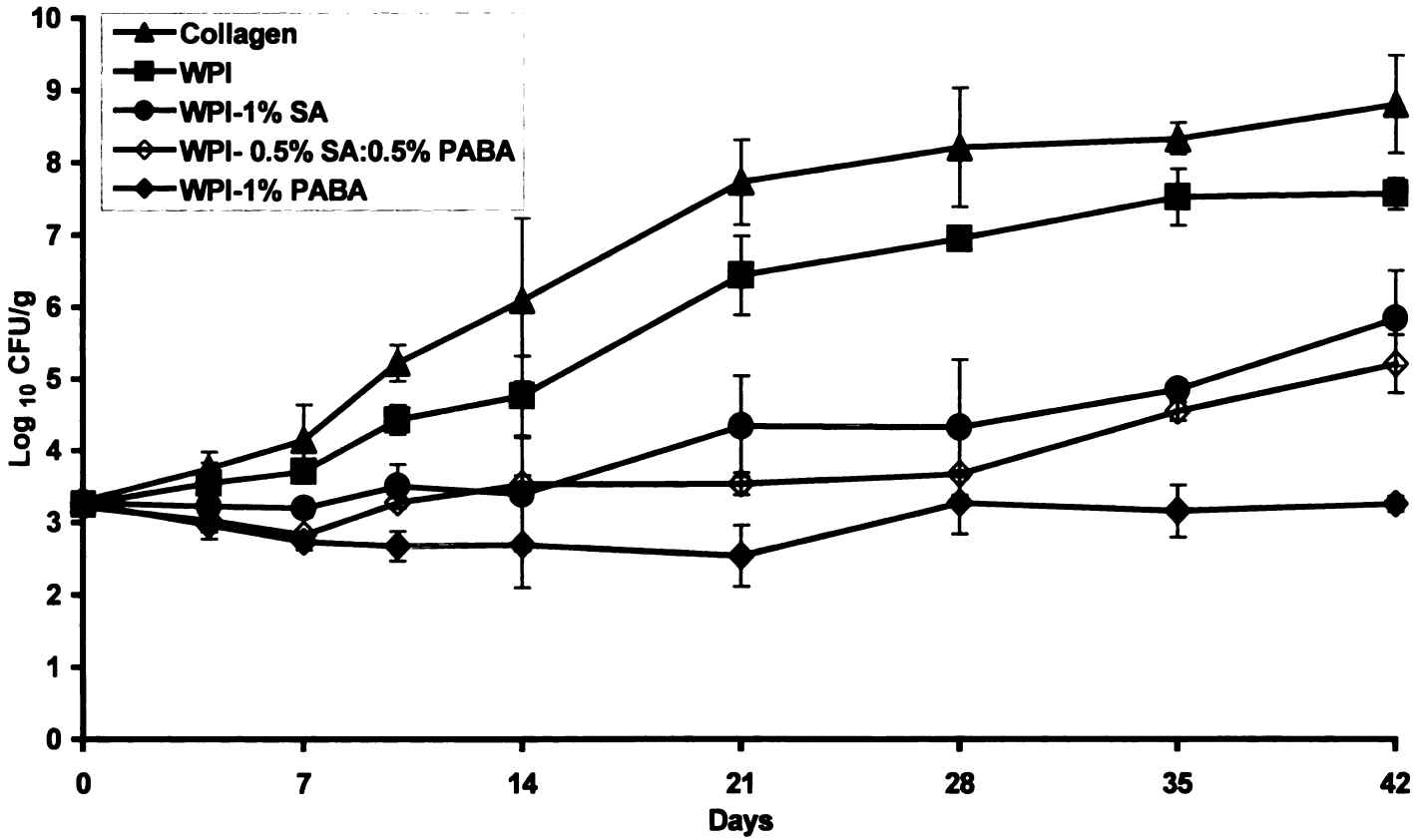


Figure 4.1. Preliminary study: Growth of *L. monocytogenes* on hot dogs prepared with different casings. Means ± standard deviation (n=3).

are able to grow in vacuum-packaged product at refrigeration temperatures. Common spoilage defects during long term storage (10-12weeks) of hot dogs include various off-odors, -flavors and -tastes, as well as color defects. These defects appear when the bacterial count has exceeded to 10^8-10^{10} CFU/g) (Silla and Simonsen, 1985). In the present study, none of the hot dogs exhibited any noticeable defects after 42 days of storage. Since shelf-life of commercial hot dogs may exceed 42 days, some defects could eventually appear on hot dogs prepared with collagen, natural and WPI-casings (antimicrobial-free) as the storage time is increased. However, WPI casings containing PABA may reduce the risk of spoilage by retarding growth of mesophilic bacteria even after opening the package.

Lactic Acid Bacteria: Casings containing 1% PABA were also most effective at retarding the growth of lactic acid bacteria on hot dogs (Figure 4.4). Populations of lactic acid bacteria were 1.7 to 2.2 logs lower on hot dogs cased in WPI-1.0% PABA compared to the other casings. Based on analysis of variance, casing type and storage time had an interactive effect on growth of LAB (Table 4.4). Cagri et al. (2002) reported that WPI films containing 1.0% PABA also partially inhibited lactic acid bacteria on bologna and summer sausage slices. Lactic acid bacteria comprise the major group of spoilage organisms in vacuum-packaged meat products (Borch et al., 1996; Holzapfel, 1998; Johnston and Tompkin, 1992). In the present study, lactic acid bacteria grew to high levels on hot dogs prepared with collagen, natural and antimicrobial-free WPI casings with WPI casings containing PABA helping to delay spoilage and extend product shelf-life.

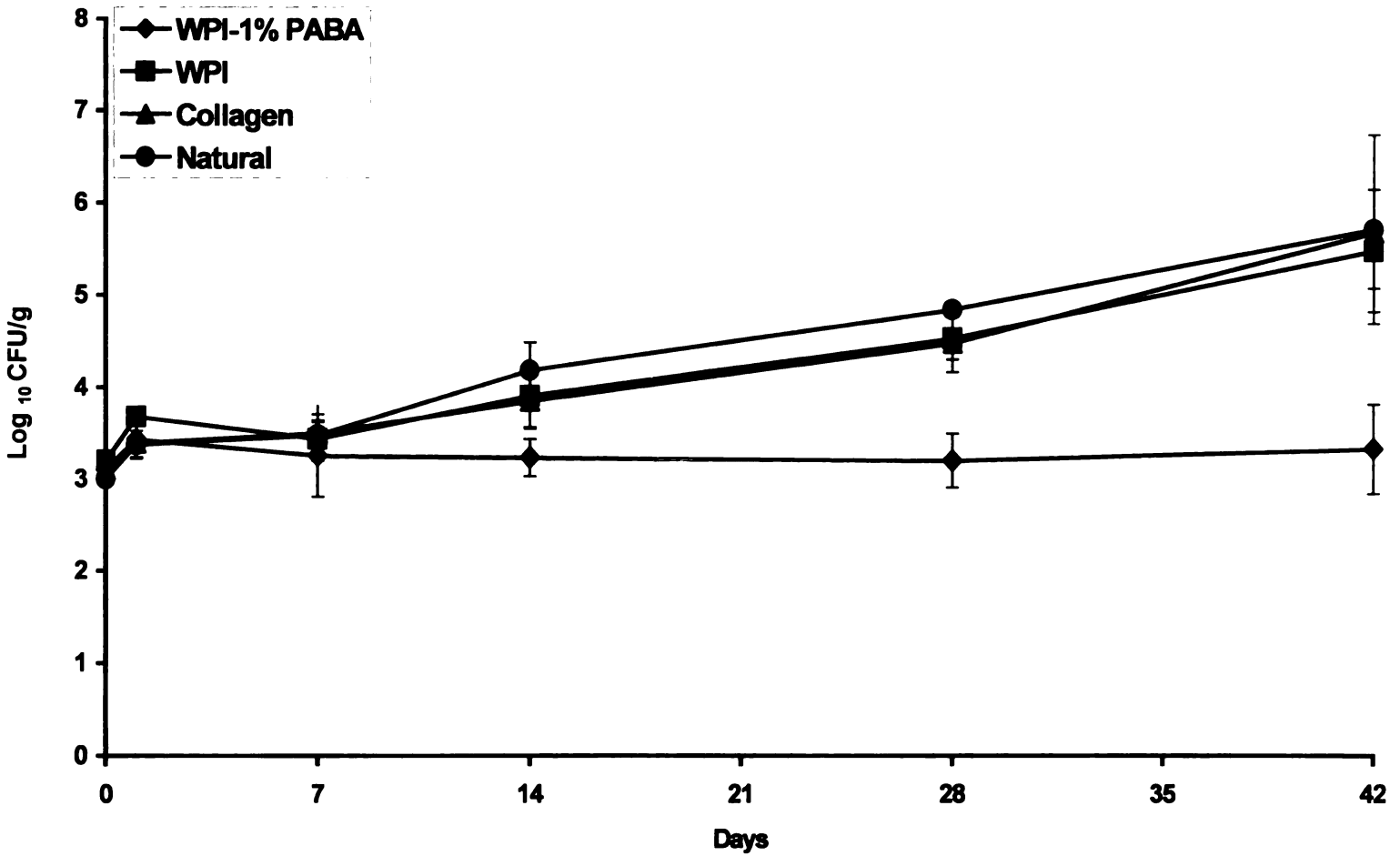


Figure 4.2. Growth of *Listeria monocytogenes* on hot dogs prepared with different casings. Geometric means ± Standard deviation (n=3).

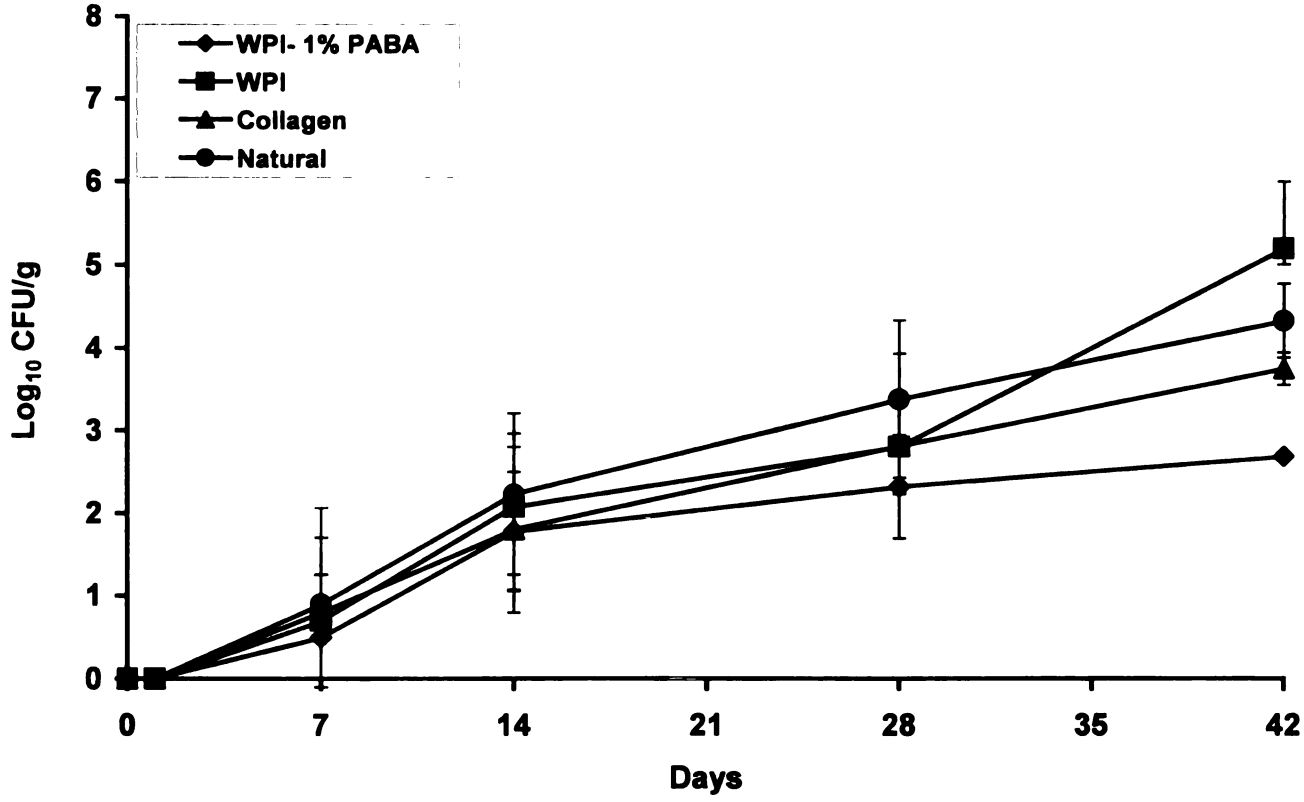


Figure 4.3. Growth of mesophilic aerobic bacteria on hot dogs prepared with different casings. Geometric mean ± standard deviation (n=3).

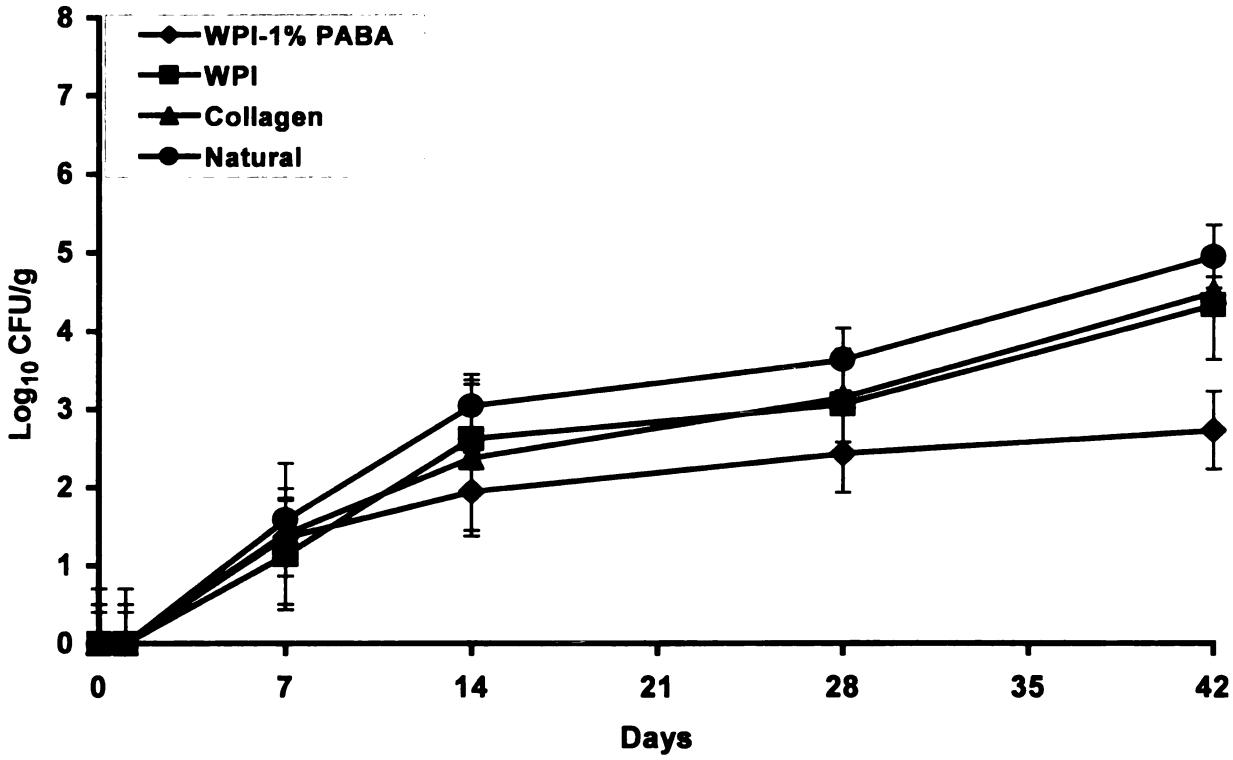


Figure 4.4. Growth of lactic acid bacteria on hot dogs prepared with different casings. Geometric mean ± standard deviation (n=3).

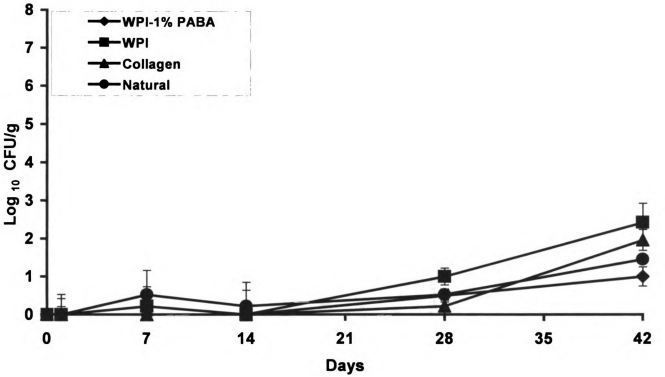


Figure 4.5. Growth of mold on hot dogs prepared with different casings. Geometric mean ± standard deviation (n=3).

Mold/ Yeasts: Mold counts increased only 1.0 to 2.4 logs on hot dogs during 42 days due to the combination of vacuum packaging and storage at refrigeration temperature (Figure 4.5). Mold growth was least using WPI-1.0% PABA casings. Although benzoic acid is a well known inhibitor of yeasts and molds (Balatsouras et al., 1963), some mold growth was observed on WPI casings containing 1.0% PABA. Although all hot dogs were vacuum-packaged, some air diffusion through inadequateheat seals could explain the mold growth that was detected on a limited number of samples. No yeasts were found during 42 days of refrigerated storage.

4.4.6. Mechanical Properties

Tensile strength of WPI casings remained unchanged after cooking and smoking (Table 4.5), whereas tensile strength of the collagen and natural casings decreased significantly from 34.8 and 43.3 to 10.2 and 9.6 MPa, respectively ($p<0.05$). Percent elongation of WPI casings containing 1.0% PABA was also unchanged after cooking and smoking (Table 4.5); however, percent elongation increased from 12.4 to 23.5% in WPI casings without PABA and decreased from 41.9 and 56.7 to 24.7 and 21.4% in collagen and natural casings, respectively.

In this study wet strength of casings were not measured. However, compared to natural and collagen casings, WPI casings required extra care to avoid breakage during stuffing and smoking. A hand stuffer was used for WPI casings adjusted to avoid breaking the heat seal or rupturing. In addition, the rate of air circulation and the humiditywere reduced during the first stage of smoking and cooking in order to keep the hot dogs on the smoke house tract.

4.4.7. Cook yields and purge loss

Hot dogs prepared with WPI casings had cook yields of 88.9 -89.4% compared to 94.3 and 95.3% for hot dogs prepared with collagen and natural casings, respectively (Figure 4.6A). Purge loss for hot dogs prepared with WPI-PABA or collagen casings did not significantly change during 42 days (Figure 4.6). However, purge loss for hot dogs prepared with WPI and natural casings decreased and increased after 14 days, respectively. WPI and WPI-PABA casings exhibited higher purge losses (0.60 and 0.69%) than did hot dogs with collagen (0.24%) and natural casings (0.30%). WPI casings are hydrophilic even after incorporation of candellila wax to decrease water vapor permeability. This explains the higher liquid release through hydrophilic WPI casings compared to other commercial casings.

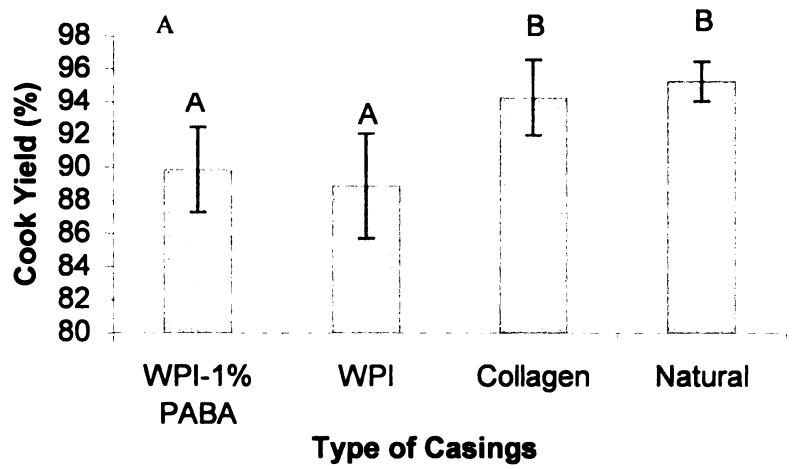
4.3.8. Shear Force

Hot dogs are suitable for shear testing since they have a constant diameter and homogeneous structure. The resulting shear forces for hot dogs prepared with WPI-1% PABA (0.75 kg/g), WPI (0.80 kg/g) and collagen (0.59 kg/g) were similar (Figure 4.6B), whereas hot dogs prepared with natural casings had a significantly higher shear force (1.71 kg/g) ($p<0.05$). When consumed, hot dogs are normally first bitten through with front teeth and then ground on the molars (Boyar and Kilcast, 1986). This biting action was used as the basis of texture measure

Table 4.4. Tensile strength (TS) and percent elongation (% E) of WPI-p-aminobenzoic acid (PABA), WPI, collagen, and natural casings before and after cooking and smoking of hot dogs.

	Casings	Before cooking and smoking	After cooking and smoking
TS (MPa)	WPI-1% PABA	19.5 ± 0.2^{c}	16.9 ± 0.3^{c}
	WPI	6.5 ± 0.4^{a}	5.4 ± 0.6^{a}
	Collagen	34.8 ± 0.1^{d}	10.2 ± 0.4^{b}
	Natural	43.3 ± 0.3^{c}	9.6 ± 0.2^{b}
% E	WPI-1% PABA	40.7 ± 0.2^{c}	39.8 ± 0.3^{c}
	WPI	12.4 ± 0.3^{a}	23.6 ± 0.1^{b}
	Collagen	41.9 ± 0.5^{c}	24.7 ± 0.3^{b}
	Natural	56.7 ± 0.3^{d}	21.4 ± 0.5^{b}

Mean ± standard deviation (n=3). Means with different superscript are significantly different ($p<0.05$).



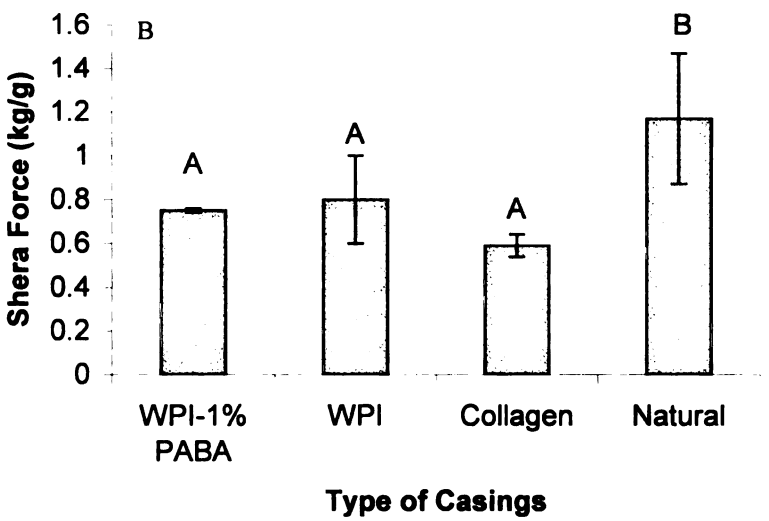


Figure 4.6. Cook Yield (A) and Shear force (B) of hot dogs prepared with WPI- p-aminobenzoic acid (PABA), WPI, collagen, and natural casings.

the texture meat which most closely relates to human assessment. Consequently, our shear force results suggest that hot dogs prepared with WPI and collagen casings will be more chewable, softer and thus more acceptable to consumers than hot dogs prepared with natural casings.

4.3.9. Color

The color of hot dogs plays an important role in consumer acceptance. L*, a*, and b* Hunter color values of interior and exterior hot dog surfaces are shown in Table 4.7. L* (lightness) and a* (redness) values significantly increased in interior samples from all hot dogs during storage ($p<0.05$); however, b* (yellowness) only increased for hot dogs encased in WPI-1.0% PABA after 28 days. Exterior yellowness of hot dogs with WPI casings was significantly higher than for hot dogs prepared with collagen and natural casings (Table 4.8). Exterior lightness (L*) of hot dogs prepared with WPI and collagen casings increased with storage time while lightness of hot dogs prepared with natural casing remained unchanged during 42 days of storage. Exterior redness (a*) and yellowness (b*) of WPI-1.0% PABA, collagen, and natural casings did not change throughout storage, whereas both of these attributes significantly increased for WPI-0% PABA casings. Based on analysis of variance, exterior lightness and yellowness of hot dogs significantly changed with casing type; however, storage time and casing type interaction only had effect on the lightness value ($p<0.001$) (Table 4.4).

Lightness refers to the relation between reflected and absorbed light, without regard to a specific wavelength. Yellowness or redness results from differences

Table 4.6. Purge loss of hot dogs prepared with WPI-p-aminobenzoic acid (PABA), WPI, collagen, and natural casings during 42 days of refrigerated storage.

		Type of Casings			
	Days	WPI-1.0% PABA	WPI	Collagen	Natural
Purge loss (%)	1	0.69 ± 0.0^{a}	0.60 ± 0.1^{a}	0.24 ± 0.0^{b}	0.30 ± 0.1^{b}
	14	0.87 ± 0.1^{a}	0.57 ± 0.1^{a}	0.25 ± 0.1^{b}	0.37 ± 0.1^{b}
	28	0.80 ± 0.0^{a}	0.37 ± 0.1^{b}	0.25 ± 0.1^{b}	0.43 ± 0.1^{b}
	42	0.76 ± 0.1^{a}	0.42 ± 0.1^{b}	0.22 ± 0.0^{b}	0.42 ± 0.1^{b}

Mean ± standard deviation (n=3). Means within the same row with different superscript are significantly different ($p<0.05$).

Table 4.7. Interior color of hot dogs prepared with WPI-p-aminobenzoic acid (PABA), WPI, collagen, and natural casings during 42 days of refrigerated storage.

		Type of Casings			
Hunter Color	Days	WPI-1.0% PABA	WPI	Collagen	Natural
	1	47.7 ± 0.6^{a}	47.7 ± 0.7^{a}	48.3 ± 0.3^{ab}	48.4 ± 0.3^{b}
L*	14	47.9 ± 0.5^{a}	48.2 ± 0.6^{b}	48.2 ± 0.5^{c}	48.5 ± 0.5^{d}
(lightness)	28	49.6 ± 0.3^{ab}	49.9 ± 0.6^{a}	49.7 ± 0.5^{ab}	49.4 ± 0.3^{b}
	42	48.9 ± 0.6^{a}	49.7 ± 0.4^{b}	49.3 ± 0.2^{ab}	48.9 ± 0.5^{a}
	1	21.0 ± 0.2^{a}	21.3 ± 0.3^{a}	21.5 ± 0.6^{a}	21.6 ± 0.6^{a}
a*	14	21.6 ± 0.3^{a}	21.5 ± 0.4^{a}	21.6 ± 0.2^{a}	21.8 ± 0.1^{a}
(redness)	28	22.5 ± 0.4^{a}	22.5 ± 0.8^{a}	22.1 ± 0.1^{a}	22.3 ± 0.8^{a}
	42	22.1 ± 0.6^{a}	22.5 ± 0.1^{ab}	22.1 ± 0.4^{ab}	21.8 ± 0.3^{b}
b*	1	6.5 ± 0.2^{a}	6.6 ± 0.1^{a}	6.7 ± 0.1^{a}	6.7 ± 0.1^{a}
(yellowness)	14	6.9 ± 0.3^{a}	6.7 ± 0.3^{a}	6.8 ± 0.2^{a}	6.9 ± 0.1^{a}
	28	7.7 ± 0.1^{a}	7.4 ± 0.3^{ab}	7.2 ± 0.2^{b}	7.1 ± 0.1^{b}
	42	7.6 ± 0.5^{a}	7.4 ± 0.2^{ab}	7.2 ± 0.2^{b}	7.1 ± 0.3^{b}

Mean ± standard deviation (n=3). Means within the same row with different superscript are significantly different ($p<0.05$).

Table 4.8. Exterior color of hot dogs prepared with WPI-p-aminobenzoic acid (PABA), WPI, collagen, and natural casings during 42 days of refrigerated storage.

		Type of Casings			
Hunter Color	Days	WPI-1.0% PABA	WPI	Collagen	Natural
	1	40.8 ± 0.2^{a}	41.7 ± 0.1^{c}	41.2 ± 0.1^{b}	41.9 ± 0.0^{d}
L*	14	40.8 ± 0.1^{a}	41.7 ± 0.0^{c}	41.4 ± 0.1^{b}	41.8 ± 0.2^{c}
(lightness)	28	41.3 ± 0.0^{a}	41.9 ± 0.0^{c}	41.6 ± 0.0^{b}	41.9 ± 0.1^{c}
	42	41.2 ± 0.1^{a}	42.7 ± 0.4^{c}	42.1 ± 0.2^{bc}	42.0 ± 0.4^{b}
	1	22.4 ± 0.4^{a}	23.1 ± 0.1^{b}	22.8 ± 0.2^{ab}	22.7 ± 0.2^{ab}
a*	14	22.8 ± 0.0^{ab}	22.9 ± 0.1^{bc}	22.7 ± 0.1^{c}	22.9 ± 0.0^{a}
(redness)	28	23.2 ± 0.0^{a}	22.9 ± 0.0^{a}	23.3 ± 0.6^{a}	23.0 ± 0.0^{a}
	42	22.8 ± 0.5^{a}	23.9 ± 0.3^{b}	22.9 ± 0.1^{a}	23.0 ± 0.8^{a}
b*	1	9.3 ± 0.1^{a}	9.3 ± 0.6^{a}	9.1 ± 0.4^{a}	9.1 ± 0.1^{a}
(yellowness)	14	9.3 ± 0.1^{a}	9.4 ± 0.8^{a}	9.0 ± 0.9^{c}	9.1 ± 0.9^{b}
	28	9.5 ± 0.5^{b}	9.8 ± 0.6^{a}	8.9 ± 0.1^{d}	9.1 ± 0.9^{c}
	42	9.5 ± 0.8^{a}	10.4 ± 0.2^{b}	9.0 ± 0.5^{a}	9.3 ± 0.6^{a}

Mean ± standard deviation (n=3). Means within the same row with different superscript are significantly different ($p<0.05$).

in absorption of radiant energy at various wavelengths. Nitrite is the agent added to hot dogs to produce a pink color. Nitrosylhemochrome-cured meat pigment is formed by heating of nitric oxide and myoglobin complex. The most significant commercial problem associated with cured meat color is rapid fading in air and light (Walch and Rose, 1956), which can be delayed by vacuum-packaging. Light induced dissociation of nitric oxide from heme followed by oxidation of heme and nitric oxide. Addition of erythorbate stabilizes pigment fading (deHoll, 1981). In the present study, no fading was observed. Although not measured, similarity in exterior color of hot dogs also suggests that a similar amount of smoke was absorbed during smoking and cooking by the WPI and commercial casings. These findings may indicate that the interior and exterior color of hot dogs prepared with WPI-1% PABA would be acceptable to customers since their color values were not significantly different from the color of hot dogs prepared with commercial casings.

4.4.10. Sensory Analysis

Sensory characteristics of hot dogs prepared with different casings are presented in Table 4.9. Using a hedonic scale (1-8) juiciness (6.1) and flavor (6.3) attributes of hot dogs with WPI-1% PABA casings were preferred ($p<0.05$) and scored higher by panelist than hot dogs with WPI, collagen and natural casings. Similarly, texture (5.9) and overall desirability (5.4) attributes of hot dogs with WPI-1% PABA were not significantly different from hot dogs with other casings ($p<0.05$). However, the casing tenderness score for WPI hot dogs (4.2-4.4) was significantly less ($p<0.05$) than the other casings (4.8-5.4). Collagen casings were preferred based casing tenderness. Panelists scored tenderness by biting vertically through the casing with their front teeth. Many panelists

Table 4.9. Sensory attributes and chemical content of hot dogs prepared with WPI-p-minobenzoic acid (PABA), WPI, collagen, and natural casings after 35 days of refrigerated storage.

	Type of Casings			
Attribute	WPI-1% PABA	WPI	Collagen	Natural
Casing tenderness	4.4 ± 0.1^{c}	4.2 ± 0.2^{c}	5.4 ± 0.6^{a}	4.8 ± 0.4^{b}
Juiciness	6.1 ± 0.2^{a}	5.9 ± 0.1^{ab}	5.6 ± 0.1^{b}	5.6 ± 0.2^{b}
Texture	5.9 ± 0.1^{a}	5.7 ± 0.2^{ab}	5.5 ± 0.1^{b}	5.6 ± 0.1^{ab}
Flavor	6.3 ± 0.1^{a}	5.8 ± 0.1^{b}	5.6 ± 0.2^{b}	5.8 ± 0.0^{b}
Overall desirability	5.4 ± 0.3^{a}	5.0 ± 0.2^{b}	5.3 ± 0.2^{ab}	5.2 ± 0.1^{ab}
Chemical content (%)				
Moisture	71.5 ± 0.3^{a}	70.3 ± 0.5^{a}	71.5 ± 0.7^{a}	71.2 ± 0.5^{a}
Fat	6.3 ± 0.3^{b}	6.5 ± 0.2^{ab}	6.3 ± 0.1^{b}	6.9 ± 0.3^{a}

Mean ± standard deviation (n=3). Means in same row with different superscripts are significantly different ($p<0.05$). A Hedonic scale was used for sensory evaluation of hot dogs from 1 (extremely undesirable) to 8 (extremely desirable).

dentified all the hot dogs as being tough and chewy since most consumers prefer skinless hot dogs. Sealed packages of hot dogs were heated in hot water to avoid flavor changes and loss of antimicrobial with this heating likely making the hot dogs tougher. Overall, the sensory characteristics of hot dogs prepared with WPI casings containing 1.0% PABA were acceptable to the150 panelists.

Moisture content of hot dogs prepared with different casings used in the sensory panel was not significantly different ($p<0.05$) (Table 4.8); however, fat content was higher in hot dogs with natural casings. Since a low-fat frankfurter formulation was used in this study, scores given for overall hot dog desirability were predictably low. Reduction of fat is an important aspect of consumer acceptance because fat adds flavor to frankfurters. Pearson et al. (1987) suggested that acceptable low-fat frankfurters could be produced by substituting water for fat. Park et al. (1990) reported that frankfurters with 14% fat and a high moisture content (~70%) were considered as undesirable as control products with 29% fat.

In summary, WPI edible casings containing 1% PABA were more inhibitory towards *L. monocytogenes* than other commercial casings with *Listeria* growth suppressed on hot dogs during 42 days of refrigerated storage. These casings also showed considerable promise in extending the shelf-life of hot dogs. Mechanical properties of WPI casings remained unchanged; however, tensile strength and percent elongation of commercial casings decreased during hot dog manufacture. Sensory panelists gave hot dogs with WPI-PABA casings superior scores for texture, juiciness, flavor and overall desirability compared to hot dogs prepared with collagen or natural casings. Given these

overall findings, WPI casings containing 1.0% PABA may provide a promising *Listeria* intervention strategy to prevent growth of this post-processing contaminant on hot dogs.

CONCLUSION

Low pH (5.2) whey protein isolate–based edible films containing p-aminobenzoic acid (PABA) or sorbic acid (SA) inhibited the growth of *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium DT104 on TSAYE. Increased concentrations of PABA or SA in WPI film increased the percent elongation (%E) and water vapor permeability (WVP). While tensile strength (TS) of WPI films decreased with increasing levels of SA, TS of films was not affected addition of PABA.

Subsequently, WPI films containing 1.0% SA, PABA and 1.0% SA:PABA (0.5:0.5) were tested between commercially produced bologna and summer sausage slices to observe whether the antimicrobial and mechanical properties remained constant while being contact with slices. Populations of *L. monocytogenes*, *E. coli* and *S.* Typhimurium decreased on bologna and summer sausage slices in contact with the film during 21 days at 4°C. Mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB) and yeasts/mold were inhibited using antimicrobial films compared to antimicrobial-free controls. Film tensile strength decreased while % E remained unchanged following 72 h of product contact.

After heat curing to modify mechanical properties, WPI films (pH 5.2) containing 0.0 or 1.0% (w/v) PABA were heat-sealed to form casings. A commercial-type hot dog batter was stuffed into WPI, collagen or natural casings. After cooking, the hot dogs were surface-inoculated to contain 10^3 *Listeria monocytogenes* CFU/g. *Listeria* populations on hot dogs prepared with WPI-1.0% PABA casings remained relatively unchanged; however, numbers of *Listeria* on hot dogs prepared with WPI-0.0% PABA, collagen, and natural casings increased ~2.5 logs during 42 days of refrigerated storage. Populations of

MAB, LAB, and mold on WPI-1.0% PABA casings were 1-3 logs lower compared to the other casings. TS and % E of WPI casings remained unchanged after cooking and smoking; however, TS and % E of the collagen and natural casings decreased. TBA and pH values, which decreased during storage, were similar for hot dogs with different casings. Shear force of hot dogs prepared with WPI and collagen was lower than that for natural casings. Purge loss was higher in hot dogs prepared with WPI rather than collagen or natural casings. Sensory attributes (overall desirability, flavor, texture, juiciness) of hot dogs with WPI-1% PABA casings were scored higher than hot dogs prepared with commercial collagen and natural casings.

WPI casings containing 1.0% PABA offer another strategy to prevent growth of *Listeria* on hot dogs and extend shelf-life. However, one of the main challenges ahead will be to develop a continuous extrusion process for WPI casing production since the current casting method is not economically viable for commercial hot dog production. One important hurdle to overcome will be the reduction of water activity in casing formulation to enhance the extrusion process. In addition, the WPI casing formulation will need to be modified for fast heat drying since heat drying is an integral step in the extrusion process.

Antimicrobial activity of WPI casings containing PABA against *Listeria* and spoilage organisms may be enhanced by minimizing the increase in pH during hot dog manufacture. For example, addition of a buffering agent into the casing formulation may prevent increase in the WPI casings pH from 5.2 to ~ 6.0. Since PABA is more active at low pH, stabilizing the casing pH at 5.2 will help maintain optimal antimicrobial the

activity of PABA. In addition, testing different antimicrobials such as lysozyme which is heat resistant and active over a wide pH range is highly recommended.

According to our diffusion test results, most of the PABA was released from the WPI casing after the first day of hot dog manufacture. Diffusion of PABA from WPI casings after hot dog processing must be somehow controlled. When protein-based edible casings come into contact with food surface the high water activity results in absorption of water and swelling, which in turn leads a loosen casing structure and enhanced diffusion of any additives. In this study, the casings were heat-cured to decrease water solubility and strengthen the casing. However, heat curing alone was insufficient to slow the release of PABA from the casing. Incorporating of various crosslinking agents in WPI casing may help tighten the protein chains in the casing matrix and thus decrease water absorption and loosening of the casing structure.

Overall, this work represents only the first step towards the development of possible commercialization WPI-based antimicrobial casings to reduce the risk of *Listeria* growth on hot dogs.

APPENDIX I

MOISTURE ANALYSIS-Air Drying Method

Sample Preparation (modified from section 983.18 Meat and Meat Products, AOAC 1990)

Hot dogs were cut into very small (<1 cm squares) pieces and were added to Tekmar grinders filling the grinding chamber halfway, dry ice was added to fill the remaining space the chamber. Samples were ground for 2-3 minutes into a fine powder which was transferred to labeled whirl pack bags. The bags were loosely closed and stored in a freezer for 2 days so that dry ice could evaporate.

Moisture Analysis (modified from section 950.46B-Air drying for determination of moisture in meats)

Two grams (± .03g) of a well ground hot dog sample was added to an ashless filter paper thimble. The edge of paper thimble was fold over the top and secured with a paperclip. Samples were placed flat on a tray in a 100°C dry-air oven (Thelco model 18, GCA/Precision Scientific, Chicago, IL) and for 20 - 24 h. After drying, the samples were completely cool in a dessicator before weighing. Once cool, sample weighs were recorded. This weight was the final weight for moisture and the initial weight for fat analysis. The following formula was used to determine percent moisture:

$$\% \text{ Moisture} = \frac{\text{Wet sample weight} - \text{dry sample weight}}{\text{Wet sample weight}} \times 100$$

APPENDIX II

FAT ANALYSIS - modified from 952-47 B soxhlet ether extraction method for meat (AOAC 1990)

Samples (approximately 2 g) were weighed in ashless filter thimble paper and dried in an oven at 100°C for 24 h. Dried samples were weighed again after cooling to room temperature in a dessicator.

Dried samples were placed in extraction tubes with all samples below the level where the ether drains off (curved glass on outside of tube). Petroleum ether was added to clean boiling flasks until about full. Two or three glass beads were placed in the boiling flask (as a boiling aid) which was connected to the extraction flask. Condensing units were placed on top of extraction flasks and the Rheostat was set on high for heating. After 24 h of heating, the cooled samples were poured onto trays in a hood and the ether was allowed to dissipate. After 5 to 10 min in an air-dry oven to remove any possible moisture, samples were placed in a dessicator to cool and then weighed again. Percent fat in the sample was determined as follow:

$$\text{Fat (\%)} = \frac{\text{Dry sample weight - extracted sample weight}}{\text{Wet sample weight}} \times 100$$

APPENDIX III

PROTEIN ANALYSIS

Sample Preparation: A 1-g sample of powdered hot dog was weighted in a porcelain crucible. The weight and sample code were entered into the program in the Leco computer. The sampler were then dried for 18 to 20 h in an oven to remove residual moisture that can cause internal malfunctions with the Leco Protein Analyzer.

LECO FP-2000 Protein Analyzer

Principal: The LECO FP-2000 Protein Analyzer (LECO Corporation, St. Joseph, MI) is a non-dispersive, infrared, mocrocomputer based-instrument, designed to measure the nitrogen content in a wide variety of organic compounds. Analysis begins by weighing a 1-g-ground sample and placing it into the porcelain sample holder referred to as a boat. When "ANALYZE" is selected and the sample enters the combustion chamber (Temperature = 566°C), the furnace and flow of oxygen gas, cause the sample to combust. The combustion process converts any elemental nitrogen into N_2 and NO_x. Combustion gases are swept down and out of the inner combustion tube and then up between the inner and outer combustion tubes. Oxygen flow is measured by the Oxygen Flow rotameter before it enters the combustion chamber. After the ballast tank is filled with sample gas, the gas is permitted to equilibrate before being released through the aliquot loop. Sample gas in the aliquot doser is swept by the carrier gas to the catalyst heater where NOx gases are reduced to N_2. Lecosorb and Anhydrone are used to remove CO_2 and H_2O, respectively. This leaves N_2 and helium to flow through one side of the cell. The out side of the cell contains carrier gas scrubber filters. The gases in both sides of cell are compared, and an output voltage is generated which is fed into the computer

processed, displayed, and stored as the nitrogen content and divided by 6.4 to obtain the protein concentration.

APPENDIX IV

TBA METHOD

TBA values were determined using the extraction procedure of Tarladgis et al. (1960) and Zipser et al, (1962).

TBA reagent Preparation: Thiobarbituric acid (1.4416 g) was dissolved in distilled water (500 ml). The flask was placed in a sonic cleaner and was shaken occasionally until TBA was dissolved.

HCl Solution: Make volume as needed; 1:2, HCl: H_2O (v/v)

Sulfonamide Reagent Preparation: Sulfanilamide (0.5 g) and concentrated HCl (20 ml) were dissolved in a 200 ml volumetric flask after which the volume was brought to 200 ml with distilled water.

Sample Preparation: Ten g of frankfurters were weighed directly into homogenizer flasks. After adding 50 ml of distilled water the sample was homogenized for 1 min using homogenizer. The homogenate was quantitatively transferred to a 500 ml extraction flask with the volume brought up to 100 ml with distilled water. Glass beads, 2.5 ml of HCl solution, two sprays of antifoam solution (~1 ml), and 2 ml sulfanilamide solution were added to the flask. The extraction flask was connected to distilling tubes and tightened to heating mantles. Powerstats were turned on to line voltage and the flasks were heated rapidly. Fifty ml of distillate was collected, transferred to culture tubes, capped and kept refrigerated for the TBA reaction.

TBA Reaction and Spectrophotometric Determination: Five ml of distillate and 5 ml of TBA reagent were added to each of 3 tubes. Five ml of distilled water was pipetted into the blank tube. After thorough mixing using a Vortex Genie shaker, the

tubes were immersed in a 100°C water bath for 35 min and then cooled in ice bath for 10 min. Absorbance was read at 538 nm in Perkin Elmer UV/VIS Spectrometer Lambda 20 (Perkin-Elmer Corporation, North American Organic Division, Norwalk, CT). The reading was multiplied by 7.8 to convert absorbance to mg malonaldehyde/1000 g of sample.

APPENDIX V

Questioner of Sensory Panel for Hot Dogs

Page Number: 1

Attribute Sequence Number: 1

Attribute Type.....: Instruction Box
Attribute Description....: { Instruction Box }
Seen With Relative Sample: none

You will evaluate cooked hot dog samples for five characteristics- juiciness, texture, flavor, casing tenderness, and overall acceptability. Lift the panel door and slide the ready portion of the card under the door. You will receive a sample with a three digit random code. Answer the questions about the sample by clicking with your mouse next to your desirable answer. When finished, slide the empty container and the finished portion of the card under the door and you will receive the next sample.

Page Number: 2

Attribute Sequence Number: 2
Attribute Type.....: Hedonic
Attribute Description....: Casing tenderness
Seen With Relative Sample: none

Is the tenderness of the casing desirable, or is it too tough, too soft, or too chewy?

[] Extremely Desirable
[] Very Desirable
[] Moderately Desirable
[] Slightly Desirable
[] Slightly Undesirable
[] Moderately Undesirable
[] Very Undesirable
[] Extremely Undesirable

Attribute Sequence Number: 3
Attribute Type.....: Hedonic
Attribute Description....: Juiciness
Seen With Relative Sample: none

Is the hot dog juiciness desirable, or is it too dry?

[] Extremely Desirable
[] Very Desirable
[] Moderately Desirable
[] Slightly Desirable
[] Slightly Undesirable
[] Moderately Undesirable
[] Very Undesirable
[] Extremely Undesirable

Page Number: 3

Attribute Sequence Number: 4
Attribute Type.....: Hedonic
Attribute Description....: Texture
Seen With Relative Sample: none

How desirable is the texture of the hot dog? Is it too hard, too mushy, or too coarse?

[] Extremely Desirable
[] Very Desirable
[] Moderately Desirable
[] Slightly Desirable
[] Slightly Undesirable
[] Moderately Undesirable
[] Very Undesirable
[] Extremely Undesirable

Attribute Sequence Number: 5
Attribute Type.....: Hedonic
Attribute Description....: Flavor
Seen With Relative Sample: none

Is the flavor desirable, or is it too bland, spicy, bitter, sour, salty, or is there and after-taste?

[] Extremely Desirable
[] Very Desirable
[] Moderately Desirable
[] Slightly Desirable
[] Slightly Undesirable
[] Moderately Undesirable
[] Very Undesirable
[] Extremely Undesirable

Page Number: 4

Attribute Sequence Number: 6
Attribute Type.....: Hedonic
Attribute Description....: Overall desirability
Seen With Relative Sample: none

Your reaction to the overall satisfaction derived form the consumption of the hot dog.

[] Extremely Desirable
[] Very Desirable
[] Moderately Desirable
[] Slightly Desirable
[] Slightly Undesirable
[] Moderately Undesirable
[] Very Undesirable
[] Extremely Undesirable

Attribute Sequence Number: 7
Attribute Type.....: Comment
Attribute Description....: Comment
Seen With Relative Sample: none

Comment Type: Optional

Please write any comments about the hot dogs:

APPENDIX VI

MICHIGAN STATE
UNIVERSITY

February 8, 2002

TO: Elliot RYSER
2108 S. Anthony

RE: **IRB# 02-031** CATEGORY: EXEMPT 1-C, 1-G

APPROVAL DATE: February 8, 2002

TITLE: ANTIMICROBIAL WHEY PROTEIN ISOLATE-BASED CASING FOR HOT

The University Committee on Research Involving Human Subjects' (UCRIHS) review of this project is complete and I am pleased to advise that the rights and welfare of the human subjects appear to be adequately protected and methods to obtain informed consent are appropriate. Therefore, the **UCRIHS approved this project.**

RENEWALS: UCRIHS approval is valid for one calendar year, beginning with the approval date shown above. Projects continuing beyond one year must be renewed with the green renewal form. A maximum of four such expedited renewals possible. Investigators wishing to continue a project beyond that time need to submit it again for a complete review.

REVISIONS: UCRIHS must review any changes in procedures involving human subjects, prior to initiation of the change. If this is done at the time of renewal, please use the green renewal form. To revise an approved protocol at any other time during the year, send your written request to the UCRIHS Chair, requesting revised approval and referencing the project's IRB# and title. Include in your request a description of the change and any revised instruments, consent forms or advertisements that are applicable.

PROBLEMS/CHANGES: Should either of the following arise during the course of the work, notify UCRIHS promptly: 1) problems (unexpected side effects, complaints, etc.) involving human subjects or 2) changes in the research environment or new information indicating greater risk to the human subjects than existed when the protocol was previously reviewed and approved.

If we can be of further assistance, please contact us at (517) 355-2180 or via email: UCRIHS@msu.edu. Please note that all UCRIHS forms are located on the web: http://www.msu.edu/user/ucrihs

Sincerely,

Ashir Kumar, M.D.
UCRIHS Chair

AK:
cc: br

Arzu Cagri
1412 F Spartan Village
E. Lansing MI 48823

OFFICE OF
RESEARCH AND GRADUATE STUDIES

University Committee on Research Involving Human Subjects

Michigan State University
246 Administration Building
East Lansing, Michigan
48824-1046

517/355-2180
FAX: 517/353-2976
Web: www.msu.edu/user/ucrihs
E-Mail: ucrihs@msu.edu

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