DEVELOPMENT OF WHEY PROTEIN FILMS INHIBITORY TO SPOILAGE AND PATHOGENIC BACTERIA

BY

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A Thesis Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

Edible films and coatings are increasingly being developed as new types of packaging materials since plastic packaging materials are not easily biodegradable and create substantial amounts of solid waste. Antimicrobial edible films or coatings applied on food products have the potential to reduce the growth of spoilage and pathogenic bacteria, yeast and mold, thereby improving food quality and extending product shelf-life as well as reducing packaging waste.

Edible antimicrobial films were produced by incorporating nisin, lysozyme, ethylenediaminetetraacetic acid (EDTA) or propyl paraben (PP) into neutral or acidic whey protein isolate (WPI) films. The antimicrobial films were evaluated in agar for their ability to inhibit several spoilage and pathogenic organisms (*Brochothrix thermosphacta*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Escherichia coli* 0157:H7) that are significant contributors to food spoilage and outbreaks of foodborne illness. To determine film antimicrobial activity, 1 cm diameter discs of films were placed on bacteria-inoculated agar plates and/or tubes and the clear inhibition zones were measured in the horizontal and vertical directions. The antimicrobial activity was further quantified by enumerating bacterial numbers on the agar areas showing inhibition zones.

Results showed that nisin incorporated into acidic WPI films inhibited Gram+ bacteria better than in neutral films. While nisin incorporated into neutral WPI films inhibited *S.* Typhimurium better, nisin in either type of film had similar effects against *E. coli* 0157:H7. Lysozyme added to neutral films was more effective than when formulated in acidic films against all bacteria except for *L. monocytogenes*. Neutral or

acidic EDTA films did not inhibit *B. thermosphacta* or *L. monocytogenes*, however, they did inhibit *S.* Typhimurium and *S. aureus*. In addition, *E. coli* 0157:H7 was more effectively inhibited by EDTA when incorporated into neutral films. PP in neutral or acidic films had equivalent effects on *B. thermosphacta*, *S. aureus* and *S.* Typhimurium, while PP in neutral films inhibited *E. coli* 0157:H7 to a greater extent.

WPI films were then optimized for antimicrobial activity by incorporating a combination of the aforementioned antimicrobials at two concentrations (50% and 150% MIC) to increase the effectiveness of the antimicrobials. Film antimicrobial activity on agar was determined by the same methods against the same organisms. A two level, five factor, full factorial experimental design was used to determine if interactions between nisin, lysozyme, EDTA, PP and WPI existed.

Results showed that nisin alone reduced numbers of all microorganisms except *E. coli* 0157:H7; lysozyme alone reduced *B. thermosphacta* and PP alone reduced *L. monocytogenes*. Negative interactions were observed between: nisin and lysozyme (all organisms); nisin and EDTA (*S. aureus* and *S.* Typhimurium); lysozyme and PP (*B. thermosphacta* and *L. monocytogenes*); nisin and WPI (*S. aureus* and *S.* Typhimurium). These negative interactions were identified as those that significantly reduced inhibitory activity. The only interaction that significantly increased inhibition was between lysozyme and PP against *S. aureus*. The most effective films contained nisin at the highest test concentration with 50% MIC levels of the other antimicrobials.

Finally, the best antimicrobial WPI formulation containing 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 450 ppm PP was tested for its ability to reduce the viability of *B. thermosphacta* on refrigerated fresh pork. Fresh pork loin cubes inoculated with *B. thermosphacta* were treated with a control WPI coating (without

antimicrobials) or the best antimicrobial WPI coating, vacuum packaged and stored at 4°C for 12 days. Samples were analysed for *B. thermosphacta*, total bacterial survivors and lactic acid bacteria present at days 0, 3, 6, 9 and 12. The antimicrobial WPI coating was bactericidal and decreased *B. thermosphacta* numbers by approximately 4 log CFU/cm^2 on pork loin cubes throughout the storage period.

Overall, these results suggest that incorporating antimicrobials in edible WPI films can be an effective inhibitor of spoilage and pathogenic bacteria. Since different bacteria responded in a different manner it is likely that for each organism there will be a specific antimicrobial film-formulation that can effectively inhibit their growth. In addition, this study clearly demonstrated that antimicrobial edible coatings can be used to successfully extend the shelf-life of fresh pork by inhibiting growth of *B. thermosphacta*.

CHAPTER # 1

INTRODUCTION

Consumers are becoming more health conscious. They demand better quality foods that are safer, contain fewer chemical preservatives and are more convenient with increased shelf-life. Food technology has developed new ways to satisfy these needs. Chemical preservatives in food products are increasingly being replaced with natural preservatives that are more acceptable to consumers. In addition, the food packaging industry is introducing new packaging systems, that include edible antimicrobial films, which not only protect food commodities but also can improve food quality, extend product shelf-life and reduce plastic packaging waste.

Whey is a cheese by-product that is produced in large quantities and has created significant disposal problems. Whey contains highly functional proteins which can be used in the production of edible films and reduce the waste problem by creating a useful and economical product. Whey protein yields films with characteristics needed to function as satisfactory active packaging materials. They have good mechanical properties and are excellent oxygen, aroma and oil barriers at low to intermediate relative humidity. In addition, they can be formulated to contain antimicrobials and sustain their activity.

Antimicrobials such as nisin, lysozyme, EDTA and propyl paraben can be formulated into edible films or coatings to reduce the growth of spoilage and pathogenic bacteria as well as yeasts and molds. Antimicrobials in films or coatings can directly inhibit microbial surface growth or can migrate through the initial surface layers of the food product and reduce microbial proliferation. Nisin (produced by *Lactococcus lactis*

subspecies *lactis*) and lysozyme (obtained from hen egg white) are natural antimicrobial agents that exhibit their action on gram positive bacterial membranes and cell walls (Padgett *et al.*, 1998). EDTA is a chelating agent that disrupts the outer membrane structure of gram negative bacteria, while propyl paraben inhibits membrane transport of electrons, essential amino acids and nutrients (Baranowski and Nagel, 1983; Lück and Jager, 1997) and both are generally recognised as safe. Used in combination these agents can have enhanced antimicrobial effects against target microorganisms.

Antimicrobial compounds usually have a specific range of activity and a single agent can not effectively control all microorganisms of concern. Therefore, studies have been done to evaluate combinations of agents to increase both their effectiveness and range against the more resistant pathogenic gram negative bacteria such as *Escherichia coli*, *Salmonella*, *Campylobacter* and *Shigella*.

The hypothesis of this research was to investigate whether antimicrobials in whey protein isolate films or coatings can inhibit spoilage or pathogenic microorganisms. To test this hypothesis, the research was divided into three separate chapters with three main objectives. The first objective was to produce neutral and acidic whey protein isolate films containing nisin, lysozyme, EDTA or propyl paraben and test for their ability to inhibit spoilage and pathogenic organisms (*Brochothrix thermosphacta*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Escherichia coli* 0157:H7) in agar. Film antimicrobial activity was determined qualitatively, by the size of the inhibition zones (horizontal and vertical) produced on bacteria-inoculated agar plates and/or tubes, and quantitatively by enumerating bacterial numbers on the agar areas showing inhibition zones. The second objective was to optimize whey protein isolate films for antimicrobial activity against the above

organisms by combining the antimicrobials and determining film antimicrobial activity qualitatively and quantitatively. The second objective also involved examination for antimicrobial interactions between antimicrobials. The final objective was to determine if the optimized antimicrobial whey protein coating could successfully inhibit the growth of *B. thermosphacta* on pork loin tissue stored under vacuum at 4°C for 12d.

CHAPTER # 2

LITERATURE REVIEW

2.1. Packaging technologies

As consumers become more health conscious and demand better quality foods (Summers, 1992) the food industry has come under pressure to improve the quality and safety of its products (Pault, 1995). Food packaging is essential to prevent contamination during distribution, display and handling by consumers. In addition, packages are used to contain the product, provide convenience as well as ingredient and nutrition information (Yam, 2000). Specific packaging materials are selected to protect the food product from microorganisms, water, moisture, gases (oxygen, O_2), rodents, insects and light as well as to protect foods from loss of quality (carbon dioxide, CO_2, moisture and aroma) (Anker, 2000).

Packaging technology targets have shifted from the use of conventional (passive) packaging of protecting contents to a more active application of packaging where packaging materials can absorb or emit gas to improve shelf-life (Brody, 2000) or to a complete intelligent packaging system which informs the retailer or consumer about shelf-life potential (Rodrigues and Han, 2003).

Active packaging has attributes beyond the basic physical barrier properties (Brody, 2000) and provides extra functions which do not exist in conventional packaging systems. They contain ingredients and/or an actively functional polymer which interacts with food components to improve shelf-life (Ahvenainen and Hurme, 1997). Examples of this type of system include O_2, moisture, ethylene and taint scavengers; ethanol emission; CO_2 control; modified atmosphere packaging (MAP);

flavour and odour control as well as antioxidant release (Vermeiren *et al.*, 1999). They may include use of edible films; gas flushing; vacuum packaging or antimicrobial activity (Vermeiren *et al.*, 1999).

Packaging systems require that the process be functionally and cost effective, increase product safety and shelf-life, as well as be user friendly to benefit the processor, the distributor and to appeal consumers (Pault, 1995; Karel, 2000). While in the past, packaging systems were designed to minimize the interaction between the package and its content (Ahvenainen and Hurme, 1997), new packaging systems are designed to stabilize food quality over longer periods of time (Pault, 1995) and this often involves deliberate interaction between the packaging material and the food inside (Ahvenainen and Hurme, 1997).

In this thesis two types of packaging will be discussed, vacuum and edible antimicrobial packaging.

2.1.1. Vacuum packaging

Vacuum packaging helps extend the shelf-life of perishable foods (fruits, vegetables, fresh and frozen meats and meat products), prevents them from being contaminated and retards moisture loss or gain (García-Esteban *et al.*, 2004). With this system, an O_2 deficient atmosphere is created when the product is contained in a low O_2 permeable bag; air is removed and the bag is then heat sealed. In the U.S., approximately 90% of red meat is vacuumed packaged (Siragusa *et al.*, 1999). Although vacuum packaging and refrigeration conditions discourage the growth of aerobic spoilage and pathogenic bacteria and can maintain meat products and subprimals more shelf stable (Siragusa *et al.*, 1999), certain bacteria such as *B. thermosphacta*, *S.* Typhimurium,

Listeria and *Lactobacilli* species can still grow under these conditions (Siragusa *et al.*, 1996a; Tu and Mustapha, 2002).

2.1.2. Edible antimicrobial films

Edible films, especially antimicrobial edible films or coatings, have the potential to improve the quality of foods and could allow for the currently used multicomponent packaging materials to be converted into a single component film thereby decreasing the use of packaging materials and increasing the ease of package recycling (McHugh *et al.*, 1994, Krochta, 1997). In addition, because edible films and coatings employ renewable materials, they result in novel use of agricultural raw materials (Gennadios *et al.*, 1996; Dawson, 1998; Debeaufort *et al.*, 1998).

2.2. Edible and biodegradable packaging films and coatings

2.2.1. Definition

Edible films are thin layers of edible materials which can be either formed directly on a food product surface as a coating or preformed separately as thin sheets then wrapped around a food item (Krochta and DeMulder-Johnston, 1997; Guilbert *et al.*, 1996; Gennadios and Weller, 1990). Edible films and coatings (hereinafter referred to as simply films) can be identical in composition and these terms have been used interchangeably.

2.2.2. Why use edible films?

In 1993, the United States (U.S.) generated approximately 8 million tonnes of plastic packaging, corresponding to about 12% of the total packaging manufactured, and

making up 4% of the solid municipal waste (Dawson, 1998). Edible films and coatings can lessen this problem. Although they are not designed to substitute for non-edible packaging materials, they can be used as an added hurdle to retain overall food quality and stability, extend shelf-life and reduce the use of synthetic packaging materials (Kester and Fennema, 1986; Guilbert *et al.*, 1996; Krochta, 1986). Consumer interest in natural high quality foods serves to stimulate interest in research to improve perishable food shelf-life using edible packaging materials. Edible films may be eaten with the packaged food, may improve sensory properties, add nutritional benefits and can be designed to deliver antimicrobials and antioxidants (Guilbert *et al.*, 1996; Gennadios and Weller, 1990).

2.2.3. Historical and current uses of edible films and coatings

The use of edible films and coatings dates back several centuries (Donhowe and Fennema, 1994). Waxes and fats were used to delay moisture loss and control respiratory gas exchange (Donhowe and Fennema, 1994; Kester and Fennema, 1986; Park, 1999). Hot-melt paraffin wax coatings were used on citrus fruits; carnauba wax on fruits and vegetables, and gelatin films were used to coat meats (Kester and Fennema, 1986). More recently, edible collagen films have been used as casings for sausages (Donhowe and Fennema, 1994). Waxes are currently applied on fresh fruits and vegetables to: retard moisture loss, decrease weight loss, provide an O_2 barrier, impart a glossy appearance, decrease mechanical damage or to deliver fungicides (Krochta, 1986; Anonymous, 1997; Baldwin *et al.*, 1997). Chitosan coatings have been applied on kiwi fruits, cucumbers, tomatoes and bell pepper fruits to decrease respiration rates, inhibit the development of fungus, delay ripening (by decreasing ethylene and CO_2 production)

and control enzymatic browning (Gontard *et al.*, 1996; Park, 1999; Shahidi *et al.*, 1999). Chocolates, chewing gum, drug tablets coated with corn zein and nuts coated with whey protein isolate (WPI) are less likely to gain or lose moisture during storage. The coatings on these food products also provide O_2 barrier, enhance structural integrity and surface gloss (Krochta, 1986; Maté and Krochta, 1996; 1998; Fu *et al.*, 1999). Pharmaceutical tablets are often coated with corn zein to mask the flavour and improve ingestibility (Krochta, 1986). Edible films and coatings have also been used to extend the shelf-life and quality of fresh, frozen and processed meat, poultry, fish and other seafood products by preventing moisture loss, oxidation, decreasing drip loss and preserving organoleptic properties (Kester and Fennema, 1986; Krochta, 1986; Klose *et al.*, 1952; Farouk *et al.*, 1990; Stuchell and Krochta, 1995; Gennadios *et al.*, 1997). Carbohydrate, cellulose, casein, corn zein, soy and chitosan coatings have been applied on fruits (eg. apples) and vegetables (eg. carrots) to control internal gas composition and to decrease dehydration (Park, 1999; Dabrowska and Lenart, 2001; Cisneros-Zevallos *et al.*, 1997). Polysaccharide coatings (eg. hydroxypropyl methyl cellulose; HPMC) are often used on battered and breaded foods to improve coating adherence, decrease oil uptake and retain moisture during deep-fat-frying (Balasubramaniam *et al.*, 1997). In addition, other edible coatings such as chocolate coatings have a long history of use on confectionary products (Kester and Fennema, 1986).

2.2.4. Functions

The functions and useful properties of edible films are dependent on food characteristics (pH, moisture content, surface charge) and on the nature of their decay (moisture loss or gain, chemical or enzymatic reactions, microbial susceptibility,

sensitivity to gas transmission losses, oxidation, physical damage and infestations by insects or rodents) (Kester and Fennema, 1986; Guilbert *et al.*, 1996; Petersen *et al.*, 1999). Edible films are able to minimize and control these problems. In general, most edible films have good barrier properties. They can act as barriers to O_2 and CO_2, aromas and especially water vapour, therefore preventing moisture transfer between the food product and its surrounding environment (Donhowe and Fennema, 1994; Kester and Fennema, 1986). These films can control the respiration rates of fresh fruits and vegetables (Kester and Fennema, 1986; Guilbert *et al.*, 1995; Baldwin *et al.*, 1997; Park, 1999). Edible hydrophilic films are used to inhibit fat and oil migration (Kester and Fennema, 1986; Donhowe and Fennema, 1994; Anonymous, 1997). Food products that are susceptible to oxidation of lipids, pigments, vitamins, flavour and aroma components benefit from edible films since they stabilize and inhibit migration (Kester and Fennema, 1986; Donhowe and Fennema, 1994; Sothornvit and Krochta, 2000). Edible films may also help maintain food quality once the outer package has been opened (Anonymous, 1997). They can indirectly improve the mechanical handling properties and physical structural integrity of foods during processing, storage and transportation (Kester and Fennema, 1986; Donhowe and Fennema, 1994). In multi-component food systems they are used to separate the food components (eg. pizzas, pies) (Gennadios and Weller, 1990). Furthermore, they have the ability to carry food ingredients such as flavours, colourings, sweeteners, vitamins, antioxidants and antimicrobials and hence can supplement the nutritional value of the treated foods (Kester and Fennema, 1986; Donhowe and Fennema, 1994). Volatile components can be encapsulated by edible films (Debeaufort *et al.*, 1998; Fu *et al.*, 1999). These films can also improve organoleptic properties and retard spoilage processes (Gennadios and Weller, 1990). Antioxidant and

antimicrobial agents can be used in smaller amounts and in localized areas, instead of being used in large concentrations which are distributed throughout the food (Kester and Fennema, 1986; Gennadios and Weller, 1990; Guilbert, 2000). It is also an advantage that edible film ingredients make these films "environmentally friendly" since they degrade easily (Gennadios and Weller, 1990).

2.2.5. Edible film components

Edible films consist of at least one high-molecular weight edible polymer (Kester and Fennema, 1986; Mahmoud and Savello, 1992). They can also be composed of more than one polymer in a single or bilayer fashion (Guilbert *et al.*, 1996). The food matrix dictates the film composition. Film components, formation methods and additives dictate film functionalities and properties (Kester and Fennema, 1986). Edible films can consist of hydrocolloids, lipids and composites of these.

2.2.5.1. Hydrocolloid films

Generally hydrocolloid films prevent O_2, CO_2 and lipids from migrating and have good mechanical and optical properties, especially under low to intermediate relative humidity (RH), however, they are poor moisture barriers due to their hydrophilic nature (Donhowe and Fennema, 1994; Guilbert *et al.*, 1996; Guilbert *et al.*, 1995). Films or coatings in this category are mainly composed of polysaccharides or proteins. Polysaccharide films (alginate, pectin, carrageenan, starch and starch derivatives such as chemically modified starches, gums, chitosan, cellulose and cellulose derivatives such as carboxymethylcellulose, methylcellulose (MC) and hydroxypropylmethyl cellulose (HPMC)) have been more extensively studied compared to other types of films

(Donhowe and Fennema, 1994; Kester and Fennema, 1986; Guilbert *et al.*, 1996; Krochta, 1986; Baldwin *et al.*, 1995; Gennadios *et al.*, 1997; Debeaufort *et al.*, 1998). Protein films have not been as widely studied, nonetheless, several polypeptides including collagen, gelatin, soy protein, casein, whey protein, serum albumin, ovalbumin, wheat gluten, keratin and corn zein have been used to make films or coatings (Donhowe and Fennema, 1994; Gennadios *et al.*, 1994; Kester and Fennema, 1986). In general, protein films have better mechanical and barrier properties compared to polysaccharide films (Guilbert *et al.*, 1997a) but can further undergo physical, chemical and enzymatic treatments to improve their barrier and mechanical properties. Some of these treatments include, heat curing, cross-linking (with acids), denaturation with alkali and exposure to ultrasound and ultraviolet radiation processing (Kester and Fennema, 1986; Gennadios *et al.*, 1996, 1998; Micard *et al*, 2000; Banerjee *et al.*, 1996; Were *et al.*, 1999; Yildirim and Hettiarachchy, 1997). Furthermore, the addition of lipids to protein films increases their moisture barrier capability, however, if too much is added it can lead to the development off-flavours or can cause oxidation (Guilbert *et al.*, 1997a).

2.2.5.2. Lipid films

Although hydrocolloid films are poor moisture barriers, lipid films provide good moisture barrier properties (Donhowe and Fennema, 1994; Kester and Fennema, 1986; Krochta, 1986). Lipid films are very common and have been used for many years to coat food products such as fresh produce or confectionary products to provide gloss (Kester and Fennema, 1986; Donhowe and Fennema, 1994; Krochta, 1986). Examples of lipids used in making films are natural waxes (carnauba, candelilla, beeswax and rice bran wax), oils (mineral, vegetable such as corn, soybean or palm and paraffin oil), petroleum

based waxes (paraffin and polyethylene wax), acetylated monoglycerides, fatty acids, oleic acids and others including shellac, fatty alcohols, surfactants, fatty-acid esters, stearic and lauric acids (Krochta, 1986; Donhowe and Fennema, 1994; Guilbert *et al.*, 1996; Baldwin *et al.*, 1995, 1997; Debeaufort *et al.*, 1998). Though lipid films are good moisture barriers, they do not tend to form good stand-alone films (Krochta, 1986). They lack structural integrity and are less flexible, durable and are usually opaque (Guilbert *et al.*, 1996; Baldwin *et al.*, 1997). To improve these films they are made into composite films or coatings (Donhowe and Fennema, 1994).

2.2.5.3. Composite films

Composite films are protein-lipid or polysaccharide-lipid in nature, made as a bilayer or as an emulsion (Donhowe and Fennema, 1994; Baldwin *et al.*, 1997). Composites have the combined advantages of both the lipid and the hydrocolloid film properties (Kester and Fennema, 1986; McHugh and Krochta, 1994b). The hydrocolloid portion of the film provides the structural integrity and optical and barrier properties (at low RH), while the lipid component provides the moisture barrier properties.

2.2.6. Film additives

Several compounds can be introduced into edible films to alter or provide better functional, mechanical, protective, organoleptic, sensory or nutritional characteristics (Kester and Fennema, 1986; Donhowe and Fennema, 1994; Guilbert *et al.*, 1995). Film additives include plasticizers, surface-active agents, antioxidant and antimicrobial agents, flavouring and texturing agents, cross-linking agents, pigments or nutritional ingredients

and vitamins (Kester and Fennema, 1986; Donhowe and Fennema, 1994; Guilbert *et al.*, 1996).

2.2.7. Film manufacturing processes

Films can be produced using a dry or a wet process (Guilbert *et al.*, 1997a). Biopolymers, in a dry process, must be thermoplastic and are heat treated above their glass transition temperatures (Tg) and extruded to form films (Guilbert *et al.*, 1997a). While in a wet process, ingredients are dissolved in a liquid medium to generate a film-forming solution, then are allowed to dry to make the edible film (Guilbert, 2000). Within the wet process, three principle methods are used to produce films or coatings, solvent removal, coacervation, and solidification of melt (Donhowe and Fennema, 1994). Hydrocolloid films are usually made using the solvent removal method, where solid films are made from film-forming materials that are dissolved in an aqueous (water, ethanol or their mixture) solution (Donhowe and Fennema, 1994). The coacervation method uses heat, pH changes, the addition of solvents or changing the charge on the polymer to solidify the polymer coating material contained in the film solution (Donhowe and Fennema, 1994; Guilbert *et al.*, 1996), while in the solidification of melt method the warmed 'melted' solution is cooled in order to form the solidified film (Donhowe and Fennema, 1994; Krochta, 1986).

2.2.8. Application of film-forming solutions

Effective edible films or coatings can be applied by three main procedures, coating (dipping), spraying or casting (Donhowe and Fennema, 1994). During coating, the food is dipped into the film-forming solution, then excess is allowed to drain and the

remainder solidifies at the food surface. This method produces reasonably uniform coatings, can allow for multiple coating applications and is often used for fruits and vegetables (Donhowe and Fennema, 1994). When a thinner, more uniform layer is required, the film-forming solution can be applied by spraying (Krochta and DeMulder, 1997). This technique can be used when a food product requires only one side to be coated, such as pizza crusts which are often coated to protect them against the moisture of the sauce (Donhowe and Fennema, 1994). A casting method is used to create free-standing films. With this procedure, the film thickness is controlled by spreading the film-forming solution onto a flat surface (Donhowe and Fennema, 1994). Following spreading the solution is allowed to solidify and it is subsequently peeled off.

Other methods used are extrusion, moulding or rolling mills (Kester and Fennema, 1986; Guilbert *et al.*, 1996) however, these methods utilize conditions (eg. high temperatures) that most edible films cannot withstand (Krochta, 1986).

2.2.9. Edible film properties

Satisfactory films demonstrate desirable mechanical, barrier and optical properties. Many factors such as the nature of film ingredients, additives, fabrication methods, pH, RH and temperature affect the film's properties (Krochta, 1997; Debeaufort *et al.*, 1998). Mechanical properties such as cohesive and adhesive strength, puncture strength, Tg, tensile strength (TS), percent elongation (%E), and elastic modulus (EM) establish whether the film can safeguard the product from physical damage (Guilbert *et al.*, 1996; Krochta, 1997). Edible films should exhibit sufficient strength to resist breakage and scrapes and be flexible enough to withstand certain deformations that occur during food distribution (Guilbert *et al.*, 1995). Barrier

properties which include water vapour permeability (WVP), CO_2 and O_2 permeability and the permeability to solutes will determine if the film is able to protect the food product from prevalent environmental conditions or from other neighbouring components during storage (Krochta, 1997). Optical properties characterize whether the film is transparent enough to see the treated food or be undetectable by consumers.

2.2.9.1. Mechanical properties

2.2.9.1.1. Cohesive and adhesive forces

The film's structural integrity (cohesion strength) greatly affects the mechanical properties of an edible film (Guilbert *et al.*, 1996). Cohesion strength is the force that exists between the molecules within a polymer of a film and the adhesion strength is the force between the coating itself and the food that it coats (Guilbert *et al.*, 1996; Guilbert and Gontard, 1995). Films with increased polymer length that are arranged in an orderly manner and those with increased polarity and evenly distributed polar groups have higher cohesive strength, since more extensive bonding can occur because there is more interchain hydrogen bonding and ionic interactions (Kester and Fennema, 1986; Guilbert *et al.*, 1996). However, films with higher cohesive strength tend to be less flexible and have less effective barrier properties (Guilbert *et al.*, 1995).

2.2.9.1.2. Temperature

Film mechanical properties are affected by the temperature during film fabrication and storage (Guilbert *et al.*, 1997b). As film components during manufacturing are exposed to temperatures above or below the Tg, the material state changes. The Tg is the temperature at which a polymer changes from a brittle "glass"

state to a highly viscous or rubbery solid state (McHugh and Krochta, 1994a). The Tg is affected by plasticizer concentrations and RH (Guilbert, 2000). The addition of plasticizers tend to decrease the Tg. (Guilbert and Gontard, 1995; Krochta, 1986).

The temperatures used during film manufacturing and during storage also affect the TS, WVP and O_2 permeability (McHugh *et al.*, 1994). McHugh and Krochta, (1994b) showed that increasing heat denaturation of WPI films increased the WVP of whey protein emulsion films. Gennadios *et al.* (1993) demonstrated that as the storage temperature increased (5-40°C), the O_2 permeability of films made with corn zein, wheat gluten and wheat gluten-soy protein isolate increased. Miller *et al.*, (1997) exposed WPI films to several heat curing temperatures (60, 70 and 80°C) at different RH (40, 60, 80%) and verified that the film's TS increased, while the %E and the WVP decreased. In addition, increasing storage temperature also increased TS and decreased WVP and %E of soy protein films (Gennadios *et al.*, 1996).

2.2.9.1.3. Tensile strength (TS), percent elongation or elongation at break (%E) and elastic modulus (EM)

The TS, %E and EM of a film represent respectively, the maximum pulling stress that a film can endure, the maximum percent change in length that a film can stretch before breaking and the measurement of film stiffness (McHugh and Krochta, 1994a; Banerjee and Chen, 1995; Krochta and DeMulder, 1997). These parameters are affected by each other and all are affected by temperature, RH and plasticizers (McHugh and Krochta, 1994a; Krochta, 1986; Gennadios *et al.*, 1993; Gennadios *et al.*, 1996). Decreasing the TS of edible films decreases the EM (stiffness) and increases the %E (stretchability), therefore films becomes more flexible, however, this also tends to

increase the WVP (McHugh and Krochta, 1994b; Krochta, 1986; Gennadios *et al.*, 1996).

2.2.9.2. Barrier properties

Barrier properties of films affect the film's functional ability to retard gas, vapour or solute transfer through the film. Film composition, storage temperatures and RH influence the barrier properties (Gennadios *et al.*, 1993, 1996; McHugh and Krochta, 1994a, 1994b; Krochta, 1986; Banerjee and Chen, 1995; Baldwin *et al.*, 1995; Guilbert *et al.*, 1997b). Films that are low to moderately water soluble are better water barriers compared to those that are very water soluble (Donhowe and Fennema, 1994). Hydrophilic films are usually polar, and hence water, vapours and gases tend to interact with them making them poor moisture barriers (Kester and Fennema, 1986). The essential barrier properties are the WVP and the permeability to CO_2 and O_2.

At high RH, hydrophilic films are generally poor moisture barriers because they have higher water content. Films that contain lipids retard moisture transmission better and are less permeable than protein films (Guilbert *et al.*, 1996; Banerjee and Chen, 1995). The WVP and the permeability of CO_2 and O_2 are affected by the water content in the film, the storage RH and the temperature (Guilbert *et al.*, 1996). Increased RH causes films to be more hydrated, molecules within the polymer become more mobile and in turn films have higher WVP and increased gas (CO_2 and O_2) permeabilities (Gennadios *et al.*, 1993; McHugh and Krochta, 1994a; Krochta, 1986; Guilbert *et al.*, 1995, 1997b; Gontard *et al.*, 1996; Baldwin *et al.*, 1995). Gontrad *et al.* (1996) and McHugh and Krochta, (1994a) demonstrated that as the RH increased (especially $\geq$

60%), the WVP and the permeabilities to O_2 and CO_2 also increased, in WPI films and in wheat gluten films, respectively. In general, protein films have good WVP, O_2 and CO_2 barrier properties, however at low RH (McHugh and Krochta, 1994a).

2.3. Protein-based edible films and coatings

2.3.1. Protein edible film manufacture and properties

Protein films are usually made from protein solutions after the solvent is evaporated (Kester and Fennema, 1986). To form a cohesive protein film, globular proteins must be first denatured by heat, acid, base and/or solvents to open up their structures, and then are allowed to form protein chains via hydrogen, ionic, hydrophobic and covalent bonding (Brandenburg *et al.*, 1993; Krochta, 1997). A cohesive film structure will be produced depending on how extensive the interactions have been.

Protein films tend to be excellent O_2 barriers at low to moderate RH and in general have a higher content of hydrophilic amino acid groups, therefore their moisture barrier properties are restricted. Increasing the number of hydrophobic groups present on the polymer chain, improves the moisture barrier characteristics of the film, however the film becomes less effective as an O_2 barrier (Krochta, 1997). Introducing a lipid component causes the permeability to both CO_2 and O_2 to decrease, because the film becomes more hydrophobic. As the water content of the film decreases, the solubility of these gasses through the film decreases (Gontard *et al.*, 1996). Plasticizers are often added to protein films to increase film flexibility by decreasing the interactions between polymer chains. However, regrettably, this also increases the permeability. In addition, protein films serve as adequate barriers to aromas and flavours since most of these compounds are hydrophobic in nature (Krochta, 1997).

There are many proteins (animal and plant origin) that are used in the formation of edible films and coatings. Proteins of plant origin used include: corn zein, soy protein, peanut protein, wheat gluten and cottonseed. Proteins of animal origin used include: gelatin, keratin, collagen, milk proteins (whey, casein, albumin) and myofibrillar proteins from muscle tissues (Gennadios *et al.*, 1994; Krochta, 1997; Micard *et al.*, 2000). For the purpose of this section the focus will be mainly on whey protein films. These will be reviewed in terms of: protein nature and recovery, film formation methods, film properties, factors affecting film properties and potential applications.

2.3.2. Whey protein nature and recovery

Milk has a protein content of about 33 g/L (Gennadios *et al.*, 1994) and proteins present are highly functional, have good water solubility and emulsification properties. For these reasons, milk proteins tend to be good candidates for edible film production (Gennadios *et al.*, 1994). The two major protein components of milk are casein and whey protein fractions. Casein represents 80% of the total proteins and whey accounts for the remaining 20%. Liquid whey is a cheese manufacturing by-product that is produced in large quantities and has created a serious disposal problem (Gennadios *et al.*, 1994). In 1989, it was estimated that 6.5 billion kg/year of whey were disposed of in the U.S. (Banerjee and Chen, 1995). As a consequence, this has lead investigators to find new ways to utilize the excess whey such as in the production of edible films in order to improve the waste disposal problem (Banerjee and Chen, 1995; McHugh *et al.*, 1994).

2.3.3. Whey protein fractions

There are five highly functional protein types in the whey protein fraction, β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), immunoglobulins and the proteose-peptone fraction (Gennadios *et al.*, 1994; Krochta, 1997; Banerjee and Chen, 1995). These proteins are globular, heat labile and produce useful films (Krochta, 1997). The major protein in the whey fraction is β-lactoglobulin which accounts for 50-70% of whey protein (Gennadios *et al.*, 1994; Krochta, 1997). α-lactalbumin makes up approximately 25% (Gennadios *et al.*, 1994; Krochta, 1997). The immunoglobulins and the proteose-peptone fractions are smaller fractions however, they substantially influence the functional properties of the film (Gennadios *et al.*, 1994).

2.3.4. Whey protein purification methods

Whey protein separation involves several steps. Acid hydrolysis separates the casein and whey protein fractions. This is achieved by adjusting skim milk to pH 4.6 which causes casein to precipitate while the whey fraction remains soluble (Gennadios *et al.*, 1994; Krochta, 1997). Once the casein fraction is removed, various methods can be employed to separate the whey protein from the other whey components. These methods include: complexing whey protein solution with a dilute solution of carboxymethylcellulose (CMC), ultrafiltration, electrodialysis, ion exchange with sodium hexametaphosphate, cellulose acetate membranes and reverse osmosis (Gennadios *et al.*, 1994). The individual whey protein components can further be separated by electrophoretic, differential solubility or chromatographic techniques (Gennadios *et al.*, 1994). However, fractionating the individual whey proteins is more

costly and they are not frequently used in film formulations, however, the whole whey protein fraction has been broadly studied.

Commercially, whey is processed to yield whey protein concentrates (WPC) and further purified to yield whey protein isolates (WPI). Both have been extensively studied for their usefulness in producing edible films (Mahmoud and Savello, 1992; Gennadios *et al.*, 1994; McHugh *et al.*, 1994; McHugh and Krochta, 1994a, 1994b; Banerjee and Chen, 1995; Stuchell and Krochta, 1995; Shellhamer and Krochta, 1995; Maté and Krochta, 1996, 1998; Miller *et al.*, 1997, 1998; Yildirim and Hettiarachchy, 1997; Pérez-Gago and Krochta, 1999; Han and Krochta, 1999; Anker, 2000). WPI is a highly purified product containing more than 90% (dry basis) whey protein (Maté and Krochta, 1996). It is purified using high performance hydrophilic ion-exchangers (Gennadios *et al.*, 1994; Krochta, 1997) and ultrafiltration methods (Maté and Krochta, 1996). WPC contains whey protein in amounts ranging between 25-80% (Gennadios *et al.*, 1994; Krochta, 1997).

2.3.5. Whey protein film formation

The formation of whey protein films starts with a denaturation step to open up the protein's globular structure. Heating whey protein at temperatures above 65°C causes the native disulfide bonds to break and new intermolecular disulfide and hydrophobic interactions occur (Gennadios *et al.*, 1994; Krochta, 1997). The films produced are transparent, flexible (when a plasticizer is used) and tend to be water insoluble (Krochta, 1997).

Originally, whey protein films were formed by Mahmoud and Savello (1992) using transglutaminase as a cross-linking agent. This enzyme, in the presence of calcium

and glycerol at pH 7.5 induced gelation. The films produced after casting and air drying at low to intermediate RH were transparent, flexible, possessed good CO_2 and O_2 barrier properties, but had limited ability to prevent moisture migration due to their hydrophilic nature (Mahmoud and Savello, 1992). However, due to the high costs of transglutaminase it has not been possible to use it in large scale food applications (McHugh *et al.*, 1994).

More recently, McHugh *et al.* (1994), developed alternative WPI film-forming conditions and criteria. Optimal films were formed at neutral pH, with 10% (w/w) WPI when heat treated for 30 min at 90°C. Heat treatments were needed to induce intermolecular disulfide bonds by thiol-disulfide interchange and thiol oxidation reactions (Gennadios *et al.*, 1994; McHugh *et al.*, 1994). The resulting films were intact, strong and insoluble. It was found that with heat treatments alone the films were too brittle, and hence the addition of plasticizers was necessary to increase flexibility. Following this, the film-forming solution was degassed by applying a vacuum to minimize the size and numbers of air bubbles (McHugh *et al.*, 1994). The film was then cast on a petri-dish and left to dry over night (18-24h) at room temperature (40% RH). After drying, the intact films were peeled from the dishes and were tested for functional properties.

2.3.6. Whey protein film properties

There are many properties that can be examined to determine the usefulness of whey protein films. However, one must keep in mind that these films are made of biological material and are hydrophilic in nature and, therefore, are susceptible to moisture uptake or loss. In addition, factors such as RH and temperature during

manufacturing and storage can change the film's mechanical and barrier properties (Gennadios *et al.*, 1994, 1996). It is essential that edible films be kept in controlled environments during assessment. Factors that are useful for evaluation of whey protein film performance are: film thickness, mechanical and barrier properties (see section 2.2.9.1 and 2.2.9.2), storage RH and temperatures (see section 2.2.9.2 and 2.2.9.1.2), protein type and concentration, heat effects, type and amount of plasticizer and pH effects.

2.3.6.1. Film factors that are assessed and affect film performance

2.3.6.1.1. Film thickness

Uniform film thickness is a prerequisite for accurate evaluation of film performance. Thickness depends on the viscosity of the film-forming solution and the method of application (Debeaufort *et al.*, 1998) and it is measured using a micrometer (Banerjee and Chen, 1995; Mahmound and Savello, 1992). Most hydrophilic films have increased WVP as the film thickness increases (McHugh *et al.*, 1993).

2.3.6.1.2. WPI concentration effects

The amount and type of whey protein drastically affects the performance of the film. McHugh *et al.* (1994) studied the properties of films made with 8-12 % w/w WPI. Whey protein concentrations below 8% did not produce intact films. It was speculated that not enough intermolecular bonds were formed. Furthermore, above 12% the solution formed gels too thick to be spread. Therefore, 10% w/w WPI was chosen as the optimum protein concentration to form adequate films.

In addition, WPI and WPC produce films with different characteristics. Since WPI contains a higher level of protein purity, there are more covalent interactions between the protein molecules which produce stronger, more rigid films (Gennadios *et al.*, 1994). However, although they exhibited higher TS, they also exhibited higher WVP compared to WPC films. WPC contains a lower protein content and hence more impurities (fat and carbohydrate) are present which weaken the film strength (Gennadios *et al.*, 1994). However, WPC films possess better moisture and gas barrier properties (Krochta, 1997; Banerjee and Chen, 1995). Mahmoud and Savello (1992) also noted that higher protein concentrations caused film thickness to increase.

2.3.6.1.3. Heat effects

The temperature at which protein film-forming solutions are heated is important. Treatment at 70-100°C for 15-45 min was needed to induce formation of intermolecular disulfide bonds (Gennadios *et al.*, 1994; McHugh *et al.*, 1994). Without heat treatment, the films cracked (McHugh *et al.*, 1994). The minimum temperature needed to form WPI films was 75°C for 30 min, since β-lactoglobulin denatures at ~78°C (McHugh *et al.*, 1994). At temperatures < 75°C weaker films were formed due to fewer intermolecular interactions. The optimum time-temperature combination was determined to be 90°C for 30 min (McHugh *et al.*, 1994).

2.3.6.1.4. Plasticizer effects

Different composition, molecular weight and concentrations of plasticizers play an important role in formulating edible protein films. Plasticizers by nature are hydrophilic, of low molecular weight and reduce film brittleness by reducing the internal

hydrogen bonding between polymer chains which increases the intermolecular spacing and relaxes film structure generating increased flexibility (Gennadios *et al.*, 1994; McHugh *et al.*, 1994; Mahmoud and Savello, 1992; Sothornvit and Krochta, 2000). There are several types of plasticizers that are used in making edible protein films. These include glycerine, glycerol, sorbitol, sucrose, mannitol, polyethelene glycol, propylene glycol, fatty acids and monoglycerides (Donhowe and Fennema, 1994; Kester and Fennema, 1986; Krochta, 1986; Gennadios *et al.*, 1994; McHugh *et al.*, 1994, Mahmoud and Savello, 1992; Maté and Krochta, 1996; McHugh and Krochta, 1994a; Sothornvit and Krochta, 2000).

Glycerol is a good plasticizer since it is non-volatile, polar, soluble in water, miscible in protein, has a high boiling point and increases water holding capacity (Mahmoud and Savello, 1992). The higher the glycerol concentration used the greater is the stretchability of the film (Mahmoud and Savello, 1992). On the hard, films which lack plasticizers or have low concentrations become very brittle (Pérez-Gago and Krochta, 1999).

In general, increasing the plasticizer concentration causes the brittleness to decrease by increasing the flexibility, however, the WVP and the permeability to O_2 and CO_2 across the film also increases (McHugh *et al.*, 1994; McHugh and Krochta, 1994a; Baldwin *et al.*, 1997; Sothornvit and Krochta, 2000). Furthermore, as the amount of plasticizer increases in the film, the overall cohesion is decreased, and is seen by a decrease in TS and EM (McHugh and Krochta, 1994a; Krochta, 1986; Sothornvit and Krochta, 2000; Cha *et al.*, 2003). As a consequence the film becomes weaker but more stretchable (Krochta, 1986; Gennadios *et al.*, 1994; McHugh and Krochta, 1994a).

The size of the plasticizer molecule is also important. Plasticizers with lower molecular weight like glycerol play a more interesting role than do larger plasticizers like sorbitol. The smaller molecules have the ability to more readily influence the mechanical and barrier properties, since they can better interact with the molecules in the film structure (McHugh and Krochta, 1994a). The permeability through films where smaller plasticizers are used is usually greater than in films where larger molecular weight plasticizers are used. Sorbitol containing WPI films had lower WVP (McHugh *et al.*, 1994) and lower O_2 permeability (McHugh and Krochta, 1994a) than those containing glycerol. β-lactoglobulin and casein films containing sorbitol had a lower O_2 permeability than the same films containing glycerol (Sothornvit and Krochta, 2000; Chick and Ustunol, 1998).

2.3.6.1.5. pH effects

The pH of the whey protein solution was found to be optimal for film formation when between pH 6-8 (McHugh *et al.*, 1994). The pH of aqueous WPI is ~7.0. McHugh *et al* (1994) adjusted the pH between 5-10, before and after heat denaturation and the solution was allowed to cool at room temperature. Films from solutions with pH $\leq$5 or $\geq$ 10 could not be formed since whey protein precipitated at $\leq$5 and very strong gels were formed that could not be poured at $\geq$10. Adjusting the pH after denaturation yielded films with a higher WVP compared to those adjusted before heat treatments (McHugh *et al.*, 1994).

2.3.7. WPI film or coating applications

The application of WPI films has not been extensive and WPI is more commonly applied as coatings. In addition, commercially, in the food industry, it has not been used very much because of high costs. WPI coatings have been applied on dry roasted peanuts to delay O_2 uptake, which decreases lipid oxidation and the development of rancidity (Maté and Krochta, 1996, 1998). WPI as a spray, with a 5% antioxidant overspray or as a bilayer with acetylated monoglyceride has been applied on frozen King Salmon, to delay lipid oxidation onset and moisture loss (Krochta, 1997; Stuchell and Krochta, 1995). Green bell peppers have also been coated with WPI (Krochta, 1997). Furthermore, WPI has been used to micro-encapsulate food additives and to coat raisins, frozen peas, breakfast cereal and cheese (Gennadios *et al.*, 1997).

2.4. Spoilage and pathogenic microorganisms

There are many spoilage and pathogenic gram positive (Gram+) and gram negative (Gram-) microorganisms that are of concern in the food industry, some of which include: *Listeria* (*L.*) *monocytogenes*, *Staphylococcus* (*S.*) *aureus*, *Brochothrix* (*B.*) *thermosphacta*, *Escherichia* (*E.*) *coli* O157:H7 and *Salmonella* (*S.*) Typhimurium.

Gram- bacteria possess an outer membrane (OM) composed of lipopolysaccharides (LPS), lipoproteins and divalent cations that coat the peptidoglycan layer (Nakamura *et al.*, 1991; Stevens *et al.*, 1992). This membrane provides a hydrophilic protective surface in which only small hydrophilic molecules are able to penetrate through, other macromolecules and hydrophobic molecules are usually obstructed (Helander *et al.*, 1998). The divalent cations (calcium, Ca^{2+} and magnesium, Mg^{2+}) impart stability to the LPS, linking them electrostatically (Stevens *et al.*, 1992;

Schved *et al.*, 1994). In general, Gram- bacteria are resistant to many detergents, disinfectants, hydrophobic antibiotics and toxic drugs due to their OM (Helander *et al.*, 1998; Shefet *et al.*, 1995). Hence, the OM, must first be destroyed before antimicrobials can penetrate and exert their action on the inner cytoplasmic layer (Helander *et al.*, 1998; Padgett *et al.*, 1998). Chelating agents and osmotic shock treatments are used to disrupt the stabilizing effects of the cations (Padgett *et al.*, 1998) which will then allow antimicrobial agents to penetrate the OM (Stevens *et al.*, 1992). Gram- bacteria are often pathogenic and are the major cause of foodborne illness outbreaks, therefore, controlling their contamination of foods is important (Nakamura *et al.*, 1991).

Gram+ bacteria are often responsible for food spoilage problems, however, some are also pathogenic (Chung and Hancock, 2000). They are more susceptible to antimicrobial agents since they do not posses an OM over their peptidoglycan protective layer. Microbial food spoilage usually starts at the surface of solid foods and as the bacteria grow a slime layer is produced, food discolouration and off-odours occur (Vojdani and Torres, 1990; Labuza, 1996), and eventually the bacteria proliferate and penetrate into the food product (El-Khateib *et al.*, 1993).

In the U.S., foodborne illnesses due to pathogens account for 76 million illnesses a year, in 325,000 hospitalizations and 5000 deaths (Franklin *et al.*, 2004). Foodborne illness outbreaks are mostly linked with improper cooking, temperature abuse, use of contaminated raw ingredients and cross-contamination (Natrajan and Sheldon, 2000b). Fifty percent of foodborne illnesses are related to the consumption of contaminated meat and poultry (raw and processed) products which result in mild to severe illnesses and in some cases death (Chung *et al.*, 1989; Natrajan and Sheldon, 2000b). Although pathogen growth in foods can yield illness, bacterial growth on food contact surfaces leading to

the production of biofilms can also indirectly lead to foodborne illness outbreaks due to cross-contamination (Daeschel *et al.*, 1992).

2.4.1. *L. monocytogenes*

L. monocytogenes is a Gram+, psychrotrophic, facultatively anaerobic, rod-shaped bacterium which is pathogenic to humans (Fang and Lin, 1994). It can cause listeriosis which can be life threatening to fetuses, the newborn, infants, pregnant women, the elderly and immuno-compromised individuals (Bruno *et al.*, 1992; Oh and Marshall, 1992). In more severe cases it causes meningitis, septicemia and abortions (Oh and Marshall, 1992). It is found in many environments, has been isolated from sewage, plants, soils and water (Abee *et al.*, 1994) and is difficult to control in foods since it can grow at temperatures from 1-45°C, is relatively heat resistant, has high tolerance to salt (sodium chloride, NaCl), can initiate growth at low pH and can survive in dry settings (Bruno *et al.*, 1992; Crandall and Montville, 1998). In addition, although they do not grow at freezing temperatures, viable cells after freezing/thawing can survive and cause problems (Dean and Zottola, 1996). The pathogen has been detected and is associated with raw, processed and ready-to-eat (RTE) red meats (El-Khateib *et al.*, 1993), poultry products (Fang and Lin, 1994), smoked fish products (Nykänen *et al.*, 2000), raw milk and other dairy products (Zapico *et al.*, 1998) causing health problems and economical losses to the food industry (Janes *et al.*, 2002). Most listeriosis outbreaks are due to consuming *L. monocytogenes* contaminated foods (Liberti *et al.*, 1996) and therefore the organism is usually considered a foodborne pathogen (Thomas and Wimpenny, 1996). In the U.S., in 2000 there were 2300 cases reported of foodborne listeriosis, costing

approximately $2.33 billion, making listeriosis the second most expensive foodborne disease (USDA-FSIS, 2001b).

2.4.2. *S. aureus*

S. aureus is a Gram+ facultatively anaerobic, cocci-shaped bacterium that is resistant to high NaCl concentrations (Thomas and Wimpenny, 1996) and is more heat resistant than *Salmonella* (Ng and Garibaldi, 1975). Although they are part of the human microflora, they can lead to skin and soft tissue infections and in more serious cases systemic infections (Bergsson *et al.*, 2001). They are considered foodborne pathogens (Thomas and Wimpenny, 1996) and have consistently caused cases of food poisoning. Poor sanitation and cross-contamination have led to the transfer of these organisms and their enterotoxins to processed foods. *Staphylococcus* species are becoming more important because of the development of antibiotic resistance, for example to methicillin and gentamicin.

2.4.3. *E. coli* O157:H7

E. coli is a Gram-, facultatively anaerobic, rod-shaped bacterium. It is commonly found in the lower gastrointestinal tract of mammals (Francis *et al.*, 1999). Not all strains of *E. coli* are pathogenic, but the enterohemorrhagic *E. coli* 0157:H7 is a significant foodborne pathogen. It has been frequently associated with contaminated, undercooked beef and dairy products and in many countries has caused major outbreaks and several deaths (Zhang and Mustapha, 1999). The pathogen is mainly transmitted via the fecal-oral route. It can be transmitted to foods by fecal material containing the pathogen or by cross-contamination through food, soil and food preparation surfaces

(Cieslak *et al.*, 1993). Fresh fruits and vegetables have also been associated with *E. coli*, being contaminated by other foods and utensils (Cagri *et al.*, 2002). Contaminated drinking and swimming water have also been reported to be involved in the transmission of the organism to humans (Dev *et al.*, 1991). In 2000, in the U.S., approximately 62,460 *E. coli* 0157:H7 infections were reported costing nearly $ 660 million (USDA-FSIS 2001a).

2.4.4. *S.* Typhimurium

Salmonellae are Gram-, facultatively anaerobic, rod-shaped bacteria. Most serovars are considered pathogenic to humans and animals (Harris *et al.*, 1997), sometimes causing large foodborne illness outbreaks. They grow best at pH's of 6-8 (Stevens *et al.*, 1992) and at temperatures of 35-43°C, and although they do not tend to grow under modified atmosphere packaging (MAP) at low temperatures ($\leq$7°C) they can survive and consequently cause health problems (Skandamis *et al.*, 2002). *Salmonella* species are present in nature and have been isolated from mammals, poultry, fish, amphibians, reptiles and insects (Francis *et al.*, 1999), and are found in animal intestinal tracts where they may not cause clinical symptoms (Leyer and Johnson, 1997). *Salmonella* have been associated with improperly fermented foods (Leyer and Johnson, 1997), but they are principally associated with poultry products which are considered to be the leading source of *Salmonella* in foodborne illnesses (Natrajan and Sheldon, 2000a). The pathogen is often transferred from contaminated poultry skin, feathers or intestinal tract content to other carcasses, equipment and employees (Natrajan and Sheldon, 2000a). In addition, it has also been associated with roast beef, ham, pork sausage, salami and cooked meats (Cagri *et al.*, 2002). *Salmonella* species and

Campylobacter jejuni together are responsible for being the largest cause of bacterial gastroenteritis in the U.S. and other developed countries (Natrajan and Sheldon, 2000a). In 1990, in the U.S., approximately 2.3 to 16 million people became ill from *Salmonella* (Leyer and Johnson, 1997) and nearly 4000 deaths a year occur in the U.S. due to this organism (Shefet *et al.*, 1995). It is the most costly foodborne organism in the U.S., costing ~ $2.38 billion each year (USDA-FSIS, 2001b).

2.4.5. *B. thermosphacta*

B. thermosphacta is a Gram+ facultatively anaerobic, psychrotrophic organism able to grow at temperatures of 0-30°C. The optimum temperature for growth is between 20-25°C and above 30°C growth rarely occurs. Optimum pH for growth is 7, however it is able to grow from pH 5-9 (Holley, 1999). *Brochothrix* species are naturally found on meat carcasses and although do not survive cooking temperatures they recontaminate meat products during packaging operations (Holley, 1999). *B. thermosphacta* is non-pathogenic to humans but has received attention because it often causes the spoilage of fresh and cured meats. It is the principal spoilage organism found on fresh and cured packaged meats (red meat, poultry and pork) (Chung and Hancock, 2000; Samelis *et al.*, 2000; Tu and Mustapha, 2002) that have not been properly vacuum packaged and refrigerated. This is because the organism has the ability to tolerate high salt concentrations and in the presence of low O_2 (>0.2%) levels can grow at low water activity and low temperature (Holley, 1999). Although the organism does not produce meat discoloration it leads to objectionable odours and quickly leads to meat spoilage. However, if meat products are properly vacuum packaged with good O_2 barrier films, such as polyvinylediene chloride (PVDC)-based packaging bags, *B. thermosphacta*

should not cause problems (Holley, 1999). Nonetheless, meat products in the retail industry are packaged and distributed on trays that are overwrapped with high gas-permeable films providing *B. thermosphacta* optimal conditions to grow, if present.

2.5. Antimicrobial packaging

Consumers demand more 'natural' foods with lower contents of fat, salt, sugar and additives. They insist foods be more convenient, safer and with extended shelf-life. Spoilage and pathogenic organisms always find a way of contaminating food products, especially during slicing and packaging procedures. Therefore, new ways of deterring their contamination are being developed.

Antimicrobials incorporated into edible films can enhance food safety and increase shelf-life by controlling surface microbial contamination, a major source of refrigerated food spoilage, particularly in fresh meats (Han and Floros, 1997; Padgett *et al.*, 1998). Traditional food preservation methods used low temperatures, organic acids (citric, ascorbic, propionic, sorbic, benzoic acetic, and lactic acid) to lower the pH or chemicals to increase the osmolarity and lower the water activity to safeguard foods (Golden *et al.*, 1995; Datta and Benjamin, 1997; Cagri *et al.*, 2004).

Later on, preservative sprays and dips were used to apply these preservatives on the surface of foods (Vojdani and Torres, 1990; Samelis *et al.*, 2001; Cutter and Siragusa, 1994b; El-Khateib *et al.*, 1993; Golden *et al.*, 1995; Lim and Mustapha, 2004; Pierson *et al.*, 1980; Stivarius *et al.*, 2002; Ariyapitipun *et al.*, 2000; Cutter and Siragusa, 1996a; Weng *et al.*, 1997).

Now, more natural antimicrobials (lysozyme, nisin) applied directly in the food or as solutions are being assessed for their ability to safeguard foods as well as for their

safety during consumption (Padgett *et al.*, 1998). However, the incorporation of naturally occurring antimicrobial compounds into packaging materials, including edible films is considered more desirable and has received much attention to control certain spoilage and pathogenic bacteria (Cutter and Siragusa, 1997). Greater use of edible antimicrobial films would also result in packaging waste reduction through their increased biodegradability (Padgett *et al.*, 1998; Siragusa *et al.*, 1999). In addition, antimicrobial films or coatings may have advantages over antimicrobials sprays or dips or direct application of the agents (Chen *et al.*, 1996; Padgett *et al.*, 1998) since the latter two, over time may not be as effective since agents may either diffuse too quickly into the food or can be degraded by food components (Ouattara *et al.*, 2000b). The addition of natural antimicrobial agents in edible films will permit antimicrobial compounds to be in higher concentrations at the surface of the food product to exhibit a relatively constant inhibitory effect, while minimizing their concentrations within the food product (Lee *et al.*, 1998). Their release from the film can also be controlled. Therefore, only the required quantity would be used and the direct addition to the food product could be avoided (Siragusa *et al.*, 1999), furthermore, the agents could be slowly released into the food during storage and distribution (Han and Floros, 1997) reducing or eliminating microbial growth (Weng *et al.*, 1997).

2.5.1. Examples of antimicrobial compounds

Various natural compounds exhibit antimicrobial properties against spoilage and pathogenic bacteria and have the potential of being effective natural preservatives. Some examples include those extracted from raw foods, medicinal herbs and from edible plants such as: thymol, carvacrol, cinnamaldehyde, (+)-carvone, eugenol, cinnamic acid,

diacetyl, crytotanshione, kushenol H, coumarin and various spices and their essential oils, including, garlic, clove, curry, ginger, basil, mustard, cinnamon, thyme, rosemary, and oregano (Helander *et al.*, 1998; Ohr, 2000; Kim *et al.*, 2001; Olasupo *et al.*, 2003; Walsh *et al.*, 2003; Alzoreky and Nakahara, 2003; Skandamis *et al.*, 2002). Other natural agents include allyl isothiocyanate and wasabi extract (Lee *et al.*, 1998).

Several antimicrobial agents such as benzoic acid, sodium benzoate, sorbic acid, potassium sorbate, propionic acid, parabens, acetic acid, lactic acid, bacteriocins (nisin, pediocin, curvaticin 13, leuconocin, brochocin-C), lysozyme, free fatty acids and their esters (monolaurin) and ethylenediaminetetraacetic acid (EDTA) have been investigated for their ability to be incorporated into a variety of protein-, polysaccharide- and lipid-based edible films (Han, 2000, 2002, 2003, 2005; Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002; Cagri *et al.*, 2004).

2.5.2. Natural antimicrobial agents

Nisin and lysozyme are natural antimicrobial agents that have been extensively studied, alone or in combination with other agents including chelators, as antimicrobial solutions or incorporated into edible films, in liquid, in model foods and in real food systems for their ability to inhibit the growth of spoilage and pathogenic microorganisms.

2.5.2.1. Nisin

Nisin is a biologically active peptide which contains 34 amino acids and is produced by various strains of *Lactococcus lactis* subspecies *lactis* (Winkowski *et al.*, 1996; Crandall and Montville, 1998; Verheul *et al.*, 1997). It is non-toxic, non-antigenic in humans (Bower *et al.*, 1995), digestible by enzymes in the human intestinal tract

(Chung *et al.*, 1989) and does not impart negative sensory characteristics to foods (Buncic *et al.*, 1995). It was discovered in the 1920's (Verheul *et al.*, 1997) and was allowed for use in foods as a biopreservative in 1969 by the Joint Food and Agriculture Organization/ World Health Organization Committee (Davies and Adams, 1994).

Nisin prevents the growth of vegetative Gram+ cells such as *L. monocytogenes* and *S. aureus*, and the outgrowth of *Bacillus* and *Clostridium botulinum* spores (Thomas and Wimpenny, 1996). It was first used as a food preservative to decrease "clostridial blowing" or gas production in Swiss cheese (Ming and Daeschel, 1993) and has now been used as an antimicrobial on other cheeses, in dairy desserts, fresh and canned evaporated milk, canned vegetables (potatoes, peas and mushrooms), soups, cereal puddings, meats (Chung *et al.*, 1989) and in pasteurized liquid egg ingredients (Siragusa *et al.*, 1999).

Nisin is naturally found in two forms, nisin A and Z. These are virtually identical, however in nisin Z asparagine is substituted for histidine at position 27 (Abee *et al.*, 1994). Nisin is a positively charged, amphiphilic molecule containing 4 atypical amino acids: dehydroalanine, dehydrobutyrine, lanthionine and ß-methyllanthionine (Winkowski *et al.*, 1996). The N terminus contains a large number of hydrophobic residues while the C terminus is more hydrophilic (Verheul *et al.*, 1997). Nisin is antimicrobial because it disrupts the phospholipids in the cytoplasmic membrane of vegetative cells and the sulfhydryl membrane groups of germinating spores (Hanlin *et al.*, 1993; Winkowski *et al.*, 1994, 1996; Crandall and Montville, 1998; Padgett *et al.*, 1998; Rose *et al.*, 1999). There are different suggested modes of action but the most accepted one is the barrel or pore formation model (Winkowski *et al.*, 1994, 1996). In this model nisin monomers first bind to the membrane, then insert themselves into it and

eventually oligomerize and form barrel-shaped pores (Winkowski *et al.*, 1994, 1996; Carneiro de Melo *et al.*, 1996; Crandall and Montville, 1998; Padgett *et al.*, 1998). The pores increase the membrane's permeability and allow the efflux of small cytoplasmic components such as amino acids, monovalent cations (K^+) and ATP (Davies and Adams, 1994; Verheul *et al.*, 1997). As a result, the membrane potential and pH gradient become demolished leading to the destruction of the proton motive force (PMF) (Bruno *et al.*, 1992). Biosynthesis stops and cell inactivation and death occur (Verheul *et al.*, 1997; Davies and Adams, 1994; Winkowski *et al.*, 1994).

Nisin can also act on Gram- microorganisms if the OM's integrity or its barrier functions are disrupted prior to its addition (Verheul *et al.*, 1997). Chemical and physical treatments such as heat, freezing, thawing, acid treatments, salt and chelating agents (EDTA) (Kalchayanand *et al.*, 1992; Coma *et al.*, 2001) can disrupt or injure the OM, rendering Gram- bacteria susceptible to nisin.

2.5.2.1.1. Factors affecting nisin activity

Nisin activity is affected by pH, salt concentration and temperature. Nisin at neutral and alkaline pH is more unstable and more insoluble (Harris *et al.*, 1991) while at lower pHs it dissolves with greater ease and is more stable (Thomas and Wimpenny, 1996). Thus, it has been shown to be more inhibitory at acidic pHs (Okereke and Thompson, 1996; Ukuku and Shelef, 1997; Parente *et al.*, 1998; Bouttefroy *et al.*, 2000). Harris *et al.*, (1991) and Okereke and Thompson, (1996) observed that nisin's activity increased by decreasing the medium's pH from 6.5 to 5.5 and below 4.75, respectively. In addition, lowering the pH from 7.9 to 5 increased the effectiveness of nisin against *S. aureus* and *L. monocytogenes* (Thomas and Wimpenny, 1996). Datta and Benjamin

(1997) observed that *L. monocytogenes* cells were only inhibited by nisin (250 μg/ml) when in low pH medium (~ pH 3), while at neutral pH (~ 7.3), the viability of *L. monocytogenes* was not reduced.

The addition of salt (2.5% w/v NaCl) or increasing its concentration (2.1 to 7% w/v) in the medium has also been shown to increase the inhibitory activity of nisin against *L. monocytogenes* (Harris *et al.*, 1991; Thomas and Wimpenny, 1996). In addition, both nisin and leucocin F10 had increased activity against the same organism in broth when salt concentrations were increased from 0.7 to 4.5% w/v (Parente *et al.*, 1998). Nilsson *et al.* (1997) observed that nisin applied on cold-smoked salmon inoculated with *L. monocytogenes* had increased antimicrobial activity in the presence of 100% CO_2 and increased NaCl (0.5 to 5% w/v) concentrations. However, Bouttefroy *et al.* (2000) showed that the presence of salt decreased the activity of nisin against *L. monocytogenes* in broth. In addition, Knight *et al.* (1999) demonstrated that nisin (10 μg/ml) in unsalted liquid whole eggs (LWE) reduced *L. monocytogenes* by 4 logs, however in salted LWE it minimally inhibited the organism (Knight *et al.*, 1999).

Nisin coupled with lower temperature treatments had enhanced activity. De Martinis *et al.* (1997) showed that nisin used with low temperatures (10°C) and pH (5.5) had increased action against *L. monocytogenes* in broth. Thomas and Wimpenny (1996) also showed that decreasing temperatures from 35 to 20°C and increasing NaCl concentrations (2.1 to 7% w/v) enhanced the activity of nisin against *S. aureus* growth on gradient plates. In addition, the growth of *L. monocytogenes* in broth grown in the presence of a CO_2-enriched atmosphere was better inhibited at 4°C compared to 12°C (Szabo and Cahill, 1998).

However, Budu-Amoako *et al.* (1999) observed that nisin (25 mg/kg) added to the brine solution in cans of cold-pack lobster meat that was inoculated with *L. monocytogenes*, retorted at 60°C for 5 min or 65°C for 2 min, cooled and stored at -20°C reduced the population by 3-5 log CFU/ml, whereas heat or nisin treatments alone only reduced numbers by 1-3 log CFU/ml. In addition, nisin and heat treatments conducted at pH 5 were seen to be more inhibitory than at pH 8.

2.5.2.1.2. Nisin as an effective antimicrobial for use in foods

Nisin has been extensively studied to control the proliferation of organisms. In liquid media nisin has been observed to inhibit *S. aureus* (Carneiro de Melo *et al.*, 1996; Sebti and Coma, 2002), *E. coli* (Carneiro de Melo *et al.*, 1996) and *L. monocytogenes* (Ukuku and Shelef, 1997). When immobilized onto hydrophobic and hydrophilic silicon surfaces its activity remained stable and it exhibited antimicrobial activity against *Pediococcus pentosaceus* (Daeschel *et al.*, 1992) and *L. monocytogenes* (Bower *et al.*, 1995). In addition, it has been used to treat the surface of several food products (Cutter and Siragusa 1994a, 1996a; Chung *et al.*, 1989; El-Khateib *et al.*, 1993; Murray and Richard, 1997; Mahadeo and Tatini, 1994; Carneiro de Melo *et al.*, 1998).

Nisin (5000 activity units (AU)/ml)) applied as a spray on beef carcass surfaces inoculated with *B. thermosphacta*, *Carnobacterium divergens* or *L. innocua* achieved reductions of 2.8, 3.1 and 3 log CFU/cm^2, respectively after 24h (Cutter and Siragusa, 1994a). Further reduction of *B. thermosphacta* (4.5 log CFU/cm^2) and *L. innocua* (2.83 log CFU/cm^2) on beef carcass tissue was achieved when a nisin spray treatment was followed by vacuum packaging and refrigeration for 4 weeks (Cutter and Siragusa, 1996a).

Chung *et al.*, (1989) showed that when beef cubes were dipped into a nisin solution, the numbers of *L. monocytogenes* and *S. aureus* cells attached on the beef were decreased, however, nisin had no effect on *S.* Typhimurium cells. A nisin solution also reduced (>99.9%) *S. aureus* on chicken skin (Carneiro de Melo *et al.*, 1998). *L. monocytogenes* inoculated on beef cubes was also inhibited by 40000 international units (IU)/ml nisin, 3200 AU/ml pediocin PO2 or 2% lactic acid solutions (El-Khateib *et al.*, 1993). The organism was reduced by ~ 1.1, 1.7 and 0.6 log, respectively. However, Murray and Richard, (1997) observed that pork cubes inoculated with a *Listeria* suspension, treated with a nisin solution (300, 1500 and 3000 IU/ml) and ground, only had bacterial numbers reduced for the first 2d, after which the bacteria were able to resume growth (Murray and Richard, 1997).

Some researchers have shown that the fat content in food products such as in milk or in adipose tissues in meat can protect certain bacteria from the inhibitory action of nisin. Mahadeo and Tatini (1994) showed that nisin (100 IU/ml) was more effective in inhibiting *L. monocytogenes* cells suspended in the scald water (> 4 log CFU/ml reduction), than those attached on turkey skin (1 log CFU/ml reduction). Nisin (14 mg/kg) in 3% fat ice-cream mix inoculated with *L. monocytogenes*, stored for 3 months at -18°C, was more inhibitory against the organism than in 10% fat ice-cream mix (Dean and Zottola, 1996). Cutter and Siragusa (1996a) found that *B. thermosphacta* and *L. innocua* survived better on adipose tissue than on lean tissues when treated with a nisin (5000 AU/ml) solution. These studies suggest that bacteria can be more protected against the action of bacteriocins by fatty meat tissues or by high fat content ice-cream.

Although nisin can be an effective antimicrobial agent, Abdalla *et al.* (1993) did not show reduction in numbers of *L. monocytogenes*, *S. aureus* or *S.* Typhimurium by nisin (25 μg/ml) when used during the manufacture and storage of white pickled cheese.

2.5.2.2. Lysozyme

Lysozyme is a muramidase enzyme composed of 129 amino acid residues in a polypeptide chain containing four disulfide bridges (Yang and Cunningham, 1993). It is a positively charged protein that is found in various animal and human secretions, such as tears, milk, saliva and serum (Payne *et al.*, 1994). Although human milk contains 400 μg/ml lysozyme and is more active than hen egg white lysozyme (Maga *et al.*, 1998), the latter is the major source of the enzyme (Payne *et al.*, 1994; Maga *et al.*, 1998).

Lysozyme has the ability to inhibit the growth or lyse gram+ bacteria (Padgett *et al.*, 1998). It hydrolyses the β(1,4)-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglycosamine of the peptidoglycan in the cell wall of Gram+ bacteria (Payne *et al.*, 1994; Liberti *et al.*, 1996; Ibrahim *et al.*, 1996a, 1996b; Appendini and Hotchkiss, 1997).

2.5.2.2.1. Factors affecting lysozyme activity

Lysozyme activity is influenced by pH, ionic strength and salt concentrations. Lysozyme is normally stable between pH of 5 to 10, but the optimum pH for its activity is between pH 5.3 to 6.4 (Razavi-Rohani and Griffiths, 1996). In addition to the pH, lysozyme's activity is also affected by the ionic concentration of the medium, which in turn is pH dependent (Chang and Carr, 1971; Davies *et al.*, 1969). Chang and Carr (1971) determined that increasing concentrations of salt decreased the activity of

lysozyme. Yang and Cunningham, (1993) observed that lysozyme activity was stable over a pH of 6.5 to 9 at ionic strengths between 0.06 to 0.14, however, above pH 9 and ionic strength >0.14, lower lysozyme activities were seen. Furthermore, since lysozyme is an enzyme its stability during storage is decreased due to enzyme degradation (Yang and Cunningham, 1993).

2.5.2.2.2. Lysozyme as an effective antimicrobial for use in foods

Verheul *et al.* (1997) observed that lysozyme (5 mg/ml) reduced *L. monocytogenes* in BHI broth by ~1 log CFU/ml (Verheul *et al.*, 1997). Lysozyme has also been shown to inhibit *L. monocytogenes* cells in suspension (Carneiro de Melo *et al.*, 1998), the growth of *S. aureus* in shaken liquid whole egg (Ng and Garibaldi, 1975) and in broth (Ibrahim *et al.*, 1997). In addition, Alakomi *et al.* (2000) showed that lysozyme (10 μg/ml) was only slightly inhibitory in buffer against *S.* Typhimurium and *E. coli.* However, Carneiro de Melo *et al.* (1998) showed that suspended *S. aureus* cells were not inhibited by lysozyme, and lysozyme ($\leq$200 μg/ml) was not inhibitory against *S.* Typhimurium in UHT milk (Payne *et al.*, 1994).

2.5.2.2.3. Lysozyme modifications

It has been shown that lysozyme can be more effective in inhibiting Gram-bacteria when it is chemically modified. The antimicrobial activity of lysozyme can be enhanced by: interacting it with hydrophobic peptides, fatty acids (FAs) (Ibrahim *et al.*, 1994), hydrophobic phenolic aldehyde compounds (by covalently attaching perillaldehyde residues) (Ibrahim *et al.*, 1994), by heat-denaturing the enzyme to increase its surface hydrophobicity (Ibrahim *et al.*, 1996a, 1996b), or by forming

lysozyme-dextran conjugates (Nakamura *et al.*, 1991) in order for it to have better access to the OM of Gram- bacteria.

Modified lysozyme was shown to be more active than native lysozyme against *S. aureus* and *E. coli* (Ibrahim *et al.*, 1994, 1996a, 1996b, 1997) and against *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Proteus mirabilis* and *B. cereus* (Nakamura *et al.*, 1991) in phosphate buffer.

2.5.2.3. Ethylenediaminetetraacetic acid (EDTA)

EDTA is a chelating agent that disrupts the OM structure of Gram- bacteria by complexing with Mg^{2+} and Ca^{2+} ions that stabilize the membrane (Garibaldi *et al.*, 1969; Shefet *et al.*, 1995). It has been shown that EDTA releases 30-50% of the surface LPS by interacting with the divalent cations (Ibrahim *et al.*, 1997). As a result, it loosens and weakens the bacterial cell structure (Gao *et al.*, 1999). The membrane no longer acts as a barrier (Helander and Mattila-Sandholm, 2000), consequently, there is an increase in the cell's susceptibility to other antimicrobial agents which may more easily penetrate to the peptidoglycan layer (Hughey and Johnston, 1987; Padgett *et al.*, 1998).

Few studies have used EDTA alone to inhibit bacterial growth, since by itself EDTA has little antagonistic effect (Hoffman *et al.*, 2001). Razavi-Rohani and Griffiths (1996) showed that EDTA did not inhibit *E. coli* 0157:H7 unless concentrations were > 3 mg/ml, but it was effective against *L. monocytogenes* and *S. aureus*. Payne *et al.*, (1994) observed that EDTA ($\leq$2.5 mg/ml) alone decreased *E. coli* 0157:H7 numbers by 4 log CFU/ml in UHT milk, however it had no effect on *S.* Typhimurium. Generally, in most studies EDTA has been used in combination with nisin, lysozyme and other antimicrobial agents to enhance their effectiveness (Padgett *et al.*, 1998).

2.5.2.4. Propyl paraben (PP)

Propyl paraben contains a benzene ring, is an ester of *p*-hydroxybenzoic acid (paraben) and a derivative of benzoate (Thompson, 1994). It has antifungal activity and is most effective against Gram+ bacteria and fungi as opposed to Gram- bacteria (Thompson, 1994). Parabens inhibit membrane transport of electrons, essential amino acids and nutrients (Baranowski and Nagel, 1983; Lück and Jager, 1997), and can act over a wide range of pHs (3-8) (Payne *et al.*, 1989; Chung *et al.*, 2001; Cagri *et al.*, 2004). It is generally recognised as safe (GRAS) and maximum levels currently allowed in foods are ≤1000 parts per million (ppm) (Pierson *et al.*, 1980; Chung *et al.*, 2001).

As with nisin, the activity of PP increases as the acidity in the medium increases (Thompson, 1994). In addition, longer chain parabens (butyl and propyl paraben) have increased antimicrobial activity compared to shorter chain parabens (Thompson, 1994; Cagri *et al.*, 2004). Furthermore, PP is more soluble in alcohol than in water (Chung *et al.*, 2001), but for use in food PP is dissolved in water.

Pierson *et al.* (1980) reported that PP (300 ppm and 500 ppm) reduced numbers of *S. aureus* by 3 log CFU/ml after 48h, however, it minimally inhibited *S.* Typhimurium during the first 6 days in broth. Butylated hydroxyanisole (BHA), a structurally similar phenolic compound, also decreased the initial growth of the same organisms (Pierson *et al.*, 1980). *L. monocytogenes* has also been reduced (by 4 log CFU/ml) using 1000 μg/ml PP in a milk model system (Payne *et al.*, 1989).

Furthermore, Thompson (1994) observed that combining parabens (butyl, ethyl, methyl and propyl paraben) in pairs had increased antifungal activity against *Aspergillus*, *Fusarium* and *Penicillium* species.

2.5.3. Resistance of organisms to antimicrobials

Antimicrobial agents used at concentrations that are too low, and repeatedly exposing microorganisms to the antimicrobials can lead to the development of antimicrobial-resistant organisms. Nisin resistant *L. monocytogenes* have been observed to be 12 times more resistant than sensitive cells (Verheul *et al.*, 1997). In addition, these resistant organisms were shown to contain a different bacterial membrane structure, where FA or phospholipid composition and levels, or protein distribution were altered (Ming and Daeschel, 1993, 1995; Verheul *et al.*, 1997; Crandall and Montville 1998). Juneja and Davidson (1993) also observed that *L. monocytogenes* cells that contained an altered FA composition (contain increased levels of saturated FA) exhibited more resistance to PP (1000 ppm), methyl paraben (1500 ppm) and tertiary butylhydroquinone (TBHQ, 200 ppm). Bargiota *et al.* (1987) showed that *S. aureus* cells that were resistant to parabens had an increased total lipid content, more phosphatidyl glycerol and reduced levels of cyclopropane FA compared to sensitive cells. Nisin resistant *L. monocytogenes* cells were also shown to contain higher levels of phosphatidylglycerol and lower levels of diphosphatidylglycerol and phosphoglycolipid (Verheul *et al.*, 1997).

In addition, others have shown that nisin resistant organisms had reduced numbers of accessible nisin absorption sites (Davies and Adams, 1994; Verheul *et al.*, 1997).

Furthermore, antimicrobial resistant organisms can develop cross-resistance to other antimicrobials. Crandall and Montville (1998) observed that nisin resistant *L. monocytogenes* cells displayed cross-resistance to pediocin PA-1 and leuconocin S and were more resistant to the effects of lysozyme than were sensitive cells.

As well, others have shown that organisms that are subjected to certain treatments such as when acid-adapted, can become resistant to the action of antimicrobials (Datta and Benjamin, 1997; Okereke and Thompson, 1996; van Schaik *et al.*, 1999). *L. monocytogenes* cells which were tolerant of pH 5.4 (Okereke and Thompson, 1996) or of 5.5 (van Schaik *et al.*, 1999) in BHI broth were seen to be more resistant to nisin than unadapted *L. monocytogenes* cells. The acid tolerant cells were also more resistant to lacticin 3147, than the unadapted cells. In addition, the bacterial cell membranes of acid-adapted cells and coincidently the nisin resistant cells were also seen to have more saturated straight chain FAs than the unadapted cells (van Schaik *et al.*, 1999).

2.5.4. Combining antimicrobials

Combining antimicrobials with refrigeration, vacuum or modified atmosphere packaging or with food ingredients such as NaCl, chelating agents, organic acids or with other antimicrobial compounds can further increase the microbial safety of food products (Murray and Richard, 1997; Parente *et al.*, 1998; Siragusa *et al.*, 1999). However, the overall effectiveness depends on: the bacteria in question, the antimicrobials and treatments used, the specific food product and the storage conditions (Stevens *et al.*, 1992; Payne *et al.*, 1994). Antimicrobials with different modes of action can be combined to increase their effectiveness and broaden the range of inhibition which may include otherwise resistant Gram+ and Gram- organisms. For example, agents that are able to disrupt the OM can be used in conjunction with agents that attack the cytoplasmic membrane to increase their effectiveness.

2.5.4.1. Combinations with nisin

Many authors have shown that nisin has increased antimicrobial activity against Gram- and + organisms in the presence of agents such as EDTA (Stevens *et al.*, 1991, 1992; Schved *et al.*, 1994; Cutter and Siragusa, 1995a, 1995b; Gao *et al.*, 1999; Zhang and Mustapha, 1999; Boziaris and Adams, 1999; Helander and Mattilla-Sandholm, 2000; Cutter *et al.*, 2001; Tu and Mustapha, 2002).

Helander and Mattilla-Sandholm, (2000) observed that nisin and 1mM EDTA in buffer destabilized the OM of *E. coli*, *P. aeroginosa*, *P. marginalis* and several strains of *S.* Typhimurium. Stevens *et al.* (1991) showed that 50 μg/ml nisin plus 20 mM EDTA inhibited the growth of *E. coli* 0157:H7, *Shigella flexneri*, *Citrobacter freundii*, *Enterobacter aerogenes* and *Salmonella* species by ~3 to 7 log CFU/ml in buffer at 37°C, during 30 min to 1 h exposures, however neither agent used alone inhibited the organisms. In addition, a 100 IU/ml nisin plus 10 mM EDTA combination inhibited *E. coli*, *Pseudomonas aeruginosa* and *S.* Enteritidis in broth (pH 6.5), much better than when EDTA was used alone (Boziaris and Adams, 1999). Schved *et al.* (1994) showed that the numbers of viable *E. coli* and *S.* Typhimurium cells in buffer at 30°C were reduced by ~ 4 and 3 log CFU/ml, respectively, when a 3200 IU/ml nisin and 20 mM EDTA combination was used, while the individual antimicrobials had no effect on the organisms.

Bacteriocins have been used in combination to obtain increased antimicrobial activity (Hanlin *et al.*, 1993; Bouttefroy and Millière, 2000). Hanlin *et al.* (1993) combined nisin and pediocin ACH and produced greater antimicrobial activity ($\leq$3 log reduction) than when either bacteriocin was used individually against various Gram+ psychrotrophic spoilage or pathogenic organisms (*L. monocytogenes*, *Leuconostoc*

mesenteroides and *Lactobacillus* species). Bouttefroy and Millière (2000) used nisin and curvaticin 13 to inhibit *L. monocytogenes* in broth and although each bacteriocin inhibited the organism, re-growth occurred. The use of both bacteriocins simultaneously produced synergistic bactericidal effects and re-growth was prevented. The authors also observed that the addition of the antimicrobials simultaneously displayed a greater inhibitory effect than when the agents were added sequentially together.

Nisin or brochocin-C combined with 20 mM EDTA decrease viable counts (by ~2 log CFU/ml) of *E. coli* and *Salmonella enterica* in BHI broth, while each agent alone did not show any inhibitory effects (Gao *et al.*, 1999).

Buncic *et al.* (1995) observed that 400 IU/ml nisin initially ($\leq$3d) reduced the growth of *L. monocytogenes* in broth at 4°C by ~1 log CFU/ml, however when nisin was used with 0.3% w/v potassium sorbate the bacterial population was reduced by 4.5 log CFU/ml during the 5 weeks of storage.

Nisin and CO_2 have also been shown to act synergistically against *L. monocytogenes* cells (Nilsson *et al.*, 2000). It was observed that cells grown in broth with added nisin (2.5 μg/ml) at 4°C in the presence of air were reduced by 2 log CFU/ml, while those grown in the same conditions in 100% CO_2 had greater reductions (4 log CFU/ml).

Schved *et al.* (1994) and Cutter and Siragusa (1995b) observed that the addition of Ca^{2+} and Mg^{2+} ions to EDTA or to buffer that contained nisin, eliminated the effects of EDTA and the antimicrobial activity of nisin was decreased against *E. coli* and *S.* Typhimurium cells in buffer. It was suggested that the addition of ions such as Ca^{2+} and Mg^{2+} to EDTA or to buffers, caused EDTA or the buffer to chelate the added ions and

hence they were not able to chelate a critical level of divalent cations from the LPS layer of the OM of Gram- bacteria (Schved *et al.*, 1994; Cutter and Siragusa, 1995b).

Nisin used with other antimicrobial agents in solutions have also been applied on the surface of food products to inhibit bacterial proliferation (Cutter and Siragusa, 1994a, 1994b, 1995a, 1996a, 1996b, 1997; Murray and Richard, 1997; Shefet *et al.*, 1995; Zhang and Mustapha, 1999; Tu and Mustapha, 2002; Ariyapitipun *et al.*, 2000; Fang and Lin, 1995; Natrajan and Sheldon, 2000a; Scannell *et al.*, 2000). However, antimicrobial treatments on meat tissues have resulted in less inhibition than when used in buffers, broths or agar media (Cutter and Siragusa, 1995a; Mahadeo and Tatini 1994; Carneiro de Melo *et al.*, 1998). Cutter and Siragusa, (1995a) observed that beef cubes treated with 50 μg/ml of pure nisin and 50 mM EDTA only slightly reduced (0.42 log CFU/cm^2) numbers of attached *S.* Typhimurium or *E. coli* 0157:H7 populations, however, the organisms in a buffer system containing the same agents were inhibited to a greater extent (~2 and 4 log CFU/ml, respectively) (Cutter and Siragusa, 1995b).

Zhang and Mustapha, (1999) effectively inhibited *E. coli* 0157:H7 and *L. monocytogenes* on fresh beef cubes using 5000 IU/ml nisin (1.02 and 2.01 log CFU/cm^2, respectively) and a 5000 IU/ml nisin plus 20 mM EDTA solution (0.8 and 0.99 log CFU/cm^2, respectively) when followed by vacuum packaging and storage at 4°C for 30 days. In addition, under the same conditions *B. thermosphacta* was completely inhibited (by 6.76 log CFU/ml) by nisin and by nisin plus EDTA, however neither of the solutions inhibited *S.* Typhimurium (Tu and Mustapha, 2002).

When beef cubes were treated with a solution of 400 IU/ml nisin alone or in combination with 2% (w/v) lactic acid or 2% (w/v) low-molecular weight polylactic acid, vacuum packaged and stored at 4°C for 42d, *L. monocytogenes* numbers were decreased

from ~5.33 log CFU/cm^2 to 2.21, 0.89 or 0.84 log CFU/cm^2, respectively (Ariyapitipun *et al.*, 2000).

Shefet *et al.* (1995) observed that fresh turkey drumstick skin dipped in a solution of 100 μg/ml nisin, 5mM EDTA, 3% w/v citric acid and 0.5% Tween 20 for 30 min reduced *S.* Typhimurium cells by ~5 log CFU/ml more than a 20 ppm chlorine solution. In addition, the former antimicrobial solution was able to increase the shelf-life of whole broiler drumsticks at 4°C by 1.5 to 3 days.

Nykänen *et al.*, (2000) applied nisin alone or in combination with sodium lactate to cold-smoked rainbow trout prior to smoking or to the finished smoked fish that was then inoculated with *L. monocytogenes*, vacuum packaged and stored either at 3°C for 29d or at 8°C for 17d, respectively. Smoked trout (finished product) that was injected with nisin and lactate together decreased the population of *L. monocytogenes* from 3.3 to 1.8 log CFU/g after 17d at 8°C. However, when the agents were injected into the rainbow trout prior to smoking, greater inhibition was observed over 29d at 3°C. In either case, the use of the agents together was more inhibitory than when used individually.

The use of treatments such as MAP, vacuum packaging with salt and low temperature storage combined with antimicrobials, tends to increase the overall microbial stability of food products. However, Fang and Lin (1994) observed that the combined effect of nisin (10,000 IU/ml) and MAP on the growth of *L. monocytogenes* and *Pseudomonas fragi* on cooked pork tenderloin was not much better than the individual treatments. Nisin used alone was bactericidal against *L. monocytogenes*, while MAP inhibited the growth of *P. fragi*, and although both bacteria were inhibited by the

combined treatments, the effects were not much greater than when each treatment was used alone.

Fang *et al.*, (1997) showed that *S. aureus* and *B. cereus* inoculated in a vegetarian food model were inhibited to a greater extent with the use of 5000 IU/g nisin plus 0.12% sodium benzoate or with 3% (w/v) potassium sorbate (than either agent alone). Inhibition was greater at 4°C than 30°C, and with vacuum packaging than without.

Beef steaks, surface treated with 1000 IU nisin/ml plus 1% sorbate, inoculated with *L. monocytogenes*, vacuum packaged and stored for 4 weeks at 4°C showed greater bacterial reductions (96.5% lower) than beef steaks that were prepared in the same manner but packaged under 100% CO_2 (89.3% lower) (Avery and Buncic, 1997).

Zapico *et al.* (1998) observed that nisin (10 or 100 IU/ml) combined with lactoperoxidase acted synergistically against *L. monocytogenes* in ultra-high temperature (UHT) processed milk, reducing the organism by 5.6 log CFU/ml. Lactoperoxidase combined with nisin (100 or 200 IU/ml) also decreased the organism in skim milk (Boussouel *et al.*, 2000). *Listeria* numbers were reduced from 10^4 CFU/ml to undetectable levels and re-growth did not occur.

Nisin (500 or 1000 IU/ml) applied to CO_2-packaged cold-smoked salmon inoculated with *L. monocytogenes* reduced the population of the organism by 1-2 log and levels remained < 3 log CFU/g for 27d at 5°C (Nilsson *et al.*, 1997).

Although some researchers have shown that EDTA enhances the effectiveness of nisin, others like Zhang and Mustapha (1999) did not observe increased inhibition against *L. monocytogenes* and *E. coli* on fresh beef cubes using a nisin (5000 IU/ml) plus EDTA (20 mM) combination compared to a nisin solution alone. In another study, the

same combination and concentration of the agents was found to be ineffective against *S.* Typhimurium on the same product (Tu and Mustapha, 2002).

2.5.4.2. Combinations with lysozyme

Lysozyme as well has been shown to have increased antimicrobial activity in the presence of EDTA (Garibaldi *et al.*, 1969; Hughey and Johnson, 1987; Payne *et al.*, 1994; Razavi-Rohani and Griffiths, 1996; Ibrahim *et al.*, 1997). Hughey and Johnson, (1987) showed that although lysozyme was able to decrease the growth of *L. monocytogenes* on fresh vegetables, a lysozyme and EDTA combination had better inhibitory effects on the organism. *L. monocytogenes* was also reduced (6 log CFU/ml) by a lysozyme ($\leq$200 μg/ml) plus EDTA ($\leq$2.5 mg/ml) combination in UHT processed milk; however, a combination of the agents did not inhibit *S.* Typhimurium and did not yield increased inhibition over EDTA against *E. coli* in the same food product (Payne *et al.*, 1994). In addition, *L. monocytogenes and S. aureus* in broth have been shown to be inhibited to a greater extent using a lysozyme plus EDTA solution than with either agent used alone (Razavi-Rohani and Griffiths, 1996). Nevertheless, the same agents used together did not yield greater inhibition against *S.* Typhimurium and *E. coli* (Razavi-Rohani and Griffiths, 1996). On the other hand, Garibaldi *et al.* (1969) and Ibrahim *et al.* (1997) showed that lysozyme used with EDTA decreased viable *S.* Typhimurium cells in egg white and *E. coli* cells in broth, respectively.

The effectiveness of lysozyme can be enhanced when used in combination with lactic acid or with lipase. Alakomi *et al.*, (2000) observed that the activity of lysozyme (10 μg/ml) was slightly more inhibitory against *E. coli* and *S.* Typhimurium when used with lactic acid (10 mM) compared to when lysozyme was used alone. In addition,

lysozyme was much more effective against *P. aeruginosa* in combination with the acid. Lysozyme (0.3 mg/ml) used with lipase (50 IU/ml) had increased action against *L. monocytogenes* in broth at 5, 15 and 32°C, however this action was pH and NaCl dependent (Liberti *et al.*, 1996). At 5°C, lysozyme plus lipase inhibited *L. monocytogenes* when the medium contained 0.5% (w/v) NaCl at pH ~5-6, but at 32°C it was only able to inhibit *L. monocytogenes* when the salt concentration was ≤0.5% (w/v) (Liberti *et al.*, 1996).

A trisodium phosphate (TSP) treatment followed by nisin reduced viable numbers of *C. jejuni*, *E. coli* and *S.* Enteritidis on chicken skin better than TSP-lysozyme treatments (Carneiro de Melo *et al.*, 1998). In addition, the treatments were better able to inhibit suspended cells in broth than those attached to chicken skin.

Chung and Hancock, (2000) demonstrated that nisin and lysozyme used together had a better inhibitory effect (decreased the MIC) against several Gram+ bacteria, including *B. thermosphacta*, *Lactobacillus sake*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides* and *Pediococcus acidilactici*, *S. aureus* or *L. monocytogenes* in broth, than each agent used alone.

2.5.5. Incorporation of antimicrobial agents into packaging materials

Antimicrobials have been added to edible and synthetic films to control bacterial proliferation and to increase product shelf-life.

2.5.5.1. Antimicrobial edible films

Antimicrobial edible films made with polysaccharides, proteins and lipids have demonstrated with some success the ability to decrease bacterial populations in broths,

on agars and on different food products. The use of antimicrobial films is quickly being adopted. Several studies have shown that antimicrobial agents incorporated in a gel structure can remain stable and their antimicrobial activities have been observed to persist longer than when not immobilized (Siragusa and Dickson, 1992; Fang and Lin, 1995; Cutter and Siragusa, 1996b, 1997; Appendini and Hotchkiss, 1997). This suggests that antimicrobial agents might be more effective when used as antimicrobial films rather than as solutions. In addition, antimicrobials applied as solutions or dips eventually diffuse into the food product, reducing the concentration of the agents at the surface and allowing organisms to start to grow. Therefore, producing successful films will require that films adequately release the antimicrobial agents, in order to inhibit surface microbial growth until treated foods are consumed. Thus, antimicrobial films and coatings provide opportunities for extended microbial stability and shelf-life of foods.

2.5.5.1.1. Antimicrobial diffusion

Antimicrobial diffusion through edible films depends on the characteristics of the film (components, formation conditions and interactions between antimicrobials and polymer components), food product and storage conditions (Cagri *et al.*, 2004). Producing films with appropriate release profiles requires the understanding of the interactions and the structural changes that occur in the polymer. For example, the release of organic acids from chitosan films is influenced by electrostatic interactions, ionic osmosis and the changes that occur in the polymer (Ouattara *et al.*, 2000b). In addition, knowing the release mechanism of an antimicrobial from a polymer is

important because it will dictate the best food applications and the nature of the antimicrobial profile required for use on the food product (Chung *et al.*, 2001).

Ouattara *et al.* (2000b) observed that pH (5.7 and 7) did not affect the diffusion of acetic and propionic acids from chitosan-based antimicrobial films in a buffer system, however increasing the temperature (4 to 24°C) caused the diffusion to increase. Chen *et al.* (1996) also observed that the release rate of potassium sorbate or sodium benzoate from chitosan-methylcellulose films was higher at 25°C than 4°C (60 and 50%, respectively).

Furthermore, the addition of 1% lauric acid or 0.5% essential oils (cinnamaldehyde or eugenol) into chitosan-based films decreased the diffusion of acetic and propionic acids from the films (Ouattara *et al.*, 2000b). Vojdani and Torres (1990) also reported that potassium sorbate was more easily released from methylcellulose (MC) or hydroxypropyl methylcellulose (HPMC) films that contained no added FA (lauric, palmitic, stearic and arachidic acids). In addition, the authors demonstrated that potassium sorbate permeability was least with increased concentrations and chain lengths of FA. The authors suggested that the addition of hydrophobic compounds into hydrophilic film matrixes decreases the diffusion of the preservatives since film hydration is decreased (Vojdani and Torres, 1990; Ouattara *et al.*, 2000b).

In addition, different antimicrobials diffuse at different rates and to different extents. The release of propionic acid from chitosan films in a buffer system was slower than the release of acetic acid, however, propionic acid release was more complete than acetic acid when the films were applied on the surface of processed meats (Ouattara *et al.*, 2000a).

Furthermore, film components may affect the release of the agents. Nisin (500 μg/ml) in 0.75% agar gel films reduced *S.* Typhimurium to a greater extent (2 log reduction) than when added into 1.25% agar gel films (Natrajan and Sheldon, 2000a). Appendini and Hotchkiss (1997) also observed that although glycerol was needed to increase the flexibility of cellulose triacetate-methylene chloride emulsion films containing lysozyme, films with the lowest concentrations of glycerol yielded films with the highest lysozyme activity. Ozdemir and Floros, (2003) also showed that increasing concentrations of WPI and beeswax in WPI films decreased the diffusivity of potassium sorbate, however increasing concentrations of sorbitol and potassium sorbate increased the diffusion of the agent through the films.

Additionally, it has been demonstrated that when films are placed in a buffer system, the antimicrobial agents present in the films have a quicker release profile compared to when they are applied on the surface of food products (Ouattara *et al.*, 2000a).

2.5.5.1.2. Polysaccharide-based antimicrobial films

Several studies have used calcium alginate gels as a method to immobilize antimicrobials and to obtain increased antimicrobial activity. Siragusa and Dickson, (1992) observed that acetic (2% v/v) and lactic (1.7% v/v) acid immobilized in a calcium alginate gel reduced viable counts of *L. monocytogenes* on lean beef tissues (1.5 and 1.3 log CFU/ cm^2, respectively) after 7d at 5°C, better than the acid solutions used alone (0.25 and 0.03 log CFU/cm^2, respectively). Nisin immobilized in a calcium alginate gel inhibited *L. monocytogenes* and *B. thermosphacta* on cooked pork tenderloins (Fang and Lin, 1995) and on the surface of lean and adipose beef tissue (Cutter and Siragusa,

1996b). In a later study, it was shown that 100 μg/ml nisin immobilized in a calcium alginate gel applied on beef surfaces and processed into ground beef was more effective in inhibiting *B. thermosphacta* (7d at refrigerated temperatures), than when nisin was applied without a gel (Cutter and Siragusa, 1997). A calcium alginate coating containing 500 μg/ml nisin, 3% citric acid, 5mM EDTA and 0.5% Tween 80 coated on broiler samples inoculated with *S.* Typhimurium, and stored at 4°C for 72h reduced the organism by 3 log (Natrajan and Sheldon, 2000a). Gill and Holley, (2000) reported that an antimicrobial gel coating containing 25.5 g/l of lysozyme-nisin (1:3) and 25.5 g/l EDTA immediately reduced, up to 4 log CFU/cm^2 *B. thermosphacta*, *Lactobacillus sakei*, *Leuconostoc mesenteroides* and *L. monocytogenes* on cooked ham and bologna sausage slices stored at 8°C. In addition, the antimicrobial gel reduced *S.* Typhimurium by 3-4 log CFU/cm^2 during 4 weeks of storage, however it had no effect on *E. coli* on both meat products.

Cha *et al.* (2002) observed that sodium (Na)-alginate films that contained nisin plus EDTA or lysozyme plus EDTA were not able to inhibit *E. coli* and *S.* Enteritidis in agar; however, films containing all three agents were effective in inhibiting the organisms. The same was not seen with κ-carrageenan films. Furthermore, Na-alginate films containing 0.1% w/w lysozyme, 100 μg/ml nisin, 0.1% w/w grapefruit seed extract (GFSE) and 5 mmol EDTA were shown to produce larger inhibition zones against *E. coli*, *S.* Enteritidis, *S. aureus* and *L. innocua*, than κ-carrageenan films containing the same antimicrobial agents at the same concentrations (Cha *et al.*, 2002). In a previous study the authors had also observed that nisin diffused faster from MC than κ-carrageenan or HPMC films (Cha *et al.*, 2001).

A number of cellulose-based films (MC, HPMC) containing antimicrobials have been produced and exhibited good antimicrobial properties. Cellulose-based inserts immobilized with nisin (7650 AU/ cm^2) placed between cheese slices packaged under MAP at 4°C, decreased the levels of *L. innocua* and *S. aureus* by 4 and 1.8 log CFU/g, respectively, in 3 weeks, while those placed between cooked ham slices decreased the same organisms by 2 log CFU/g in 3 weeks (Scannell *et al.*, 2000). Control samples packaged just with MAP decreased numbers of the organisms to a much lesser extent ($\leq$ 1.5 log CFU/g). Ming *et al.* (1997) demonstrated that cellulosic casings coated with pediocin inhibited the growth of *L. monocytogenes* $\leq$3 logs on fresh turkey breasts, ham and beef after 12 weeks at 4°C, compared to uncoated casings. Hot dogs, surface inoculated with a cocktail of *L. monocytogenes* (10^5 log CFU/ml) and vacuum packaged in an antimicrobial pouch consisting of MC/HPMC (70/30% w/w) with nisin (10,000 or 7500 IU/ml) coated onto preformed barrier bags showed bacterial reductions to undetectable levels ($\leq$2.9 log CFU/package) during 60d storage at 4°C, while those containing 2500 IU/ml nisin had numbers reduced to the same levels until 28d (Franklin *et al.*, 2004). Hot dogs packaged in pouches that contained no nisin had *Listeria* numbers increased by ~4.5 log CFU/package.

However, because polysaccharide-based films are hydrophilic and have poor water barrier properties, many have attempted to introduce FAs (Coma *et al.*, 2001; Sebti and Coma, 2002) or to use cross-linking agents (Sebti *et al.*, 2003) to increase the hydrophobicity and hence WVP properties. However, in most cases this addition negatively affects the antimicrobial activity of the films. Nisin (1000 IU/ml) incorporated into HPMC films inhibited *L. innocua*, *S. aureus* and *M. luteus* on agar, however, films with added stearic acid (15% w/w) were not able to inhibit the bacteria

(Coma *et al.*, 2001; Sebti and Coma, 2002). The authors suggested that nisin and stearic acid interacted electrostatically and this decreased the activity of nisin in the films because nisin was not able to be released. Sebti and Coma (2002) observed that the addition of calcium (80 mg/L) to nisin and stearic acid prevented nisin and stearic acid to interact, freeing nisin and allowing it to cause inhibition. Higher concentration of nisin (5000 IU/ml) added into HPMC films produced inhibition zones against *L. monocytogenes* and *S. aureus* that were 70 and 40% larger, respectively, than those produced by films containing nisin (5000 IU/ml) and stearic acid (15% w/w) (Sebti *et al.*, 2002). Although films containing stearic acid had increased moisture barrier properties they also had decreased TS, Young's modulus and ultimate elongation. Furthermore, it was observed that reducing the pH of HPMC films (containing nisin and stearic acid) to pH 3 produced better antimicrobial films than neutral films since low pH minimized interactions between nisin and stearic acid and improved the release of nisin (Sebti *et al.*, 2002). Cross-linked HPMC films with citric acid (15% w/w) containing nisin (1000 to 100,000 IU/ml) had increased hydrophobicity, however they had no antimicrobial activity against *M. luteus* on agar compared to a nisin solution used alone or compared to control HPMC films (containing nisin but not cross-linked) (Sebti *et al.*, 2003). The authors suggested the activity of nisin must have been reduced due to the denaturation by high concentration of the cross-linking agent or by high temperatures used to induce chemical modification. Furthermore, nisin might have been tightly bound to the cross-linked HPMC films, preventing its antimicrobial action. It was also observed that if HPMC films were first cross-linked and then nisin was added, the films exhibited antimicrobial activity and increased hydrophobicity (Sebti *et al.*, 2003).

Chitosan-based films have also been used to carry antimicrobial agents since they display a good range of inhibitory activity and possess good film characteristics. Antimicrobial films produced with chitosan mixed with 4% (w/w) MC and containing 4% (w/w) sodium benzoate or potassium sorbate exhibited clear inhibition zones on assay plates against *Penicillium notatum* and *Rhodotorula rubra* (Chen *et al.*, 1996). However, simple chitosan films with added sodium benzoate or potassium sorbate did not inhibit the organisms because high compatibility between the preservatives and chitosan did not allow diffusion to occur. Ouattara *et al.* (2000a) produced chitosan films with acetic or propionic acid and with or without the addition of lauric acid or cinnamaldehyde, and applied them on the surface of processed meat slices (bologna, regular cooked ham and pastrami) that were vacuumed packaged and stored at 4°C for 21 days. Cinnamaldehyde was seen to be more inhibitory than lauric acid when added into either acetic or propionic antimicrobial films. In addition, films containing acetic acid were more effective in inhibiting inoculated *Serratia liquefaciens* and indigenous *Enterobacteriaceae* than propionic acid films during storage, even though the release of propionic acid was observed to be more complete than the release of acetic acid on the surface of the meat. Chitosan film-forming solutions (8 and 10% v/v) inhibited *L. monocytogenes* and *L. innocua* on solid and liquid media for 6d, while stand-alone chitosan films showed no inhibitory activity against the organisms (Coma *et al.*, 2002). In addition, chitosan solutions coated on emmental cheese samples inoculated with *L. innocua* and stored for 36h at 37°C, reduced the organism by 10 fold and after 132h still did not show evidence of the organism. Rodríguez *et al.* (2003) demonstrated that chitosan (0.079g/100g pizza) dissolved in acetic acid and coated on precooked pizza displayed antifungal activity against *Alternaria*, *Penicillium* and *Cladosporium* species,

however, it did not inhibit fungal growth when used as an ingredient in the pizza dough. Furthermore, it was shown that the inhibition caused by the chitosan coating was similar to the inhibition caused by calcium propionate (0.103g/100g pizza) and potassium sorbate (0.034g/100g pizza) used as preservatives in pizza dough.

A starch (amylomaize) coating containing sorbitol and 0.2 g/l potassium sorbate decreased yeast/mold counts by 2.3 log CFU/g and minimally decreased mesophilic bacteria on coated strawberries, while amylomaize coatings containing sorbitol, potassium sorbate and citric acid had synergistic effects, decreasing yeast/mold and mesophilic bacteria counts by 3.8 and 4.5 CFU/g, respectively, compared to uncoated strawberries (García *et al.*, 2001). In addition, both coatings extended the shelf-life of strawberries for 28d.

2.5.5.1.3. Protein-based antimicrobial films

Antimicrobial protein films such as corn zein (Vojdani and Torres, 1990; Hoffman *et al.*, 1998, 2001; Padgett *et al.*, 1998, 2000; Dawson *et al.*, 1997; Janes *et al.*, 2002), soy protein (Padgett *et al.*, 1998; Dawson *et al.*, 1997, 2002; Eswaranandam *et al.*, 2004) and WPI films (Cagri *et al.*, 2001, 2002, 2003; Ozdemir and Floros, 2003; Ouattara *et al.*, 2002) have also been examined for their ability to inhibit bacterial growth.

Vojdani and Torres, (1990) observed that potassium sorbate incorporated in corn zein films decreased numbers of viable *S. aureus* cells at the surfaces of model food systems. Corn zein films containing 3.6% (w/w) nisin produced larger inhibition zones against *Lactobacillus plantarum* on MRS plates compared to polyethylene (PE) films containing the same concentration of nisin (Hoffman *et al.*, 1998). It was also shown that

in PE films, a higher nisin concentration was necessary to demonstrate greater bacterial inhibition. Lower nisin activities in PE films were attributed to the high processing temperatures used in the production of these films (137°C) compared to temperatures used in the production of corn zein films (100°C) (Hoffman *et al.*, 1998). Padgett *et al.* (1998) produced soy protein and corn zein films containing nisin (6 or 40 mg/g of film) or lysozyme (67 or 140.9 mg/g of film), or containing nisin plus EDTA or lysozyme plus EDTA, using cast and heat-pressed methods. It was observed that casting methods produced films with larger inhibitory zones compared to heat-pressed films. Nisin added into heat-pressed soy protein films produced inhibition zones on agar inoculated with *L. plantarum* that were clearer than the zones produced by the same film types containing lysozyme (Padgett *et al.*, 1998). Heat-pressed corn zein films containing nisin inhibited *L. plantarum*, while those containing lysozyme did not. However, lysozyme incorporated into cast corn zein films inhibited the organism on agar and lysozyme plus EDTA in the same film type effectively inhibited *E. coli*. On the other hand, lysozyme or nisin plus EDTA incorporated into heat-pressed soy protein or corn zein films did not inhibit *E. coli* (Padgett *et al.*, 1998). It was suggested that the temperatures used in heat-pressed methods, may have caused the antimicrobials to lose some activity. Corn zein and soy films containing nisin and lauric acid have inhibited Gram+ bacteria in liquid and solid media, with increased inhibition present in liquid media (Dawson *et al.*, 1997). Padgett *et al.* (2000) demonstrated that corn zein films containing nisin (5 mg/g film) or those containing nisin plus lauric acid (0, 4 and 8% w/w) inhibited *L. plantarum* on MRS plates and in peptone water, however films containing only lauric acid did not inhibit the organism. However, Hoffman *et al.*, 2001, showed that corn zein films containing lauric acid or nisin decreased *L. monocytogenes* in broth by 5 and 5.5 log

CFU/ml, respectively, while zein films containing EDTA or no added agents did not inhibit the organism. In addition, corn zein films containing EDTA plus lauric acid; EDTA plus nisin or those containing lauric acid plus nisin or films containing all three agents decreased the *Listeria* population by 8 log CFU/ml in 12h; 5.7 log CFU/ml in 48h and to undetectable levels ($\leq 10^2$) in 12h, respectively (Hoffman *et al.*, 2001). On the other hand, Hoffman *et al.* (2001) observed that none of the antimicrobial films containing only one agent inhibited a 8 log CFU/ml *S.* Enteritidis population. However, when the bacterial population was decreased by half, films containing EDTA plus nisin; EDTA plus lauric acid and films containing all 3 antimicrobials inhibited the bacteria, with the latter two exhibiting a 5 log CFU/ml reduction compared to the control films.

Corn zein coatings applied on ready-to-eat chicken inoculated with *L. monocytogenes* were observed to be more inhibitory at 4°C than at 8°C, and all corn zein coatings (dissolved in propylene glycol or in ethanol) containing nisin (1000 IU/g) inhibited the organism at both temperatures (Janes *et al.*, 2002). When inoculation levels of 6.8 log CFU/g were used, corn zein films dissolved in propylene glycol or in ethanol with added nisin reduced *L. monocytogenes* by ~3 log CFU/g on ready-to-eat chicken after 24d at 4°C (Janes *et al.*, 2002). The authors also observed that the antimicrobial effectiveness of the coatings was increased when calcium propionate (1%) was added.

Eswaranandam *et al.* (2004) determined the antimicrobial activity of soy protein films containing organic acids (2.6% w/w) and nisin (205 IU/g protein) by inoculating *L. monocytogenes*, *E. coli* 0157:H7 or *Salmonella gaminara* onto each type of film, stomaching, and plating the suspensions. *S. gaminara* was shown to be the most sensitive and numbers were reduced by soy protein films containing malic and lactic acids with nisin (3.3 and 5.6 log CFU/ml, respectively) and without nisin (6 and 5.8 log

CFU/ml, respectively). *E. coli* was inhibited by films containing malic acid without the addition of nisin (2.1 log CFU/ml) and by films containing lactic acid with (1.9 log CFU/ml) and without nisin (1.6 log CFU/ml) (Eswaranandam *et al.*, 2004). *L. monocytogenes* was inhibited by soy protein films containing malic acid without the addition of nisin (2.8 log CFU/ml). In general, it was observed that the addition of nisin decreased the action of the acids within the film against the organisms.

Soy protein films containing nisin (4% w/w), lauric acid (8% w/w) or a combination of the two agents inhibited the growth of *L. monocytogenes* in peptone water at room temperature for the first 8h. They decreased the numbers from 6 to 0.5 log CFU/ml after 48h and reduced numbers to undetectable levels ($\leq$100 CFU/ml) after 48h, respectively, while control films with no added antimicrobials had increased numbers by 3 log CFU/ml after 48h (Dawson *et al.*, 2002). When the films were applied on the surface of turkey bologna, control films slightly decreased the bacteria (< 0.05 log CFU/ml), while films containing lauric acid and those containing nisin or nisin plus lauric acid decreased the population by 0.5 and 1 log CFU/ml, respectively, after 21d at 4°C (Dawson *et al.*, 2002).

Carlin *et al.* (2001) studied the effects of various edible protein (corn zein and wheat), lipid (palm and coconut oil) and wax-based coatings with or without the addition of sorbic acid (1 mg/g of food) on cooked sweet corn stored at 10°C for 8d, against *L. monocytogenes*. It was observed that lipid-based films did not adhere properly to the kernels and that gluten-based films were unstable, however zein-based coatings had good appearance, adherence and thickness. The kernels coated with corn zein (without sorbic acid) had bacterial counts of 1.4 log CFU/g less than kernels without a coating. In addition, it was shown that sorbic acid solutions or sorbic acid added into zein coatings

applied on the kernels displayed similar antimicrobial activities, with either treatment almost completely inhibiting the bacteria.

Low pH (5.2) WPI films containing *p*-aminobenzoic acid (PABA) (1.5% w/w) produced average inhibition zones of ~22, 15 and 14 mm (dia) on *L. monocytogenes*, *E. coli* 0157:H7 and *S.* Typhimurium bacterial lawns, respectively, while films containing sorbic acid (1.5% w/w) produced average inhibition zones of ~27, 11 and 10 mm, respectively, against the same bacteria (Cagri *et al.*, 2001). Control films (containing no antimicrobials), on the other hand, did not inhibit the bacteria.

In a later study, the films were examined for their activity against the same organisms on commercially produced bologna and summer sausage slices (Cagri *et al.*, 2002). Both PABA and sorbic acid WPI films inhibited populations of *L. monocytogenes*, *E. coli* 0157:H7 and *S.* Typhimurium by 3.4 to 4.1, 3.1 to 3.6 and 3.1 to 4.1 log CFU/g, when placed between inoculated bologna and summer sausage slices, respectively, after 21d storage at 4°C (Cagri *et al.*, 2002). In addition, bacterial counts on meat samples wrapped with WPI films containing no antimicrobials remained unchanged or minimally decreased compared to samples not exposed to the films (Cagri *et al.*, 2002). Furthermore, both PABA and sorbic acid WPI films inhibited mesophilic aerobic bacteria (MAB), yeasts, molds and lactic acid bacteria (LAB), while these organisms increased on samples exposed to films without antimicrobials or in samples not in contact with a film (Cagri *et al.*, 2002).

Cagri *et al.* (2001) also showed that increasing the concentration of PABA or sorbic acid from 0.5 to 1.5% w/w in WPI films, decreased the film's TS and increased the %E. In addition, the WVP of the films increased when agent concentrations were 1 to 1.5%, however when the agents were added at concentrations $\leq$0.75%, the WVP of

the film was not affected. Furthermore, after 24h of film contact with the meat products, the TS also decreased and the %E also increased, however, neither the TS or %E changed between 24 and 72h (Cagri *et al.*, 2002).

In another study, Cagri *et al.* (2003) showed that hot dogs surface-inoculated with *L. monocytogenes* (10^3 CFU/g), vacuum packaged in WPI casings containing PABA (1% w/v) and stored at 4°C for 42d had bacterial numbers relatively unchanged, compared to control WPI (without antimicrobials), commercial collagen or natural casings which allowed *Listeria* numbers to increase (~2.5 log CFU/g). In addition, although mold, MAB and LAB increased with the use of control WPI, commercial collagen and natural casings, WPI casings containing PABA suppressed the growth of mold, MAB and LAB by 0.5 to 1.4, 1 to 2.5 and 1.7 to 2.2 log CFU/g, respectively more than the other casings.

WPI films cross-linked with calcium caseinate were made with the addition of a mixture of spice powders (3% w/w; thyme, rosemary and sage) by Ouattara *et al.* (2002). The film solutions after heating at 90°C for 30 min were coated on ground beef with or without 0.5% (w/w) ascorbic acid and half of the treatments were gamma irradiated at 1-3 kGy and all were stored at 4°C. Unirradiated ground beef samples containing no ascorbic acid displayed a 0.5 log CFU/g increase in total aerobic numbers, while unirradiated samples containing ascorbic acid or ascorbic acid plus the antimicrobial coating displayed a 0.5 log CFU/g decrease after 7d (Ouattara *et al.*, 2002). However, the irradiation process in itself was more inhibitory and it was not enhanced with the use of ascorbic acid or the antimicrobial coating. Irradiation decreased the total aerobic bacterial numbers, total coliforms, *Enterobacteriaceae*, pseudomonads, *B. thermosphacta*, presumptive LAB and *S. aureus*.

2.5.5.2. Antimicrobial synthetic films

Nisin, lysozyme, EDTA and PP have also been incorporated into synthetic films (Natrajan and Sheldon, 1995, 2000b; Sheldon *et al.*, 1996; Siragusa *et al.*, 1999; An *et al.*, 2000; Chung *et al.*, 2001; Cutter *et al.*, 2001; Kim *et al.*, 2002; Dobiás *et al.*, 2000; Grower *et al.*, 2004a, 2004b; Cha *et al.*, 2003)

Nisin incorporated into polyethylene-based films produced inhibition zones on *B. thermosphacta* inoculated streptomycin thallous acetate actidione (STAA) agar plates (Cutter *et al.*, 2001). As well, beef cubes surface inoculated with the same organism and wrapped with low density polyethylene (LDPE) films impregnated with nisin (0.1% w/v), vacuum packaged and stored at 4°C for 20d had *B. thermosphacta* counts reduced on average by 2 log CFU/cm^2 more than untreated LDPE films (Siragusa *et al.*, 1999).

An antimicrobial solution containing 300 μg/ml nisin, 5 mM EDTA, 3% citric acid and 5% Tween 20 coated on polyvinyl chloride (PVC) films reduced the growth of *S.* Typhimurium ≤4.3 log CFU/cm^2 on inoculated fresh broiler skin stored at 4°C for 72h (Natrajan and Sheldon, 1995). When nisin at 50 μg/ml was used with EDTA, citric acid and Tween 20 at the concentrations noted above, applied on absorbent pads and included with raw poultry meat in packages inoculated with *Salmonella*, the pathogen was reduced by 3-6 log CFU/ml compared to numbers in packages containing untreated absorbent pads (Sheldon *et al.*, 1996).

PVC, linear LDPE or nylon packaging films coated with an antimicrobial solution containing 100 μg/ml nisin and 3% citric acid with different concentrations of EDTA (5 or 7.45 mM) and Tween 80 (0.5% or 0.61%) and used to wrap fresh broiler drumstick skin inoculated with *S.* Typhimurium inhibited the organism during storage for 24h at 4°C (Natrajan and Sheldon, 2000b). The authors observed that the longer the

film contacted the product, the greater was the inhibition. In addition, the shelf-life of broiler drumsticks was extended by ~2d when they were dipped in the antimicrobial solution, put into a tray pack with a nisin-treated absorbent pad and over-wrapped with the nisin-treated PVC film.

Furthermore, nisin (1% w/v) incorporated in a polyamide binder and coated on LDPE films suspended in phosphate buffer inhibited *M. flavus* by 6 log CFU/ml and reduced *L. monocytogenes* by ~0.6 CFU/ml compared to uncoated LDPE after 7d (An *et al.*, 2000). In addition, when used to wrap fresh oysters and ground beef, nisin (2% w/v) suppressed the total aerobic and coliform bacterial numbers when the products were stored at 3 or 10°C, improved product quality and extended shelf-life better than plain LDPE (Kim *et al.*, 2002). The shelf-life of oysters was extended from 10 to 12d at 3°C and from 5 to 12d at 10°C, while ground beef shelf-life was extended from 9 to 13d at 3°C and 5 to 9d at 10°C, compared to uncoated LDPE films (Kim *et al.*, 2002).

Propyl paraben (5 g/kg) added to LDPE pellets and extruded into films was released from the film into olive oil during preliminary tests, but the film was not able to decrease surface mold growth when applied on cheese or sliced bread at 6°C (Dobiás *et al.*, 2000).

Nisin dissolved in acetic acid, incorporated into MC or HPMC solutions and then coated onto LDPE films to contain $\leq$10,000 IU nisin/cm^2, inhibited *L. monocytogenes* when the films were placed in peptone water at 25°C. However, nisin incorporated into MC or HPMC solutions was more effective against the organism than when these solutions were coated on the LDPE film. For example, LDPE films coated with either MC or HPMC containing 10,000 IU/cm^2 nisin produced inhibition zones similar to MC or HPMC solutions containing 2500 IU/ml nisin (Grower *et al.*, 2004b).

2.6. Summary

Consumers demand better quality foods with fewer additives. Packaging technologies have led to the development of antimicrobial films to delay microbial proliferation and to extend product shelf-life. In particular, edible antimicrobial films seem to have greater consumer appeal. In addition, these films have the added feature of being biodegradable and may decrease packaging disposal problems. WPI films exhibit the characteristics necessary to behave as satisfactory packaging materials, can contain antimicrobials and retain their activity. Nisin, lysozyme, EDTA and propyl paraben have been used alone or in combination as antimicrobial solutions on food products. It has been reported that they inhibit a variety of spoilage and pathogenic Gram+ and Gram- organisms. More recently, these antimicrobials have been formulated within edible films which have been applied on food surfaces, successfully suppressing microbial growth. Future progress in the development of antimicrobial films for use on food requires an understanding of factors affecting target bacterial vulnerability and chemical compatibility among the antimicrobial agents, their film carriers and the food to be protected. Further work is necessary to fully understand and optimize antimicrobial effects of edible antimicrobial films or coatings in order for them to be effective in inhibiting the complex microflora present in the food matrix.

CHAPTER # 3

Activity of Antimicrobial Whey Protein Isolate Films against Spoilage and Pathogenic Bacteria

3.1. Abstract

Edible antimicrobial films were produced by incorporating antimicrobial agents into whey protein isolate (WPI). To determine the antimicrobial activity, disc-shaped films were placed on bacteria-inoculated agar plates and/or tubes and the clear inhibition zones were measured in the horizontal and vertical directions (adjacent to, or underneath the film discs, respectively). To further quantify and determine the extent of action of the antimicrobials, bacterial counts were performed on the agar areas showing inhibition zones.

Nisin incorporated into acidic WPI films inhibited Gram+ microorganisms better than in neutral films. On the other hand, nisin incorporated into neutral WPI films better inhibited *S.* Typhimurium, and nisin in either type of film had similar effects against *E. coli* 0157:H7. Lysozyme added to neutral films was more effective than when formulated in acidic films against all bacteria except for *L. monocytogenes*. Neutral or acidic EDTA films did not inhibit *B. thermosphacta* or *L. monocytogenes*, however, they inhibited *S.* Typhimurium and *S. aureus* by ~0.7 and 1 log CFU/g, respectively. In addition, *E. coli* 0157:H7 was more effectively inhibited by EDTA incorporated into neutral films. Propyl paraben (PP) in neutral or acidic films had equivalent effects on *B. thermosphacta*, *S. aureus* and *S. Typhimurium*, while PP incorporated into neutral films inhibited *E. coli* 0157:H7 to a greater extent.

Further studies are required to optimize the edible antimicrobial film formulations to create an effective packaging system to control spoilage of food products and microbial populations of concern.

3.2. Introduction

Packaging materials are selected to protect food products from moisture, light, gas, odours, microorganisms, rodents and insects. In addition, they allow for convenience. The shelf-life and quality of food products are very dependent on the packaging properties. However, food packaging materials create about 30% by wt of municipal solid waste (Krochta and Mulder-Johnston, 1997) and this has become a concern. Of packaging waste, 13% is due to plastic materials. Although plastic materials are one of the more convenient, safe, strong and economical packaging materials, plastics resist biodegradation. This has led researchers to create improved biodegradable packaging systems for foods, some of which are edible.

Edible films are thin layers of edible material formed on a food product as a coating or are preformed separately and applied on or between food components (Krochta and Mulder-Johnston, 1997). In general, most edible films have good barrier properties to gases such as O_2 and CO_2, odours and especially to water vapour, preventing moisture loss or uptake. They are also used to inhibit fat and oil migration, particularly in the confectionery industry (Donhowe and Fennema, 1994) and are used to help maintain food quality once the package has been opened (Krochta and Mulder-Johnston, 1997). In addition, these films have the ability to carry food ingredients such as flavours, antioxidants and antimicrobials (Donhowe and Fennema, 1994). The properties of edible films allow the production of foods that are safe, stable, economical

and which have improved nutritional profile (Donhowe and Fennema, 1994). These films have the potential to become an important alternative approach to reduce packaging waste.

In the cheese manufacturing industry liquid whey is a waste by-product that has created a serious disposal problem since it is produced in abundant quantity (Gennadios *et al.*, 1994). Utilizing whey in the production of edible films can lessen the waste problem and create a useful and economical product. Commercially, whey is converted into whey protein concentrate (WPC) and whey protein isolate (WPI), both of which have been extensively studied for their value in producing edible films (McHugh *et al.*, 1994; McHugh and Krochta, 1994a, 1994b; Banerjee and Chen, 1995; Maté and Krochta, 1996, 1998; Shellhamer and Krochta, 1995; Pérez-Gago and Krochta, 1999; Miller *et al.*, 1997, 1998; Guilbert *et al.*, 1996; Gontard *et al.*, 1996; Anker, 2000).

In the last few years new developments have arisen in the field of edible films. Antimicrobials are increasingly being incorporated into film-forming solutions to reduce the growth of spoilage and pathogenic bacteria as well as yeast and mold, and extend product shelf-life (Han, 2000; Padgett *et al.*, 1998). The antimicrobials in the films can directly inhibit microbial surface growth or can migrate through the initial surface layers of the food product and reduce microbial proliferation (Han, 2000). Many antimicrobials such as sorbic acid, potassium sorbate, benzoic acid, lysozyme, nisin, ethylenediaminetetraacetic acid (EDTA) and propyl paraben (PP) have been used (Han, 2000; 2003; 2005; Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002; Cagri *et al.*, 2004).

Brochothrix (B.) thermosphacta, Listeria (L.) monocytogenes and *Staphylococcus (S.) aureus* are facultative anaerobic gram positive (Gram+)

microorganisms. Under refrigerated conditions and limited amounts of O_2, *B. thermosphacta* can grow and spoil fresh and cured meat products very quickly. It is also the principal spoilage organism on packaged meats (Tu and Mustapha, 2002) that have not been adequately vacuum packaged and refrigerated. *L. monocytogenes* is a pathogenic organism. It is capable of growth at high salt concentrations, at temperatures from 1-45 °C over a range of pH values (Bower *et al.*, 1995; Crandall and Montville, 1998) and is a major concern as a post-processing contaminant (Payne *et al.*, 1989). It is also frequently found on ready-to-eat poultry and meat products (Ming *et al.*, 1997) and has been linked to listeriosis outbreaks from consumption of *L. monocytogenes* contaminated foods (Liberti *et al.*, 1996). *S. aureus* is also a pathogenic organism which can cause food intoxication upon ingestion of thermostable toxins which may be present in contaminated foods. *Escherichia (E.) coli* O157:H7 and *Salmonella (S.)* Typhimurium are facultative anaerobic gram negative (Gram-) microorganisms and are foodborne pathogens. *Salmonella* are principally associated with poultry products which are considered to be the leading source of *Salmonella* in foodborne illnesses (Natrajan and Sheldon, 2000a). *E. coli* O157:H7 has been associated with contaminated undercooked beef or improperly processed dairy products and has caused major foodborne illness outbreaks involving death (Zhang and Mustapha, 1999). These organisms were chosen for study here because they are significant contributors to food spoilage and outbreaks of foodborne illness (Bower *et al.*, 1995).

Nisin and lysozyme are antimicrobials that exhibit their action by interacting with the bacterial cell membrane or cell wall (Padgett *et al.*, 1998). Nisin is a small biologically active cationic peptide produced by *Lactococcus lactis* subspecies *lactis* (Winkowski *et al.*, 1996) that disrupts the cell membrane (Bower *et al.*, 1995; Lück and

Jager, 1997; Padgett *et al.*, 1998). It is suggested that nisin causes pore formation in the cytoplasmic membrane following contact (Bower *et al.*, 1995). Lysozyme (1,4-ß-N-acetylmuramidase) is an enzyme that lyses the bacterial cell wall (Lück and Jager, 1997; Payne *et al.*, 1994) by cleaving the glycosidic linkages of the peptidoglycan which is responsible for maintaining cell wall rigidity (Payne *et al.*, 1994). These compounds have little antimicrobial action against Gram- bacteria which possess an outer membrane (OM) that affords protection from their activity. However, they are effective against Gram+ bacteria. In contrast, EDTA and PP have been shown to be active against Gram- bacteria (Razavi-Rohani and Griffiths, 1996; Payne *et al.*, 1994). Chelating agents such as EDTA alter the OM structure of Gram- bacteria by affecting cations such as magnesium and calcium (Padgett *et al.*, 1998). When these cations are chelated from the OM, the membrane becomes destabilized (Boziaris and Adams, 1999) leading to cell injury or death (Padgett *et al.*, 1998). The antimicrobial PP is effective against both Gram- and + bacteria, however it is most inhibitory against Gram+ bacteria and fungi. Parabens inhibit membrane transport of electrons, essential amino acids and nutrients (Baranowski and Nagel, 1983; Lück and Jager, 1997).

In the present study we determined the effectiveness of neutral and acidic antimicrobial WPI films containing single antimicrobials (nisin, lysozyme, EDTA or PP) against the above mentioned organisms in agar plates. Growth at the edge of the film (horizontal) and underneath the film (vertical) was measured and bacteriostatic or bactericidal effects of the films were evaluated. Vertical inhibition was determined since in some instances it was difficult to determine the relative inhibitory effect of the films in the conventional disc agar tests because the film itself obscured the measurements of the clear zones, or the inhibition seemed to be only in the vertical direction.

3.3. Materials and Methods

3.3.1. Formation of neutral and acidic film solutions

Whey protein isolate films were produced using a method adapted from McHugh *et al.* (1994). Aqueous solutions of 10 % (w/w) whey protein isolate (WPI) supplied by Davisco Foods International Inc. (Le Sueur, MN, USA) were prepared with the addition of deionized water and glycerol (GLY) (Sigma Chemical Co., St. Louis, MO, USA), as the plasticizer. The plasticizer was added to the protein and water solution in different amounts to yield ratios of 60:40, 65:35 and 70:30 (w:w) of WPI:GLY.

The solutions were gently stirred using a magnetic bar to dissolve the protein, heated at 90°C for 30 min in a water bath to denature the protein and cooled to about 15°C.

Two film-forming solutions were produced, one with neutral pH and the other was acidic. Neutral (pH 7.09 ± 0.05) film-forming solutions were used without any pH modification, however those for the acidic tests were adjusted to pH 3.02 ± 0.05 after cooling, using 1M HCl monitored with a pH meter (Accumet model 910, Fisher Scientific).

To produce the neutral or acidic antimicrobial film solutions, the antimicrobial agents (nisin, lysozyme, EDTA and PP) were added individually from stock solutions in different concentrations to each of the three WPI:GLY combinations. Control films for both pH conditions were produced without the addition of the antimicrobials.

3.3.2. Formation of antimicrobial stock solutions

The antimicrobials were individually dissolved in deionized water to produce the desired antimicrobial stock solutions. Lysozyme (> 97% pure) and nisin (30 % pure) were both provided by Canadian Inovatech Inc. (Abbotsford, BC). Nisin was added to the film-forming solutions at levels of 0, 1, 2, 3 or 4 mg/g WPI in the film. Lysozyme was added to the film-forming solutions at levels of 0, 25, 50, 75 or 100 mg/g WPI in the film for experiments using *B. thermosphacta* (neutral films using a spread plate method) and 0, 10, 20 or 30 mg/g for the remaining experiments. Ethylenediaminetetraacetic acid (EDTA) and n-propyl p-hydroxy benzoate (propyl paraben; PP) were both purchased from Sigma Chemicals Co. (St. Louis, MO, USA). EDTA was added at concentrations of 0, 10, 20 or 30 mM per kg protein and PP was added to yield 0, 300, 600 or 900 ppm.

3.3.3. Formation of neutral and acidic antimicrobial films

After the film-forming solutions were produced (control and antimicrobial; neutral and acidic), they were cast by pipetting the solutions (8.2 ml) into individual poly methylpentene petri dishes (100 x 15 mm; VWR Int., Mississauga, ON) placed on a level surface. The dishes were left to dry in a desiccator at 22-24°C using calcium nitrate tetrahydrate (Sigma Chemicals Co.) as a desiccant to produce a relative humidity (RH) of 50%. After 72h the dried films were peeled intact from the plate and put into another desiccator (50% RH) for further drying and storage. The thickness of the films was measured with a caliper micrometer (B.C. Ames Co., Waltham, MA, USA). All experiments were performed in triplicate with duplicate measurements. The day prior to the antimicrobial tests, circular discs were cut from the various films using a 1cm diameter cutting tool and kept separately.

3.3.4. Bacterial culture maintenance

The test microorganisms (*Brochothrix thermosphacta* B2, *Listeria monocytogenes*, *Escherichia coli* O157:H7 E318, *Salmonella* Typhimurium 98 and *Staphylococcus aureus* 427) were obtained from the Department of Food Science culture collection at the University of Manitoba. Cultures were transferred monthly and maintained on Brain Heart Infusion (BHI, Difco, Becton Dickinson Co. Sparks, MD, USA) agar slants at 4°C. For experimental use, cultures were streak-plated onto BHI plates once a week and a single colony of each bacterium from the BHI agar plates was inoculated in 10 ml BHI broth and incubated overnight at room temperature. From the incubated broth, BHI agar plates were re-streaked and re-incubated to ensure purity of the culture. Organisms were incubated at 35-37°C except for *B. thermosphacta* which was cultured at room temperature.

A 50 ml BHI broth culture of each bacterium was prepared by transferring a loopful of growth from pure culture plates to the broth and incubating overnight at room temperature. Following incubation the culture was adjusted to contain an approximate bacterial population of 10^8 colony forming units per ml (CFU/ml). This was done by taking 30 ml of the bacterial culture and centrifuging at 5500 rpm for 10 min, at 15°C (Sorvall RC2-B refrigerated centrifuge, Du Pont, Newtown, CT). The supernatant was discarded and the pellet re-suspended in 30 ml of autoclaved deionized water and re-centrifuged at 5500 rpm for 10 min at 15°C. The pellet was then re-suspended in sterile deionized water and diluted to an optical density (OD) of 1.0 at 600 nm (Ultrospec 2000, Pharmacia Biotech Inc., Baie D'Urfe, QC). The approximate bacterial population at this OD was 10^8 CFU/ml.

3.3.5. Horizontal inhibition assays of antimicrobial films

To determine the clear horizontal inhibition zones on inoculated plates, 0.2 ml from the adjusted individual cultures (~10^8 CFU/ml) was used to inoculate BHI plates and create a surface lawn by spread plating or used to create confluent growth throughout the agar by pour plating.

After agar solidification, the film discs were placed on the inoculated agar plates and incubated at the appropriate temperatures for 48h. After incubation, the plates were examined for the presence of clear zones around the film disc on the inoculated media. The inhibitory activity (Fig. 3.1) was calculated by measuring zones with a metric caliper and readings were made in duplicate.

Horizontal inhibitory activity (mm) was calculated as: (diameter of the clear zones – diameter of the circular film) / 2

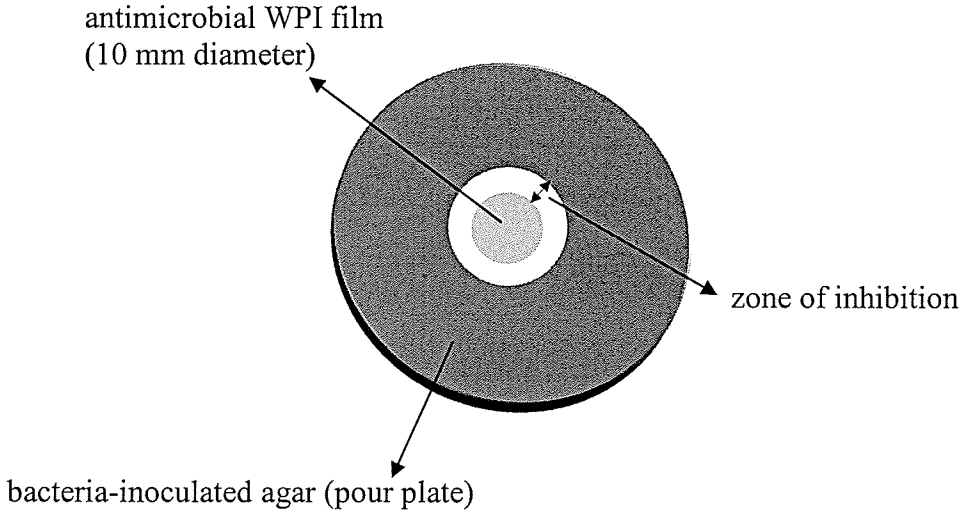

Figure 3.1. Diagram showing assessment of inhibitory activity (mm)

3.3.6. Vertical inhibition assays of antimicrobial films

The zones of inhibition underneath the film discs were measured to test the ability of the antimicrobials to inhibit bacterial growth in the vertical direction. For organisms that did not produce gas (*B. thermosphacta*, *L. monocytogenes*, and *S. aureus*), 0.2 ml from individual cultures (~10^8 CFU/ml) was transferred to molten BHI agar, mixed and poured into sterile clear polystyrene tubes with dual-position polyethylene caps to permit sample aspiration (1 cm dia., 5 cm^3; VWR Int., Mississauga, ON). Once the inoculated agar was solidified, the antimicrobial film discs were placed on top of the inoculated agar and incubated at the appropriate temperature for 48h. After incubation, tubes were examined for the presence of clear zones under the film disc on the inoculated agar. The inhibitory activity was then calculated. The experiment was performed in triplicate and all measurements were made using a metric caliper and read in duplicate.

Vertical inhibitory activity (mm) was calculated as: the clear distance measured from the bottom of the antimicrobial disc to the margin where bacterial growth was evident.

For the other organisms (*E. coli* O157:H7 and *S.* Typhimurium) that produced gas, 0.2 ml from individual cultures (~10^8 CFU/ml) was transferred to molten BHI agar that was then poured 1cm thick into petri-plates (100 x 20 mm; Fisher Scientific Ltd., Nepean, ON) and allowed to solidify. Once the inoculated agar plates solidified, antimicrobial discs were placed on the agar surface. To verify that the inhibition occurred in the vertical direction, agar plugs were cut with a sterile steel blade from the agar and placed vertically into empty sterile petri-plates and incubated at 37°C for 48h. (To prevent the agar plugs from drying the petri-plates were placed in a desiccator with

water at the bottom to keep the atmosphere humid). After incubation, the agar plugs were examined for the presence of clear zones under the film on the inoculated media. The vertical inhibitory activity (mm) was then measured as previously described. The experiment was performed in triplicate and duplicate measurements were made using a metric caliper.

3.3.7. Bacteriostatic or bactericidal effects of the 60:40 WPI:GLY antimicrobial films

Since inhibition zones on agar do not give quantitative results, an attempt was made to quantitatively calculate the inhibition of bacterial growth adjacent and beneath the film (in the horizontal and vertical direction, respectively) as well as determine the bacteriostatic or bactericidal effects of the films. Inoculated BHI agar plates with the overlaid circular antimicrobial films were prepared as in the horizontal experiments except that the agar plates were poured 1 cm thick, and inhibition was measured by counting bacterial survivors in the agar adjacent and beneath the film. The initial inoculated bacterial numbers in the agar were ~10^8 CFU/ml. After incubation, the antimicrobial film discs were removed aseptically with forceps in order to allow for the microbiological testing of the agar containing the clear zones. Two inhibition zones were tested depending on how the antimicrobial inhibition patterns occurred. The first area tested was the agar directly underneath the film disc (vertical determination) and the second was the agar around the antimicrobial film disc (horizontal determination) showing inhibition or clearing. In addition, as a control, a bacterial agar area not affected by the antimicrobial film disc was also tested. The agar plugs (0.25 g) were removed using a sterile exacto knife and added to BHI broth (2.5 ml) in a sterile stomacher bag

(~18 x 30.5 cm) (filtra-bag; VWR Int., Mississauga, ON) and stomached for 1min to produce a 10^{-1} dilution. Each sample was serially diluted with peptone water to produce 10^{-2} to 10^{-5} dilutions. Each dilution was plated in duplicate on BHI agar plates using an Autoplate 4000 equipped with a CASBA-4 automated counting system (Spiral Biotech, Inc., Bethesda, MD.), then incubated for the appropriate time and temperature. The calculated numbers of bacteria/g for the different plugs were then compared to the initial inoculated level to determine if the antimicrobials had a bactericidal or bacteriostatic effect. If the bacterial numbers recovered from test samples were lower than in the untreated agar area (agar plug not affected by a film disc) then the antimicrobial effect was considered bacteriostatic. However, if the numbers of bacteria in cleared zones of agar were lower than the initial inoculum level, then the effect was considered bactericidal.

Only the 60:40 WPI:GLY film discs were tested for effects on bacterial viability since the horizontal antimicrobial activity of the other two protein film types were not that different from each other and this protein concentration produced film characteristics that were more desirable that is, they were strong but flexible. Additional tests were performed based on the outcomes of horizontal inhibition; if no inhibition was evident viability experiments were not performed. If the clear zones around the films were too small to remove (< 2 mm), the tests were then only done in the vertical direction.

A total of four samples were tested; a control agar plug (not adjacent to a film disc), an agar plug beneath the film disc containing no antimicrobials (control film), an agar plug adjacent to an antimicrobial film disc containing the lowest concentration of

the antimicrobial and an agar plug near an antimicrobial film disc containing the highest concentration of the antimicrobial tested (Fig 3.2).

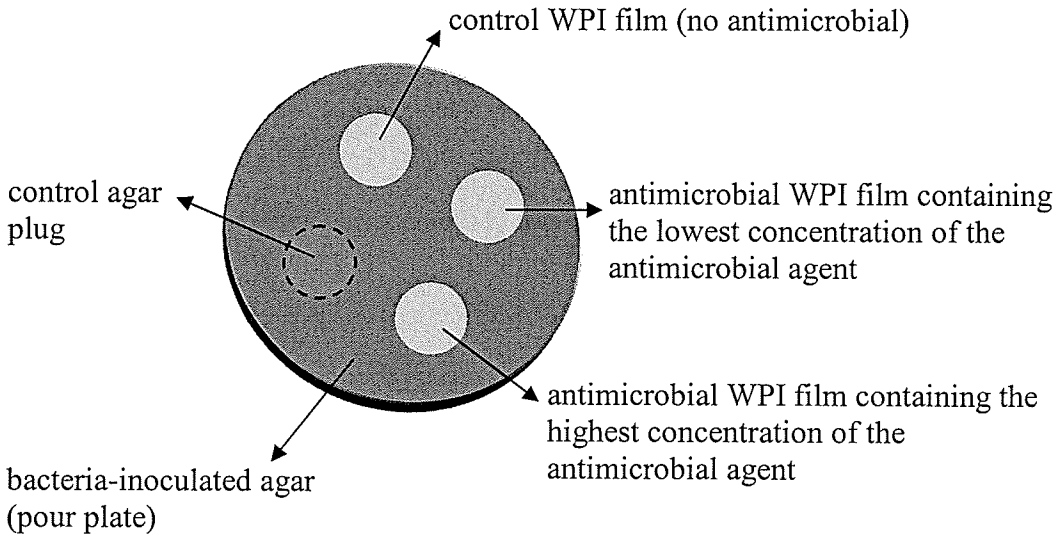

Figure 3.2. Diagram for quantification of bacterial inhibition

3.4. Results

Within the range of film ingredient and antimicrobial concentrations used, all films were transparent, relatively flexible, strong enough to be handled and maintained their integrity throughout the experiments. However in general, the 70:30 WPI:GLY antimicrobial films were more brittle than the other two film types. The thickness of the films was ~0.1 mm.

3.4.1. Spread versus pour plate evaluation of neutral antimicrobial films

To see if the antimicrobials caused inhibition only at the film contact area or if they diffused in the agar, spread versus pour plate methods were evaluated. When results in Tables 3.1 and 3.2 are compared it can be seen that all the antimicrobials had weak activity against *S. aureus*, *S.* Typhimurium and *E. coli* 0157:H7 with either method. The inhibition appeared visible only in the vertical direction. However, slight inhibition of *S. aureus* by nisin and *S.* Typhimurium by EDTA was found (Table 3.1). EDTA and PP inhibited *L. monocytogenes* at the film contact area (Table 3.1). Nisin and lysozyme inhibited *B. thermosphacta* (Tables 3.1 and 3.2) but EDTA only inhibited the organism at the film contact area as well (Table 3.1). Since pour plates provided more information, all other experiments were performed using this method.

3.4.2. Horizontal versus vertical inhibition of antimicrobial films

It was thought that the inhibitory effect of the films in the conventional disc agar tests (horizontal inhibition) could be characterized by the production of clear inhibition zones which could be measured. However, in some tests this was not the case. It was suspected that some antimicrobial agents were not able to migrate horizontally in the agar very well to be of value in measuring inhibition, and it was thought that measurements directly under the film might yield a more sensitive analysis. For this reason vertical inhibition tests were performed.

3.4.3. Inhibition zones produced by neutral versus acidic antimicrobial films

Several studies have shown that acidic environments increase the effectiveness of antimicrobial agents with pKa $\leq$6 (Harris *et al.*, 1991; Okereke and Thompson, 1996;

Datta and Benjamin, 1997; Ukuku and Shelef, 1997; Payne *et al.*, 1994; Parente *et al.*, 1998; Bouttefroy *et al.*, 2000; Razavi-Rohani and Griffiths, 1996; Thompson, 1994). Our results with pour plates agree with these findings, as seen in Tables 3.2-3.5. The acidic antimicrobial films produced substantially larger horizontal and vertical inhibition (Tables 3.3 and 3.5, respectively) compared to the neutral antimicrobial films (Tables 3.2 and 3.4, respectively). For example, the vertical inhibition zones against *B. thermosphacta* produced by films containing 4 mg/g nisin were on average 8.89 ± 0.12 mm compared to 6.02 ± 0.38 mm, for acidic and neutral films, respectively. However, this was not always seen with the quantitative studies reported in Figs. 3.3-3.11.

3.4.4. Effects of antimicrobial WPI films compared to the effects of control WPI films

Although it was expected that WPI films without antimicrobials (control films) would cause no inhibition of the organisms, in some cases they caused some inhibition (Tables 3.1, 3.2 and 3.3 and Figs. 3.3-3.11. This was not surprising, because the films restricted bacterial access to O_2. For example, neutral and acidic control films caused *S.* Typhimurium numbers to be reduced by ~0.3-1.3 and 0.6-0.9 log CFU/g, respectively, (Figs 3.8 and 3.9).

3.4.5. Antimicrobial effects against *B. thermosphacta*

Results obtained showed that nisin effectively inhibited *B. thermosphacta* (Tables 3.1-3.5). Results also indicated with this antimicrobial that the acidic films were more inhibitory than the neutral films and that the vertical inhibition zones were larger than the horizontal zones. This was also seen with the quantitative study (Figs 3.3 and

3.4), where nisin (4 mg/g) containing neutral films caused ~3.3 and 7.9 log CFU/g reductions in the horizontal and vertical directions, respectively, while complete inhibition ($\leq$2 log CFU/g) was achieved with the acidic films containing 1 and 4 mg nisin/g in both the horizontal and vertical directions. Lysozyme inhibited *B. thermosphacta* and although both horizontal and vertical qualitative tests (Tables 3.4-3.5) showed that the acidic films were more inhibitory than the neutral films, results in Figs 3.3 and 3.4 showed little difference between films containing lysozyme at different pH values. EDTA did not inhibit the organism. PP when tested horizontally (Tables 3.2 and 3.3), caused inhibition only beneath the film discs, however it was confirmed not to be inhibitory in the vertical tests (Tables 3.4 and 3.5) and very minimally inhibitory (~0.6 log CFU/g reduction) in the quantitative tests (Figs 3.3 and 3.4).

Our results suggest that nisin and lysozyme had both bactericidal and bacteriostatic effects; PP was mostly bacteriostatic and EDTA alone did not inhibit *B. thermosphacta* when the pour plate method was used.

3.4.6. Antimicrobial effects against *L. monocytogenes*

With the horizontal plate method it was shown that nisin was only inhibitory against *L. monocytogenes* when it was formulated into acidic films (Table 3.3). However, when the films were tested in the vertical direction, the acidic films exhibited good inhibition against the organism. Average vertical inhibition zones of ~3.6 $\pm$ 0.1 mm were achieved with the acidic films containing 4 mg/g nisin compared to ~1.5 $\pm$ 0.1 mm for the neutral films. It was also shown that the acidic films in the vertical direction caused a ~0.6 log CFU/g reduction (Fig 3.5). The remainder of the antimicrobials did not inhibit *L. monocytogenes* (Tables 3.2-3.5). However, using the spread plate method (Table 3.1)

it was shown that EDTA and PP inhibited *L. monocytogenes* at the contact surface. These agents may not be able to migrate with ease into the agar to inhibit confluent bacteria since no inhibition was seen with the pour plate method. Since the films containing lysozyme, EDTA or PP did not show inhibitory activity in the horizontal direction, no quantitative determinations were performed.

It appears that in this study nisin was the only agent that was effective against *L. monocytogenes*. It was bacteriostatic and may be bactericidal ≥ 4 mg/g.

3.4.7. Antimicrobial effects against *S. aureus*

The only antimicrobial that was inhibitory towards *S. aureus* was nisin (Tables 3.1-3.5). In the horizontal plate method, nisin incorporated into acidic films (Table 3.3) was able to slightly inhibit the organism. When the films were tested in the vertical direction both the neutral and the acidic films containing nisin were inhibitory (Tables 3.4 and 3.5). The acidic films containing 4 mg/g nisin caused stronger vertical inhibition than the neutral films containing the same concentration of nisin, with an average inhibition zone of ~2.0 ± 0.2 mm compared to ~0.5 ± 0.1 mm, respectively. This was also demonstrated with the quantitative study (Figs 3.6 and 3.7), where the acidic films in the vertical direction containing 4 mg/g nisin caused a ~1.5 log CFU/g reduction whereas a ~1.4 log CFU/g reduction was achieved with the neutral films. The neutral and acidic films containing, lysozyme, EDTA or PP (at their highest concentrations) showed similar inhibitory patterns (~1 log CFU/g reduction), when the agar area underneath the antimicrobial film with the highest concentration of the agent was compared with an agar area containing no film (Figs 3.6 and 3.7).

The antimicrobial effect of nisin on *S. aureus* was bacteriostatic < 4 mg/g but was bactericidal at 4 mg/g, whereas lysozyme, EDTA and PP had only bacteriostatic effects.

3.4.8. Antimicrobial effects against *S.* Typhimurium

The horizontal inhibition tests (Tables 3.2 and 3.3) showed that *S.* Typhimurium was only inhibited by agents just beneath the antimicrobial film discs. EDTA at 30 mM was inhibitory when spread plates were used (Table 3.1). To confirm these results vertical inhibition tests were conducted and antimicrobial films were not able to inhibit the organism (Tables 3.4 and 3.5). However, when bacterial counts on agar zones beneath the films were performed (Figs 3.8 and 3.9) it was shown that the antimicrobial films decreased the growth of the organism. Nisin or lysozyme formulated into neutral films were more inhibitory than when they were formulated into acidic films. Neutral films containing nisin or lysozyme caused a ~1.6 or 1.1 log CFU/g reduction, respectively, while only a ~1.1 or 0.8 log CFU/g reduction, respectively, was achieved with the acidic films (comparisons were made using agar areas without film against films with 4 mg/g nisin or 30 mg/g lysozyme). The neutral and acidic films containing EDTA or PP produced inhibitory results which were similar and were unrelated to pH reaction. Films made with EDTA reduced numbers by ~ 0.70 log CFU/g while those made with PP caused reductions of ~1 log CFU/g.

Nisin and lysozyme had bacteriostatic and bactericidal effects against *S.* Typhimurium, especially when formulated into neutral films, while EDTA and PP seemed to be bacteriostatic and may have been very slightly bactericidal depending on the concentration of the antimicrobial.

3.4.9. Antimicrobial effects against *E. coli* O157:H7

E. coli was only inhibited underneath the antimicrobial film discs using the horizontal inhibition method (Tables 3.1-3.3). In addition, from Tables 3.4 and 3.5, it was observed that the antimicrobial films did not inhibit the organism. However, when bacterial counts on agar zones beneath the films were conducted (Figs 3.10 and 3.11) it was shown that the antimicrobial films decreased the growth of the organism. Furthermore, lysozyme, EDTA and PP in neutral films were slightly more inhibitory than in acidic films.

All four antimicrobials were bacteriostatic towards *E. coli* 0157:H7, however, when lysozyme, EDTA and PP were formulated into neutral WPI films they were (slightly) bactericidal.

3.5. Discussion

3.5.1. Antimicrobial effects on *B. thermosphacta*

Similar to our study, several studies have also demonstrated that nisin successfully inhibited *B. thermosphacta* (Cutter and Siragusa, 1994a, 1996a, 1996b, 1997; Siragusa *et al.*, 1999). Nisin as a spray (Cutter and Siragusa, 1994a, 1996a) or immobilized in calcium alginate gel reduced the organism on beef surfaces (Cutter and Siragusa, 1996b, 1997). In addition, nisin incorporated into polyethylene-based films produced inhibition zones on streptomycin thallous acetate actidione (STAA) agar plates and decreased *B. thermosphacta* on beef cube surfaces more than untreated films (Siragusa *et al.*, 1999; Cutter *et al.*, 2001). Furthermore, an antimicrobial gel coating containing a combination of lysozyme, nisin and EDTA immediately reduced *B.*

thermosphacta up to 4 log CFU/cm^2 on cooked ham and bologna sausage slices and remained inhibited through the 4 week storage time (Gill and Holley, 2000).

Although studies have shown that nisin inhibits the growth of *B. thermosphacta*, Gao *et al.* (1999) showed that nisin alone did not inhibit the organism in BHI broth, but when it was used with EDTA the viable numbers decreased by 2 log CFU/ml.

In the present study, EDTA did not inhibit the organism, however, Tu and Mustapha (2002) showed that 20 mM EDTA was able to inhibit *B. thermosphacta* by 2.6 log CFU/ml on fresh beef.

3.5.2. Antimicrobial effects on *L. monocytogenes*

L. monocytogenes has been the focus of many recent studies. It has become a well known pathogen and is very difficult to control in foods because it has the ability to survive and grow in a wide range of environments. As in the present experiments, other studies have shown that nisin inhibits *L. monocytogenes* in buffer systems (Bruno *et al.*, 1992; Winkowski *et al.*, 1994); in broths (Ukuku and Shelef, 1997; Nilsson *et al.*, 2000; De Martinis *et al.*, 1997); in agars (Ming *et al.*, 1997; Harris *et al.*, 1991); in food products (Chung *et al.*, 1989; El-Khateib *et al.*, 1993; Mahadeo and Tatini, 1994; Fang and Lin, 1994; Dean and Zottola, 1996; Nilsson *et al.*, 1997; Datta and Benjamin, 1997; Zhang and Mustapha, 1999); in immobilized gels (Fang and Lin, 1995); and on food processing surfaces (Bower *et al.*, 1995).

On the other hand, others have found no inhibitory effects of nisin against *Listeria* (Abdalla *et al.*, 1993; Zapico *et al.*, 1998). Zapico *et al.* (1998) observed that nisin (10 or 100 IU/ml) after 24h at 30°C did not significantly reduce numbers of *Listeria* in UHT processed milk. As well, nisin (25 μg/ml) was not seen to be inhibitory

to the organism in white pickled cheese (Abdalla *et al.*, 1993). The authors suggested that the pH might have been too high for nisin to have optimal inhibitory action. This is plausible since several studies have shown that the activity of nisin is increased and is more stable at lower pH values (Harris *et al.*, 1991; Okereke and Thompson, 1996; Thomas and Wimpenny, 1996; Ukuku and Shelef, 1997; Budu-Amoako *et al.*, 1999; Parente *et al.*, 1998). Thomas and Wimpenny (1996) observed that lowering the pH from 7.9 to 5 increased the effectiveness of nisin against *L. monocytogenes*. In addition, Datta and Benjamin (1997) observed that *Listeria* was only inhibited by nisin (250 μg/ml) when in low pH medium (~ pH 3) and that at neutral pH (~ 7.3) the viability of the bacteria was not reduced. This was also observed in our study, where nisin incorporated into ~ pH 3 films produced larger clear inhibition zones against this organism than when nisin was incorporated into ~ pH 7 films.

Although lysozyme and EDTA did not inhibit *L. monocytogenes* in tests reported here, they have been observed to inhibit the growth of the organism (Verheul *et al.*, 1997; Hughey and Johnson, 1987; Carneiro de Melo *et al.*, 1998; Razavi-Rohani and Griffiths, 1996). Verheul *et al.* (1997) used 5 mg/ml lysozyme to reduce *L. monocytogenes* in BHI broth by ~1 log CFU/ml. On the other hand, Alzoreky and Nakahara (2003) did not observe inhibition of the organism with the use of EDTA.

Only a few studies have demonstrated that *L. monocytogenes* cells are susceptible to PP (Juneja and Davidson 1993; Payne *et al.*, 1989). Payne *et al.* (1989) observed that 1000 μg/ml PP inhibited the growth of the organism in a model milk system by 4 log CFU/ml.

Several edible films (corn zein, WPI, methylcellulose (MC), hydroxypropyl methylcellulose (HPMC) and soy) containing natural antimicrobial agents have been

used to control *L. monocytogenes*. Hoffman *et al.* (2001) reduced the numbers of these organisms by ~5.5 log CFU/ml in broth after 48h when nisin or nisin plus EDTA were incorporated into corn zein films that were added into the broth. Corn zein dissolved in propylene glycol with the addition of 1000 IU/g nisin, with or without the addition of 1% calcium propionate reduced the initial bacterial population of 6.8 log CFU/g on ready-to-eat chicken by ~5 and 3 log CFU/g, respectively, after 24d (Janes *et al.*, 2002). Low pH (5.2) WPI films containing 1.5% (w/w) para-amino benzoic acid (PABA) or sorbic acid produced average inhibition zones (dia) of ~22 and 27 mm, respectively, on bacterial lawns, whereas control films without the antimicrobials did not produce any zones (Cagri *et al.*, 2001). In addition, these antimicrobial WPI films inhibited the bacteria by 3.4 to 4.1 log CFU/g on bologna and summer sausage slices that were stored at 4°C for 21d (Cagri *et al.*, 2002). Hot dogs, surface-inoculated with *L. monocytogenes* (5 log CFU/ml) and vacuum packaged in MC/HPMC pouches containing 10,000 and 7500 IU/ml nisin showed bacterial reductions to undetectable levels ($\leq$ 2.9 log CFU/package) during 60d storage, while those containing 2500 IU/ml nisin had numbers reduced to the same levels until 28d (Franklin *et al.*, 2004). Soy protein films containing 2.6% (w/w) malic acid decreased *Listeria* numbers by 2.8 log CFU/ml, (Eswaranandam *et al.*, 2004) while those containing nisin (205 IU/g protein) and malic acid together had decreased action against the bacteria. The same films containing nisin (4% w/w) or a nisin (4% w/w) plus lauric acid (8% w/w) combination produced equally good inhibition (~1 log CFU/ml reduction) of the organism on the surface of turkey bologna (Dawson *et al.*, 2002). However, HPMC films containing nisin (5000 IU/ml) or those containing nisin (5000 IU/ml) and stearic acid (15% w/w) mixtures did not produce the same antimicrobial effectiveness. HPMC films containing nisin but without stearic acid,

produced inhibition zones that were 70% larger than the same films with the stearic acid against *L. monocytogenes* (Sebti *et al.*, 2002). Furthermore, it was observed that reducing the pH of HPMC films containing nisin to pH 3 produced better antimicrobial films than neutral films (Sebti *et al.*, 2002). In addition, 1% nisin coated on LDPE decreased numbers of the organism in phosphate buffer ~0.6 log CFU/ml after 7d compared to uncoated LDPE (An *et al.*, 2000).

3.5.3. Antimicrobial effects on *S. aureus*

Results obtained here indicated that all of the antimicrobials inhibited *S. aureus*, with nisin having the greatest inhibitory activity. Several studies have observed that nisin was effective in inhibiting the organism (Chung *et al.*, 1989; Thomas and Wimpenny, 1996; Carneiro de Melo *et al.*, 1996, 1998; An *et al.*, 2000; Sebti and Coma, 2002). Carneiro de Melo *et al.* (1996) determined that only 20 μM nisin was needed to totally inhibit *S. aureus*, and that >99.9% of the organisms on chicken skin were eliminated with 30 μM nisin (Carneiro de Melo *et al.*, 1998). On agar, nisin produced significant inhibition zones (An *et al.*, 2000) and increasing concentrations of nisin in solution (from 5000 to 10000 IU/ml), substantially reduced the growth of the organism (Sebti and Coma, 2002). However, Abdalla *et al.* (1993) observed no significant inhibitory effect of nisin on *S. aureus* in white pickled cheese.

From the present study, lysozyme (in neutral and in acidic films) decreased the numbers of the organism by ~1 log CFU/g. Very few studies have used lysozyme as the sole antimicrobial for controlling the growth of this organism (Ng and Garibaldi, 1975; Ibrahim *et al.*, 1997) and some researchers observed that native lysozyme was not very effective. Carneiro de Melo *et al.* (1998) showed that suspended *S. aureus* cells were not

killed by lysozyme. Parallel research has yielded additional approaches to increase the activity of lysozyme against *S. aureus*. Ibrahim *et al.* (1994, 1996a, 1996b, 1997) used a modified lysozyme to obtain increase inhibition of the organism in buffer.

While Razavi-Rohani and Griffiths (1996) demonstrated that EDTA was effective against *S. aureus*, Alzoreky and Nakahara (2003) did not find that EDTA was able to inhibit the organism. In the present study, 30 mM EDTA reduced *S. aureus* counts by ~1 log CFU/g.

Pierson *et al.* (1980) observed that 500 ppm PP decreased the population of *S. aureus* in broth by ~ 3 log CFU/ml after 48h. However, the films used in this study containing between 300 and 900 ppm PP on average only reduced the organism by ~ 1 log CFU/g after 48h in BHI agar.

HPMC films containing nisin (5000 IU/ml) (Coma *et al.*, 2001; Sebti and Coma, 2002) and corn zein films with potassium sorbate decreased viable *S. aureus* cells at the surfaces of model food systems (Vojdani and Torres, 1990). Cellulose-based inserts with immobilized nisin (7860 AU/cm^2) decreased the organism by 1.8 and by 2 log CFU/g when placed between cheese slices and cooked ham, respectively (Scannell *et al.*, 2000). Na-alginate films containing lysozyme (0.1% w/w), nisin (100 μg/ml), grapefruit seed extract (GFSE) (0.1% w/w) and EDTA (5 mmol) were shown to produce larger inhibition zones than κ-carrageenan films containing the same antimicrobial agents at the same concentrations (Cha *et al.*, 2002).

3.5.4. Antimicrobial effects on *S.* Typhimurium

Several researchers have observed that nisin is not inhibitory against *S.* Typhimurium in buffer (Stevens *et al.*, 1991; Schved *et al.*, 1994; Olasupo *et al.*, 2003)

or in food products (Chung *et al.*, 1989; Abdalla *et al.*, 1993; Tu and Mustapha, 2002). This is in agreement with results obtained here from the inhibition tests where films containing nisin were not able to produce clear inhibition zones in agar. However, quantitatively the neutral or acidic films containing nisin were able to reduce the organism by ~1.6 or 1.1 log CFU/g, respectively. Tested lysozyme films only reduced *S.* Typhimurium numbers by ~1 or 0.75 log CFU/g (neutral or acidic films, respectively). Alakomi *et al.* (2000) showed that lysozyme (10 μg/ml) was only slightly inhibitory against *S.* Typhimurium in buffer, however, its activity was increased when it was used with 10 mM lactic acid. The EDTA films used here (neutral and acidic) only caused marginal inhibitory effects on the organism. In fact, studies that used EDTA as an inhibitory agent have found that it is not an effective agent for inhibiting this organism (Stevens *et al.*, 1991; Schved *et al.*, 1994; Payne *et al.*, 1994). Similar to experiments reported here, Pierson *et al.* (1980) showed that 300 ppm PP decreased the population of *S.* Typhimurium in broth, however only initially (first 6h) and that subsequent growth occurred.

Gill and Holley (2000) reduced *S.* Typhimurium by 3-4 log CFU/cm^2 on cooked ham and bologna sausage slices during 4 weeks storage, using an antimicrobial gel coating containing a lysozyme-nisin (1:3) mixture and EDTA. In another study, low pH (5.2) WPI films containing 1.5% (w/w) PABA or sorbic acid produced average inhibition zones of ~14 and 10 mm (dia), respectively on TSAYE bacterial lawns, whereas control films without antimicrobials did not produce any inhibitory zones (Cagri *et al.*, 2001). Furthermore, the films used inhibited these organisms by 3.1 to 4.1 log CFU/g on bologna and summer sausage slices when stored at 4°C for 21d (Cagri *et al.*, 2002).

3.5.5. Antimicrobial effects on *E. coli* 0157:H7

The nisin-containing WPI films used here inhibited *E. coli* 0157:H7 by ~ 0.8 log CFU/g. Nisin is not normally inhibitory against Gram- bacteria, since these microorganisms posses an OM that nisin is not able to penetrate. Both Schved *et al.* (1994) and Olasupo *et al.* (2003) showed that nisin did not inhibit *E. coli*. Nonetheless, some researchers have shown that nisin does inhibit this organism (Carneiro de Melo *et al.*, 1996; Zhang and Mustapha, 1999; An *et al.*, 2000). Zhang and Mustapha (1999) reduced *E. coli* by 1.02 log CFU/cm^2 on fresh beef cubes using nisin (5000 IU/ml). Lysozyme as well has been observed to inhibit *E. coli* although it is also not usually used on Gram- bacteria (Razavi-Rohani and Griffiths, 1996; Alakomi *et al.*, 2000). To increase its effectiveness against *E. coli* modification of lysozyme by heat-denaturation or covalent attachment to perillaldehyde has been done (Ibrahim *et al.*, 1994, 1996a, 1996b, 1997). *E. coli* has been reported to be sensitive to EDTA (Razavi-Rohani and Griffiths, 1996; Boziaris and Adams, 1999; Payne *et al.*, 1994; Alakomi *et al.*, 2000). Payne *et al.* (1994) observed that EDTA ($\leq$2.5 mg/ml) alone decreased *E. coli* 0157:H7 numbers by 4 log CFU/ml in UHT processed milk. However, Gao *et al.* (1999) and Schved *et al.* (1994) observed that when EDTA was used as the only inhibitory agent it did not inhibit this organism.

A number of edible films containing antimicrobials have been used to effectively inhibit *E. coli*. A lysozyme plus EDTA combination in cast corn zein films was found to be effective against the organism (Padgett *et al.*, 1998). However, lysozyme or nisin plus EDTA incorporated into heat-pressed soy protein or corn zein films did not inhibit *E. coli* 0157:H7 (Padgett *et al.*, 1998). It was suggested that the high temperatures used in the formation of heat-pressed films may have caused the antimicrobials to lose some

activity. Low pH (5.2) WPI films containing 1.5% (w/w) PABA or sorbic acid produced average inhibition zones of ~15 and 11 mm dia, respectively, on TSAYE bacterial lawns, whereas control films without the antimicrobials did not produce any inhibition zones (Cagri *et al.*, 2001). Furthermore, these films inhibited *E. coli* 0157:H7 populations by 3.1 to 3.6 log CFU/g on bologna and summer sausage slices, stored at 4°C for 21d (Cagri *et al.*, 2002). In addition, *E. coli* 0157:H7 was inhibited by 2.1 log CFU/ml by soy protein films containing 2.6% (w/w) malic acid and by soy films containing 2.6% (w/w) lauric acid with (1.9 log CFU/ml) and without the addition of nisin (1.6 log CFU/ml) (Eswaranandam *et al.*, 2004). However, a gelatin antimicrobial coating containing a lysozyme-nisin (1:3) mixture and EDTA had no effect on *E. coli* 0157:H7 on cooked ham and bologna sausage slices (Gill and Holley, 2000). Na-alginate films that contained nisin plus EDTA or lysozyme plus EDTA did not inhibit the organism; however Na-alginate films that contained nisin, lysozyme and EDTA were able to inhibit *E. coli* 0157:H7 (Cha *et al.*, 2002).

3.6. Summary

Several studies have shown that antimicrobials incorporated into films have increased inhibition over antimicrobial solutions. For every organism a specific antimicrobial film-formulation is required to effectively inhibit their growth. Optimum antimicrobial WPI films to inhibit *B. thermosphacta* should contain nisin formulated into acid films or lysozyme in neutral films. *L. monocytogenes* and *S. aureus* can be effectively inhibited by nisin incorporated into acidic WPI films. While, nisin and lysozyme formulated into neutral films are best suited to inhibit *S.* Typhimurium. On the

other hand, *E. coli* is more of a challenge, but can be minimally inhibited by antimicrobials formulated into neutral WPI films.

Further research is required to optimize antimicrobial WPI films to include combinations of antimicrobials with additive or synergistic effects against organisms which are normally resistant.

Table 3.1. Horizontal inhibitory activity (mm) of neutral antimicrobial WPI films containing nisin, lysozyme, EDTA or propyl paraben against several spoilage and pathogenic microorganisms using a spread plate method

Microorganism	WPI:GLY	Nisin (mg/g)					Lysozyme (mg/g)					EDTA (mM)				Propyl paraben (ppm)			
		0	1	2	3	4	0	25	50	75	100	0	10	20	30	0	300	600	900
B. thermosphacta	**60:40**	0[a]	6.58 ± 0.29	7.42 ± 0.29	7.75 ± 0.66	7.50 ± 0.43	0	6.67 ± 0.58	7.33 ± 0.95	7.42 ± 0.29	7.50 ± 0.25	0.30 ± 0.05	0.19 ± 0.11	0.25 ± 0.07	0.32 ± 0.04	v	v	v	v
	65:35	0	.6.75 ± 0.43	7.17 ± 0.38	7.08 ± 0.29	7.83 ± 0.52	0	5.50 ± 0.75	6.67 ± 0.76	7.08 ± 0.80	7.33 ± 0.29	0.19 ± 0.06	0.19 ± 0.05	0.23 ± 0.13	0.14 ± 0.09	v	v	v	v
	70:30	0	6.58 ± 0.14	6.54 ± 0.71	6.83 ± 0.88	7.00 ± 0	0	4.83 ± 0.76	6.17 ± 0.88	6.25 ± 0.66	7.83 ± 0.52	0.21 ± 0.18	0.26 ± 0.09	0.28 ± 0.13	0.40 ± 0.19	v	v	v	v
							0	**10**	**20**	**30**									
L. monocytogenes	**60:40**	0	0	0	0	0	0	0	0	0		0.56 ± 0.07	0.64 ± 0.06	0.68 ± 0.06	0.67 ± 0.08	0.32 ± 0.22	0.38 ± 0.13	0.50 ± 0	0.50 ± 0
	65:35	0	0	0	0	0	0	0	0	0		v	0.92 ± 0.14	0.83 ± 0.52	1.17 ± 0.29	0.42 ± 0.26	0.53 ± 0.04	0.58 ± 0.07	0.50 ± 0
	70:30	0	0	0	0	0	0	0	0	0		0.47 ± 0.13	0.54 ± 0.07	0.54 ± 0.03	0.56 ± 0.06	0.63 ± 0.18	0.52 ± 0.03	0.58 ± 0.14	0.50 ± 0
S. aureus	**60:40**	v[b]	v	v	v	v	v	v	v	v		v	v	v	v	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v		v	v	v	v	v	v	v	v
	70:30	v	v	v	0.40 ± 0.06	0.50 ± 0.16	v	v	v	v		v	v	v	v	v	v	v	v
***S.* Typhimurium**	**60:40**	v	v	v	v	v	v	v	v	v		v	v	v	1.38 ± 0.70	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v		v	v	v	1.00 ± 0.87	v	v	v	v
	70:30	v	v	v	v	v	v	v	v	v		v	v	v	.71 ± 0.62	v	v	v	v
E. coli	**60:40**	v	v	v	v	v	v	v	v	v		v	v	v	v	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v		v	v	v	v	v	v	v	v
	70:30	v	v	v	v	v	v	v	v	v		v	v	v	v	v	v	v	v

[a] '0' represents no horizontal inhibitory activity and no vertical inhibitory activity
[b] 'v' represents vertical inhibition only (the inhibition was only visible beneath the film disc)
Each result is an average of 3 measurements read in duplicate

Table 3.2. Horizontal inhibitory activity (mm) of neutral antimicrobial WPI films containing nisin, lysozyme, EDTA or propyl paraben against several spoilage and pathogenic microorganisms using a pour plate method

Microorganism	WPI:GLY	Nisin (mg/g)					Lysozyme (mg/g)				EDTA (mM)				Propyl paraben (ppm)			
		0	1	2	3	4	0	10	20	30	0	10	20	30	0	300	600	900
B. thermosphacta	**60:40**	0[a]	3.88 ± 0.24	4.59 ± 0.24	4.80 ± 0.11	5.30 ± 0.14	0	v	0.72 ± 0.10	0.97 ± 0.10	0	0	0	0	v	v	v	v
	65:35	0	3.78 ± 0.30	4.69 ± 0.19	4.94 ± 0.23	5.35 ± 0.14	0	v	0.53 ± 0.08	0.89 ± 0.11	0	0	0	0	v	v	v	v
	70:30	0	3.44 ± 0.65	4.36 ± 0.33	4.99 ± 0.34	5.20 ± 0.02	0	v	0.64 ± 0 .27	1.05 ± 0.19	0	0	0	0	v	v	v	v
L. monocytogenes	**60:40**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	**60:40**	v[b]	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	70:30	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
***S.* Typhimurium**	**60:40**	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	70:30	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
E. coli	**60:40**	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	70:30	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v

[a] '0' represents no horizontal inhibitory activity and no vertical inhibitory activity
[b] 'v' represents vertical inhibition only (the inhibition was only visible beneath the film disc)
Each result is an average of 3 measurements read in duplicate

Table 3.3. Horizontal inhibitory activity (mm) of acidic antimicrobial WPI films containing nisin, lysozyme, EDTA or propyl paraben against several spoilage and pathogenic microorganisms using a pour plate method

Microorganism	WPI:GLY	Nisin (mg/g)					Lysozyme (mg/g)				EDTA (mM)				Propyl paraben (ppm)			
		0	1	2	3	4	0	10	20	30	0	10	20	30	0	300	600	900
B. thermosphacta	**60:40**	0[a]	4.63 ± 0.26	5.05 ± 0.08	5.17 ± 0.56	6.14 ± 0.14	0	3.57 ± 0.31	3.94 ± 0.29	4.34 ± 0.40	0	0	0	0	v	v	v	v
	65:35	0	4.41 ± 0.70	4.83 ± .21	5.34 ± .31	5.51 ± 0.42	0	3.24 ± 0.45	4.14 ± 0.34	4.24 ± 0.33	0	0	0	0	v	v	v	v
	70:30	0	4.73 ± 0.32	5.35 ± 0.37	5.29 ± 0.35	5.85 ± 0.27	0	2.86 ± 0.26	3.04 ± 0.28	3.29 ± 0.43	0	0	0	0	v	v	v	v
L. monocytogenes	**60:40**	0	0.46 ± 0.08	0.69 ± 0.26	0.67 ± 0.17	0.93 ± 0.14	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0.41 ± 0.05	0.70 ± 0.14	0.97 ± 0.21	1.04 ± 0.16	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0.34 ± 0.07	0.63 ± 0.17	0.94 ± 0.15	0.80 ± 0.22	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	**60:40**	v[b]	v	0.34 ± .12	0.55 ± 0.10	0.67 ± 0.07	v	v	v	v	v	v	v	v	v	v	v	v
	65:35	v	v	0.39 ± 0.17	0.50 ± 0.09	0.75 ± 0.06	v	v	v	v	v	v	v	v	v	v	v	v
	70:30	v	v	0.39 ± 0.20	0.63 ± 0.17	1.07 ± 0.22	v	v	v	v	v	v	v	v	v	v	v	v
***S.* Typhimurium**	**60:40**	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	70:30	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
E. coli	**60:40**	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	65:35	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	70:30	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v

[a] '0' represents no horizontal inhibitory activity and no vertical inhibitory activity
[b] 'v' represents vertical inhibition only (the inhibition was only visible beneath the film disc)
Each result is an average of 3 measurements read in duplicate

Table 3.4. Vertical inhibitory activity (mm) of neutral antimicrobial WPI films containing nisin, lysozyme, EDTA or propyl paraben against several spoilage and pathogenic microorganisms

Microorganism	WPI:GLY	Nisin (mg/g)					Lysozyme (mg/g)				EDTA (mM)				Propyl paraben (ppm)			
		0	1	2	3	4	0	10	20	30	0	10	20	30	0	300	600	900
B. thermosphacta[b]	**60:40**	0[a]	4.20 ± 0.35	4.35 ± 0.40	5.20 ± 0.10	5.86 ± 0.17	0	0	0	0.95 ± 0.17	0	0	0	0	0	0	0	0
	65:35	0	4.03 ± 0.42	5.68 ± 0.31	5.72 ± 0.49	5.68 ± 0.74	0	0	0	0.30 ± 0.17	0	0	0	0	0	0	0	0
	70:30	0	4.50 ± 0.07	5.85 ±.095	5.95 ± 0.78	6.53 ± 0.22	0	0	0.22 ± 0.19	0.30 ± 0.05	0	0	0	0	0	0	0	0
L. monocytogenes[b]	**60:40**	0	0	0	1.23 ± 0.04	1.63 ± 0.11	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0	0	1.15 ± 0.07	1.43 ± 0.09	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0	1.13 ± 0.19	1.39 ± 0.12	1.50 ± 0.07	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus[b]	**60:40**	0	0	0	0	0.41 ± 0.15	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0	0	0	0.48 ± 0.04	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0	0	0	0.53 ± 0.08	0	0	0	0	0	0	0	0	0	0	0	0
***S.* Typhimurium**[c]	**60:40**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. coli[c]	**60:40**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] '0' represents no vertical inhibitory activity
[b] vertical tube method
[c] vertical plug method (gas producing microorganisms)
Each result is an average of 3 measurements read in duplicate

Table 3.5. Vertical inhibitory activity (mm) of acidic antimicrobial WPI films containing nisin, lysozyme, EDTA or propyl paraben against several spoilage and pathogenic microorganisms

Microorganism	WPI:GLY	Nisin (mg/g)					Lysozyme (mg/g)				EDTA (mM)				Propyl paraben (ppm)			
		0	1	2	3	4	0	10	20	30	0	10	20	30	0	300	600	900
B. thermosphacta[b]	**60:40**	0[a]	7.50 ± 0.13	8.30 ± 0.09	8.63 ± 0.25	9.16 ± 0.23	0	1.47 ± 0.06	1.74 ± 0.11	1.90 ± 0	0	0	0	0	0	0	0	0
	65:35	0	7.45 ± 0.04	8.22 ± 0.03	8.40 ± 0.05	8.89 ± 0.04	0	1.19 ± 0.01	1.50 ± 0.13	1.78 ± 0.03	0	0	0	0	0	0	0	0
	70:30	0	7.03 ± 0.55	7.87 ± 0.18	8.40 ± 0.28	8.63 ± 0.10	0	1.06 ± 0.016	1.40 ± 0	1.65 ± 0.04	0	0	0	0	0	0	0	0
L. monocytogenes[b]	**60:40**	0	3.54 ± 0.17	3.65 ± 0.26	3.78 ± 0.16	3.92 ± 0.03	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	2.88 ± 0.06	2.92 ± 0.04	3.18 ± 0.05	3.48 ± 0.11	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	2.91 ± 0.09	3.01 ± 0.01	3.29 ± 0.12	3.32 ± 0.03	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus[b]	**60:40**	0	1.68 ± 0.10	1.83 ± 0.07	1.87 ± .06	2.18 ± 0.24	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0.63 ± 0.15	1.07 ± 0.08	1.62 ± 0.10	1.85 ± 0.05	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0.63 ± 0.40	1.23 ± 0.06	1.55 ± 0.05	1.83 ± 0.20	0	0	0	0	0	0	0	0	0	0	0	0
***S.* Typhimurium**[c]	**60:40**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. coli[c]	**60:40**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	65:35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	70:30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] '0' represents no vertical inhibitory activity
[b] vertical tube method
[c] vertical plug method (gas producing microorganisms)
Each result is an average of 3 measurements read in duplicate

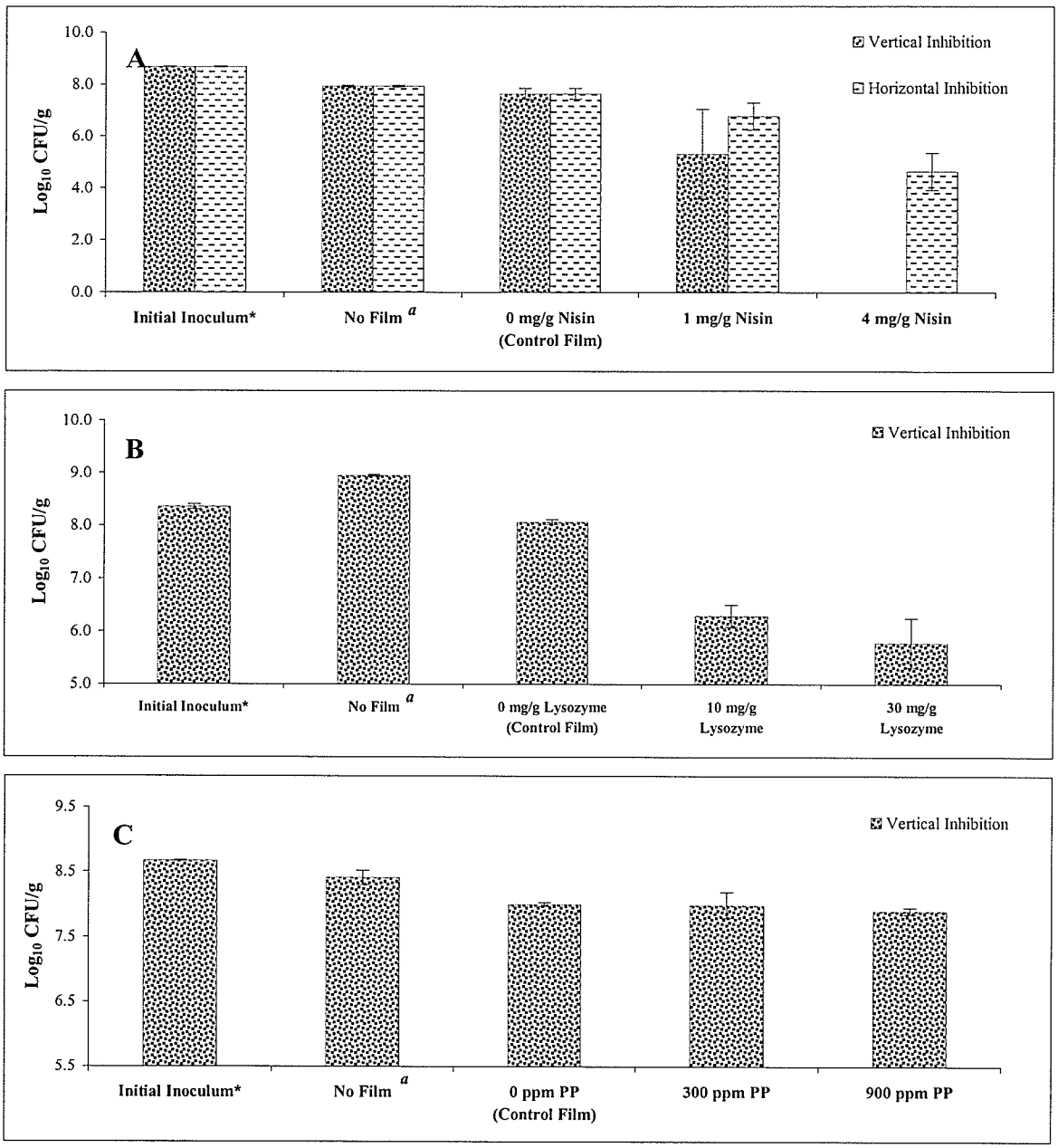

Figure 3.3. Quantification of bacterial inhibition by neutral antimicrobial WPI films containing A-nisin, B-lysozyme or C-propyl paraben (PP) against *B. thermosphacta*

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

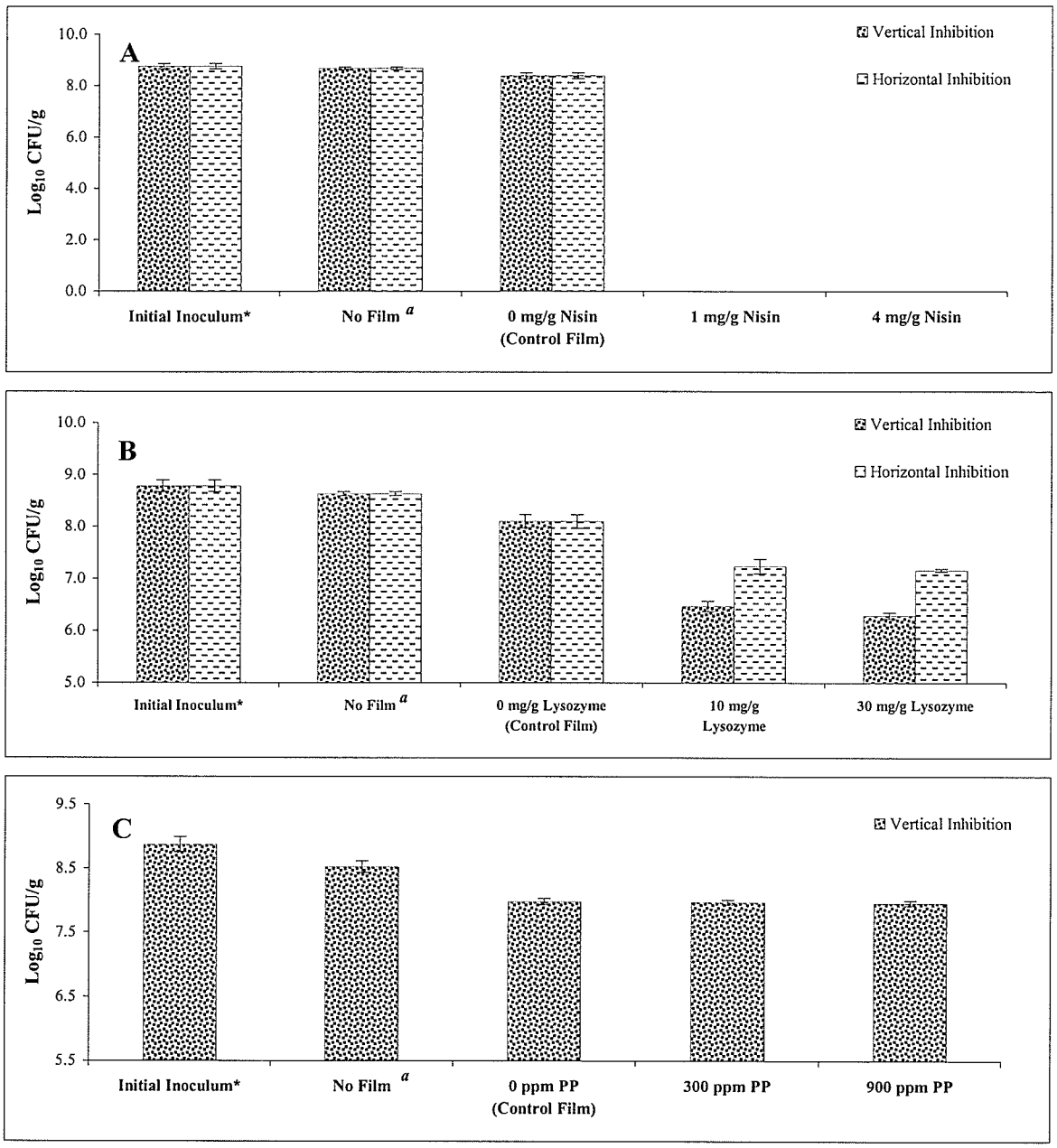

Figure 3.4. Quantification of bacterial inhibition by acidic antimicrobial WPI films containing A-nisin, B-lysozyme or C-propyl paraben (PP) against *B. thermosphacta*

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

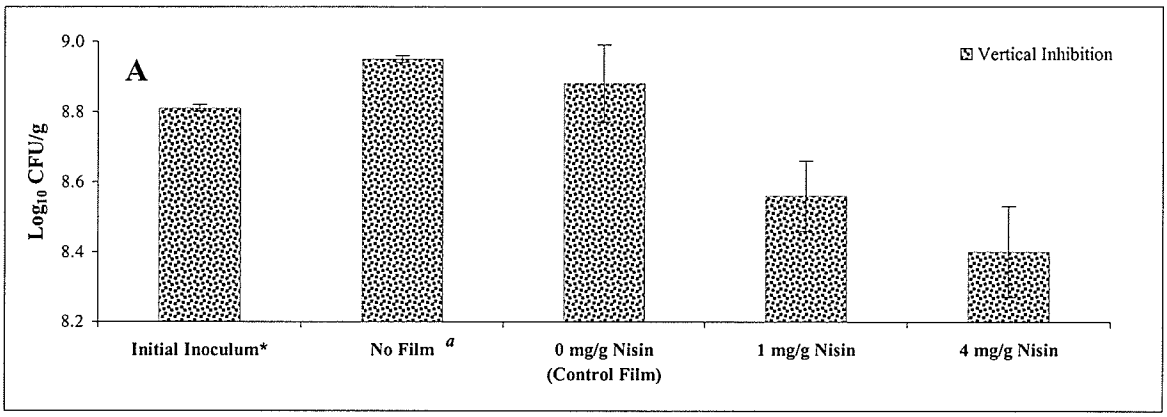

Figure 3.5. Quantification of bacterial inhibition by acidic antimicrobial WPI films containing nisin against *L. monocytogenes*

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

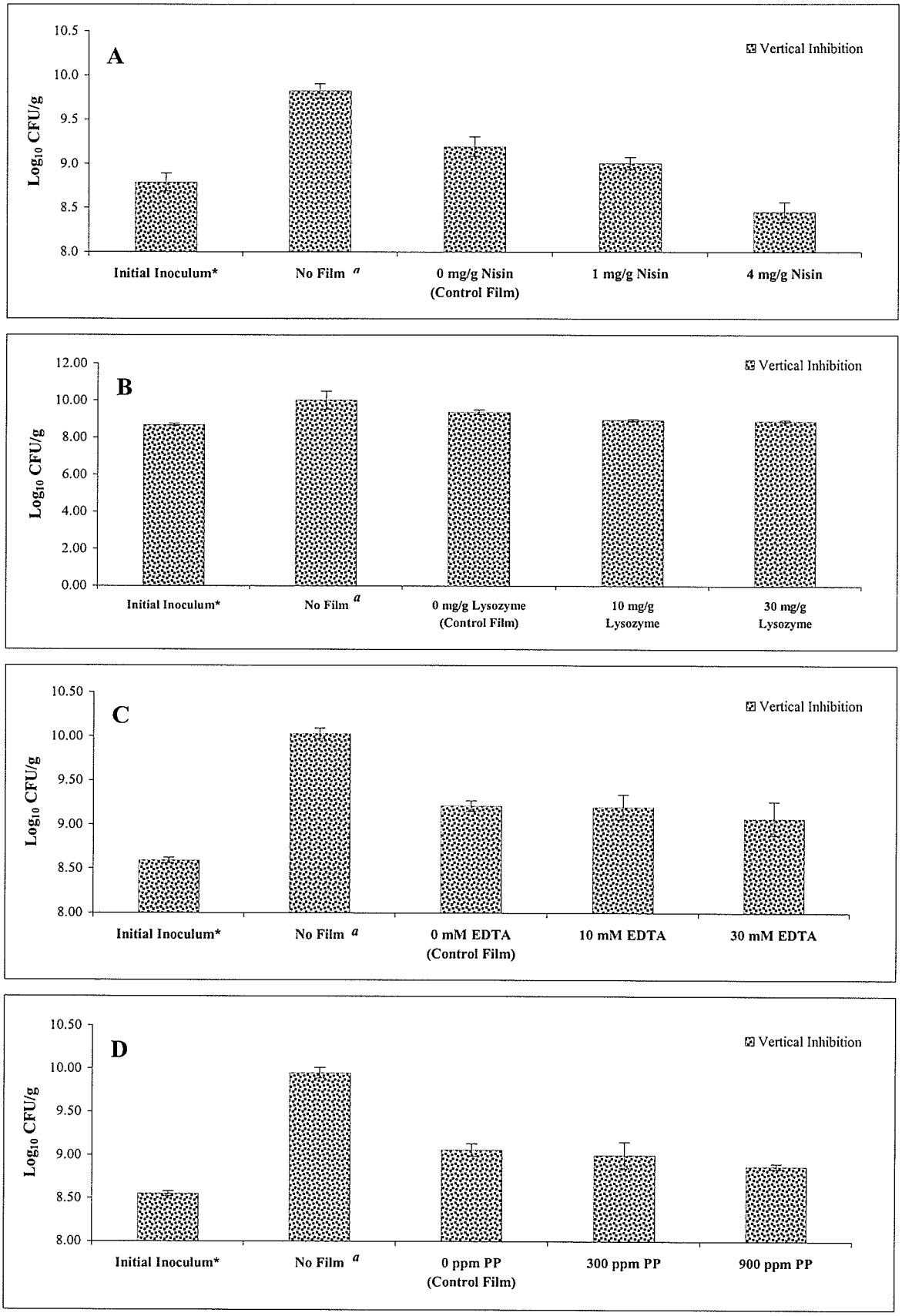

Figure 3.6. Quantification of bacterial inhibition by neutral antimicrobial WPI films containing A-nisin, B-lysozyme, C-EDTA or D-propyl paraben (PP) against *S. aureus*

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

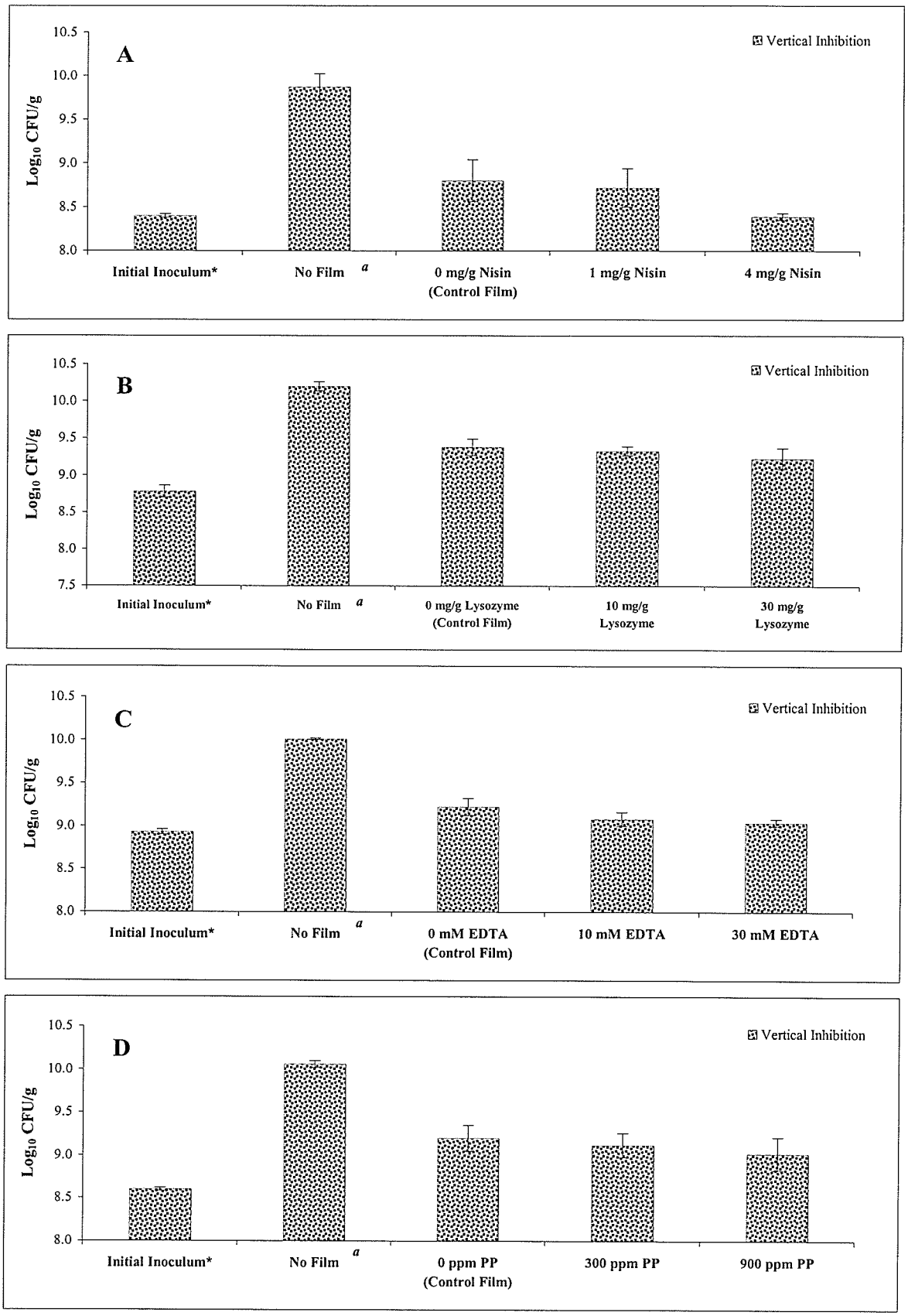

Figure 3.7. Quantification of bacterial inhibition by acidic antimicrobial WPI films containing A-nisin, B-lysozyme, C-EDTA or D-propyl paraben (PP) against *S. aureus*

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

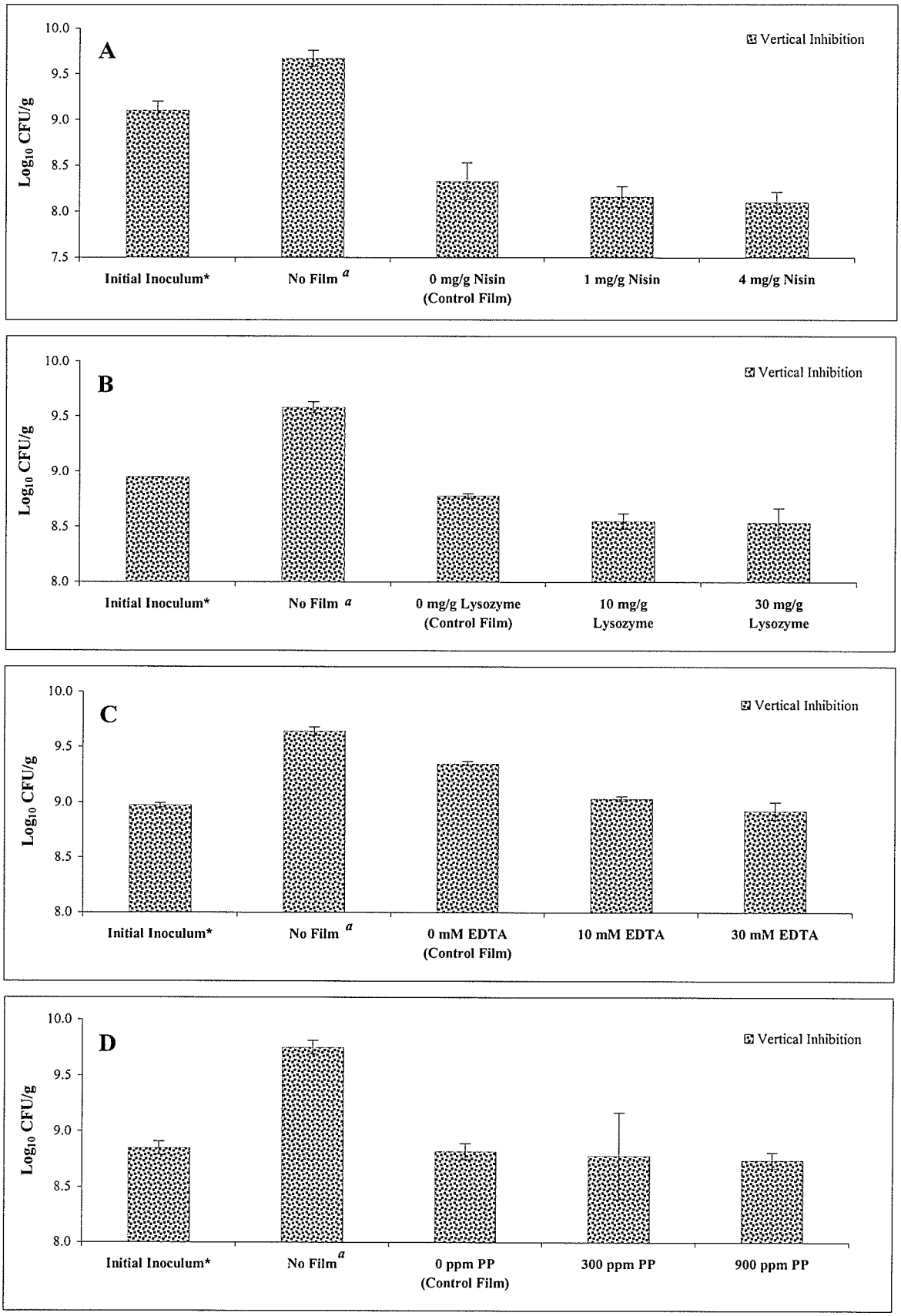

Figure 3.8. Quantification of bacterial inhibition by neutral antimicrobial WPI films containing A-nisin, B-lysozyme, C-EDTA or D-propyl paraben (PP) against *S.* Typhimurium

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

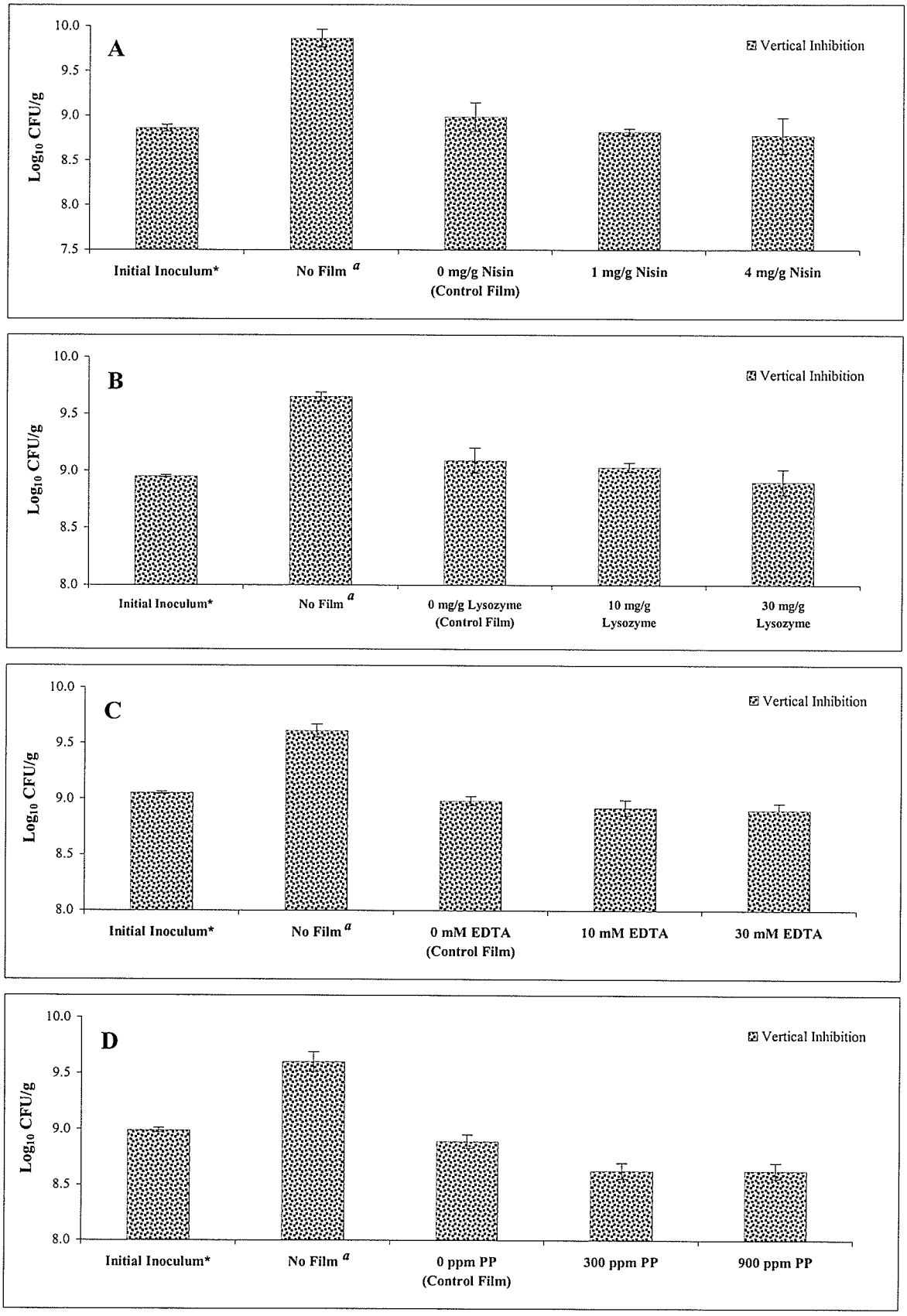

Figure 3.9. Quantification of bacterial inhibition by acidic antimicrobial WPI films containing A-nisin, B-lysozyme, C-EDTA or D-propyl paraben (PP) against *S.* Typhimurium

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

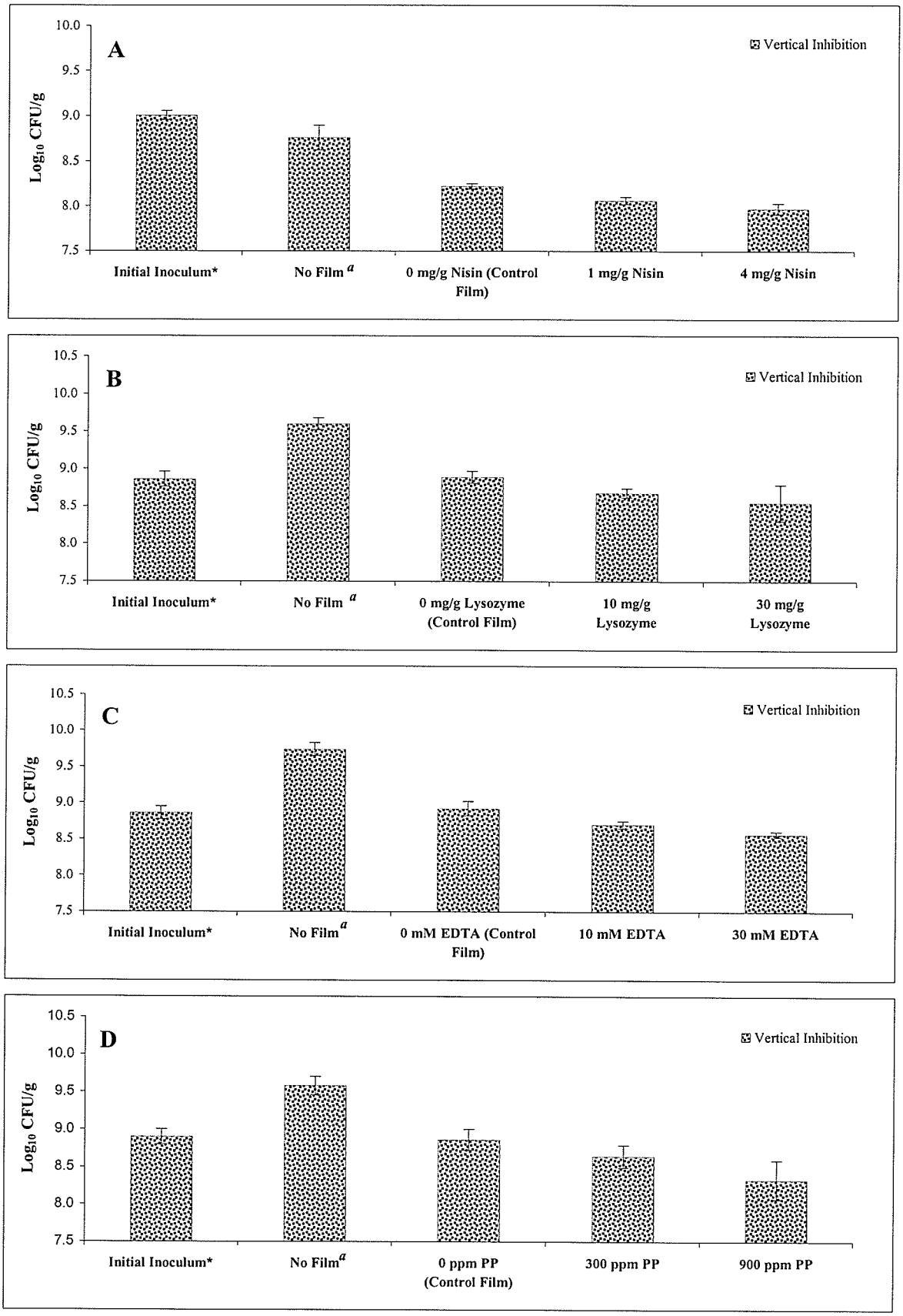

Figure 3.10. Quantification of bacterial inhibition by neutral antimicrobial WPI films containing A-nisin, B-lysozyme, C-EDTA or D-propyl paraben (PP) against *E. coli* 0157:H7

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

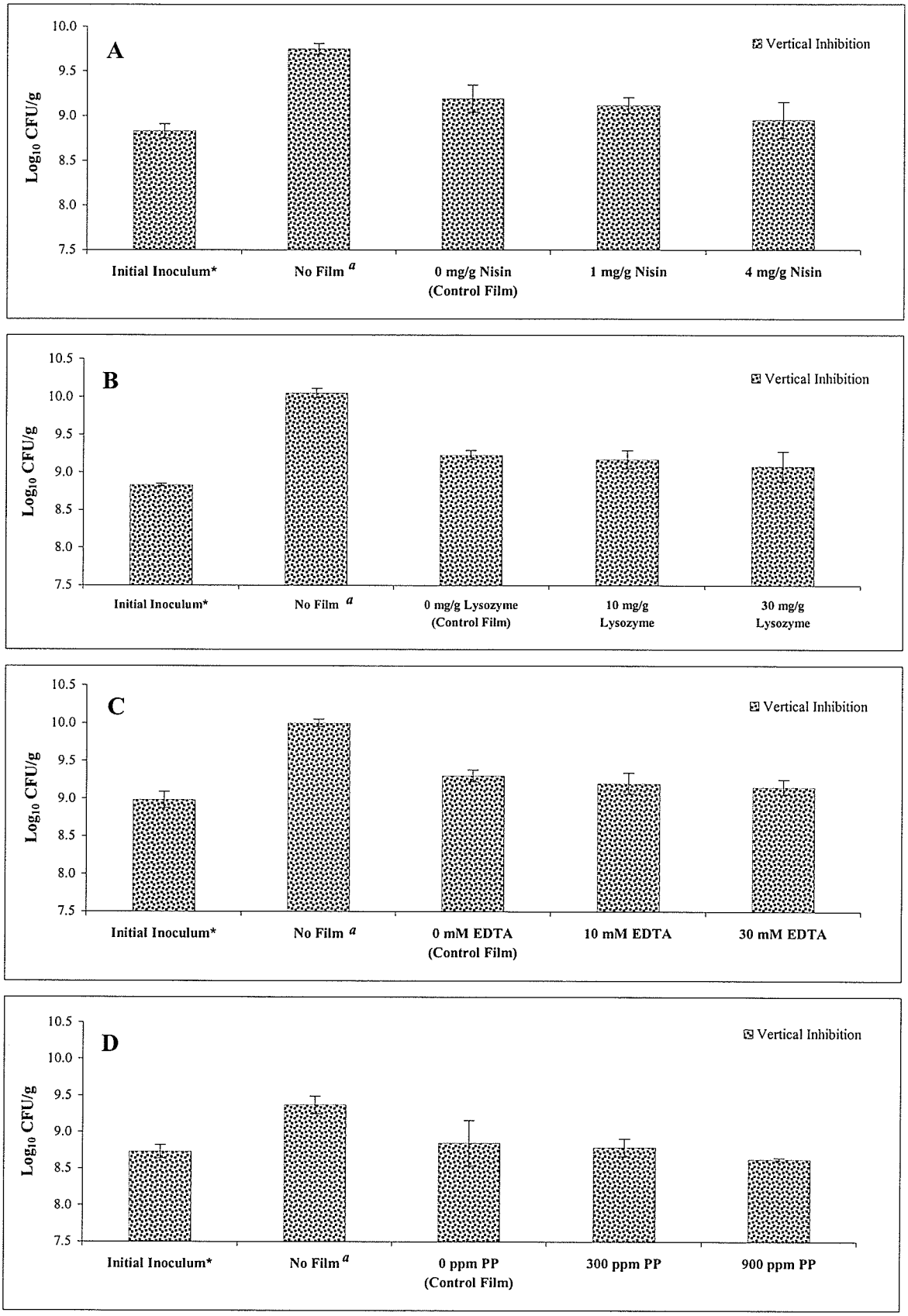

Figure 3.11. Quantification of bacterial inhibition by acidic antimicrobial WPI films containing A-nisin, B-lysozyme, C-EDTA or D-propyl paraben (PP) against *E. coli* 0157:H7

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

CHAPTER # 4

Activity of Multiple Antimicrobials in Edible Whey Protein Isolate Films against Spoilage and Pathogenic Bacteria

4.1. Abstract

Consumers demand packaging systems that not only protect food commodities, but are environmentally friendly and extend shelf-life. The minimum inhibitory concentrations (MICs) of several antimicrobials of interest for inclusion in packaging films were determined, and whey protein isolate (WPI) films were optimized for antimicrobial activity by using nisin, lysozyme, EDTA and propyl paraben (PP) at two concentrations (50% and 150% MIC). Film antimicrobial activity was determined by the size of the inhibition zones (horizontal and vertical) produced on agar plates inoculated with *Brochothrix thermosphacta*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aureus* or *Escherichia coli* 0157:H7.

A two level, five factor, full factorial experimental design was used to determine if interactions between antimicrobials existed. Results showed that nisin alone reduced all microorganisms except for *E. coli* 0157:H7; lysozyme alone reduced *B. thermosphacta* and PP alone reduced *L. monocytogenes*. Negative interactions were observed between: nisin and lysozyme (all organisms); nisin and EDTA (*S. aureus* and *S.* Typhimurium); lysozyme and PP (*B. thermosphacta* and *L. monocytogenes*); nisin and WPI (*S. aureus* and *S.* Typhimurium). These negative interactions were denoted as those that significantly reduced bacterial inhibitory activity. The only interaction that significantly increased the inhibition was between lysozyme and PP against *S. aureus*.

The most effective antimicrobial films contained nisin at the highest test concentration with 50% MIC levels of the other antimicrobials.

4.2. Introduction

Packaging materials are used to protect food products from environmental hazards, provide convenience and product information. The shelf-life and quality of foods are influenced by packaging properties. However, conventional food packaging materials, especially plastic materials, are not easily biodegradable and create substantial solid waste (Krochta and Mulder-Johnston, 1997). These factors have led researchers to develop new types of packaging materials that have improved biodegradability and will still safeguard food product quality.

Edible films are thin layers of edible material formed on a food product as a coating or are preformed separately on or used between units of food (Krochta and Mulder-Johnston, 1997). In general, most edible films have good barrier properties against gases (O_2 and CO_2), odours and water vapour. They can inhibit fat and oil migration (Donhowe and Fennema, 1994) and can help maintain food quality once the package has been opened (Krochta and Mulder-Johnston, 1997). In addition, they have the ability to carry food ingredients such as flavours, antioxidants and antimicrobials (Donhowe and Fennema, 1994).

Whey, a protein by-product from the cheese manufacturing industry (Gennadios *et al.*, 1994) represents an ongoing serious environmental problem since it is produced in abundant quantity. Excess whey has been converted into whey protein concentrate (WPC) and further purified to yield whey protein isolate (WPI), both of which have been extensively used to make edible films (McHugh *et al.*, 1994; McHugh and Krochta,

1994a, 1994b; Banerjee and Chen, 1995; Maté and Krochta, 1996; Shellhamer and Krochta, 1995; Pérez-Gago and Krochta, 1999; Miller *et al.*, 1997, 1998; Guilbert *et al.*, 1996; Gontard *et al.*, 1996; Anker, 2000).

Antimicrobials are also incorporated into film-forming solutions to form bioactive films which are used to reduce the growth of spoilage and pathogenic bacteria, yeast and mold and improve the shelf-life and safety of treated foods (Han, 2000; Padgett *et al.*, 1998). The antimicrobials in the films can directly inhibit the bacteria at the surface of the food or can diffuse through its surface layers thereby preventing further microbial development (Han, 2000). Many antimicrobials have been used to produce different types of edible antimicrobial films (Han, 2000, 2003, 2005; Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002; Cagri *et al.*, 2004).

B. thermosphacta, L. monocytogenes, *S. aureus*, *S.* Typhimurium and *E. coli* O157:H7 are major contributors to spoilage and the latter four have been implicated in outbreaks of foodborne illness (Bower *et al.*, 1995), and therefore were chosen as the test microorganisms of interest. *B. thermosphacta* is the principal spoilage organism found on packaged meats (Tu and Mustapha, 2002) that have not been properly vacuum packaged and refrigerated. *L. monocytogenes* is a pathogenic organism that is capable of growing and surviving at high salt concentrations and over wide temperature and pH ranges (Bower *et al.*, 1995; Crandall and Montville, 1998) as well as being a major concern as a post-processing contaminant (Payne *et al.*, 1989). In addition, it has been responsible for listeriosis outbreaks that have resulted in death (Liberti *et al.*, 1996). *S. aureus* is a pathogenic organism that can cause foodborne illness upon ingestion of pre-formed toxins in the food or from its toxins following establishment of the organism in a host. *E. coli* O157:H7 and *S.* Typhimurium are also foodborne pathogens. *Salmonella*

are principally associated with poultry products which are considered to be the leading food vehicle for *Salmonella*-derived illnesses (Natrajan and Sheldon, 2000a). *E. coli* O157:H7 has frequently been associated with contaminated undercooked beef and has caused major outbreaks (Zhang and Mustapha, 1999).

Nisin and lysozyme are antimicrobials that exhibit their action by interfering with the cytoplasmic membrane or wall structure of sensitive cells (Padgett *et al.*, 1998). It is suggested that nisin, due to its small size forms pores in the cytoplasmic membrane as it inserts itself in the membrane (Bower *et al.*, 1995). Lysozyme (1,4-ß-N-acetylmuramidase) is a bacteriolytic enzyme that cleaves the glycosidic linkages of the peptidoglycan in the cell wall of Gram+ bacteria (Lück and Jager, 1997; Payne *et al.*, 1994). Neither of these agents has any substantial antimicrobial action against Gram- bacteria because these possess an outer membrane (OM) which shields them from antimicrobial action.

While lysozyme and nisin are not effective against Gram- bacteria, EDTA and propyl paraben (PP) have been shown to be active (Razavi-Rohani and Griffiths, 1996; Payne *et al.*, 1994). Chelating agents such as EDTA are found to alter the OM in Gram- bacteria by affecting magnesium and calcium cations (Padgett *et al.*, 1998). When these cations are chelated from the OM, the membrane becomes destabilized and bacterial inhibition results (Boziaris and Adams, 1999; Padgett *et al.*, 1998). The antimicrobial PP is effective against both Gram- and + bacteria, however it is most inhibitory against Gram+ bacteria and fungi. Parabens inhibit membrane transport of electrons, essential amino acids and nutrients (Baranowski and Nagel, 1983; Lück and Jager, 1997).

With greater frequency antimicrobial agents have been used in combination with each other or with EDTA to increase their effectiveness against Gram- and + organisms

(Garibaldi *et al.*, 1969; Hughey and Johnson, 1987; Stevens *et al.*, 1991, 1992; Schved *et al.*, 1994; Payne *et al.*, 1994; Cutter and Siragusa, 1995a, 1995b; Gao *et al.*, 1999; Razavi-Rohani and Griffiths, 1996; Ibrahim *et al.*, 1997; Zhang and Mustapha, 1999; Boziaris and Adams, 1999; Helander and Mattilla-Sandholm, 2000; Cutter *et al.*, 2001; Tu and Mustapha, 2002). It is suggested that once EDTA destabilizes the membrane, other antimicrobials used in parallel can reach sensitive sites at cell surfaces they would not normally find accessible.

In this study the minimum inhibitory concentrations (MICs) of individual antimicrobial agents were determined by the least significant difference (LSD) test and WPI films were optimized for antimicrobial action by incorporation of a mixture of four antimicrobials (nisin, lysozyme, EDTA and PP) at two levels, 50% MIC and 150% MIC in different combinations to determine whether positive interactions were possible among the antimicrobials using BHI agar plates. Control films (without antimicrobials) as well as a film containing the antimicrobials at the 100% MIC level were also produced and used only for the purpose of comparison. Horizontal and vertical inhibition by mixed antimicrobial films against the four test organisms were qualitatively determined. The viability of treated cells taken from agar inhibition zones was also examined.

Since it has been shown that the activity of antimicrobial agents can be affected by protein concentrations in the film (Ozdemir and Floros, 2003), the antimicrobials (two levels each) were introduced into WPI films of two protein concentrations (60:40 and 70:30 WPI:glycerol (WPI:GLY)) to determine if the activity of the agents was affected by protein concentration.

A two level, five factor, full factorial experimental design was used to determine interaction effects. The two levels for each factor were: nisin (0.8 and 2.4 mg/g), lysozyme (21 and 63 mg/g), EDTA (8 and 25 mM), PP (150 and 450 ppm) and WPI protein concentration (60:40 and 70:30 WPI:GLY, w:w). The two protein levels were chosen as the high and low protein concentrations used throughout the thesis. Nisin, lysozyme, EDTA and PP concentrations were determined and based on the MIC values for each antimicrobial across genera. The two levels used were 50% MIC and 150% MIC.

The objectives of this study were to test the effectiveness of mixed antimicrobial WPI films against five spoilage or pathogenic bacteria, to identify significant variables and to suggest the optimal film compositions for maximum inhibitory activity against each organism.

4.3. Materials and Methods

4.3.1. Determination of minimum inhibitory concentrations (MICs)

After determining the effect of the antimicrobial films containing single antimicrobials against the test microorganisms (Chapter 3), their optimal concentration in WPI films was determined. This concentration was determined using a least significant difference (LSD) test and was based on the results of the horizontal inhibition experiments containing one antimicrobial in films prepared at neutrality (Chapter 3).

Using the LSD test, the critical concentration (MIC, minimum concentration that produced inhibition) was determined based on the activity of nisin and lysozyme against the Gram+ organisms, EDTA against the Gram- organisms and PP against all the organisms. A mean MIC (100% MIC) for each antimicrobial was then used across

genera. The 50 and 150% MIC values were determined based on the 100% MIC. Table 4.1 depicts the MICs (and ± 50% MIC) of each antimicrobial and the WPI:GLY used in subsequent experiments.

4.3.2. Formation of mixed antimicrobial films and bacterial culture preparations

The film-forming solutions were produced in a manner similar to that used for the acidic film-forming solutions (described in section 3.3.1). The pH reactions of the film-forming solutions were first adjusted to 2.98 ± 0.05 prior to the addition of the antimicrobials in order to maintain the solubility of the antimicrobials together. The antimicrobial stock solutions were produced by individually dissolving the antimicrobials in deionized water (Table 4.1).

To produce the antimicrobial film solutions, the antimicrobial agents (nisin, lysozyme, EDTA and PP) were added in different combinations and introduced into the acidified protein film-forming solutions to produce 32 different film solution combinations (Table 4.2). Control film solutions (containing no antimicrobials) of three protein concentrations (60:40, 65:35 and 70:30 WPI:GLY) and a film (100% MIC) containing the antimicrobials at the 100% MIC level (Table 4.1) were also produced. The film solutions were then cast, dried, cut into 1 cm circular film discs and stored as previously described (section 3.3.3).

The test organisms and the inoculated pour plates were prepared in the same manner as described in section 3.3.4.

4.3.3. Horizontal inhibition assays of mixed antimicrobial films

Pre-cut film discs were placed upon BHI agar surfaces inoculated (pour plated) with test bacteria and incubated for the appropriate time and temperature. After incubation, the plates were examined for the presence of clear zones around the film disc on the inoculated media and the horizontal inhibitory activity (mm) was calculated as previously described (section 3.3.5).

4.3.4. Vertical inhibition and bacterial inhibition assays of mixed antimicrobial films

After determining the horizontal inhibitory activity produced by the 32 different combinations of the mixed antimicrobial films, the two film combinations that produced the greatest horizontal inhibitory activity, the 100% MIC films and a film without antimicrobials (control film) were tested against each organism for vertical inhibitory activity and bacterial recoveries were performed on the clear inhibition zones (Table 4.3).

The vertical inhibitory activity (mm) was determined as previously described in section 3.3.6. Bacterial inhibition adjacent and beneath films was quantified by viable recovery of cells from cleared zones in the agar as described in section 3.3.7.

4.3.5. Statistical Analysis

Analysis of variance (ANOVA) and least significant difference (LSD) tests were performed to determine the optimum antimicrobial concentrations (based on the horizontal inhibitory activity at neutrality of the antimicrobial films containing only one antimicrobial (Chapter 3)). The experimental design used was a two level (low and high), five factor (nisin, lysozyme, EDTA, PP and WPI) full factorial experimental design

(shown in Table 4.2) which was statistically analysed by the JMP IN program (version 3.2, SAS Institute Inc.) to identify individual factors as well as two way interactions between factors which significantly affected the inhibitory activity. An effect test (F test) and parameter estimates were conducted and tabulated results are Prob>F. This is the probability that a factor or interaction between factors does not affect the inhibitory activity.

The effect of a factor or its interaction with a second factor on the inhibitory activity was determined using an alpha value of 0.05. If the factor or interaction had no effect on the inhibitory activity the response would be > 0.05. However if the factor or interaction had an effect on the inhibitory activity the response would be < 0.05; negative (-) effects were indicative of factors and interactions between factors decreasing the inhibitory activity against a particular organism, while positive (+) effects were indicative of factors and interactions between factors increasing the inhibitory activity against a particular organism.

4.4. Results

All films produced were transparent, relatively flexible, strong enough to be handled and maintained their integrity throughout the experiments.

Control films (without antimicrobials) of different protein concentrations (60:40, 65:35 and 70:30 WPI:GLY) did not visually inhibit the test bacteria on agar in the horizontal or vertical directions (Figs. 4.1 to 4.4 and Table 4.4). However, when the number of viable bacteria in the agar zone beneath the control films was counted (Fig. 4.5 and 4.6), it was shown that these films in themselves were able to reduce on average the bacterial populations by 0.69 ± 0.12 log CFU/g compared to agar areas that were not

covered by film. All control films (60:40, 65:35 and 70:30 WPI:GLY) produced similar inhibitory patterns.

In general the vertical inhibitory activity of the tested antimicrobial films (7, 14 and 100% MIC films for *B. thermosphacta* and *L. monocytogenes*; 6, 16 and 100% MIC films for *S. aureus* and 14, 16 and 100% MIC films for *S.* Typhimurium) was larger than the horizontal inhibitory activity of the respective film types (Table 4.4 and Figs. 4.1 to 4.4). Furthermore, the results suggested that the films containing the antimicrobials at the 100 % MIC levels for each organism (except for *B. thermosphacta*) were not the most inhibitory films. This was observed with the horizontal and vertical inhibition assay tests as well as with the quantitative study, where the 100% MIC films yielded smaller reductions in viability than the other two antimicrobial film types tested against the specific organism.

E. coli 0157:H7 was not inhibited by any of the mixed antimicrobial films in the traditional (horizontal) agar test method, therefore statistical analysis, vertical inhibition assays and bacterial cell recoveries were not performed.

4.4.1. Antimicrobial effects against *B. thermosphacta*

All mixed antimicrobial films inhibited the bacteria on agar and produced horizontal inhibitory zones from ~5.2 ± 0.3 to 6.3 ± 0.2 mm (Fig. 4.1). Films 7 and 14 produced the largest zones, which were similar in dimension ~6.3 ± 0.3 mm. When the areas of clear inhibition produced by these films were examined for viable bacteria it was found that both films completely prevented growth by *B. thermosphacta* ($\leq$2 log CFU/g) (Fig. 4.5A). Upon statistical analysis, it was observed that nisin and lysozyme used alone were significant inhibitory factors (Table 4.5). However, a negative

interaction was observed between these two agents, such that their combination decreased the overall inhibitory activity against *B. thermosphacta*. In addition, a negative interaction was observed between lysozyme and PP against this organism.

4.4.2. Antimicrobial effects against *L. monocytogenes*

The mixed antimicrobial WPI films minimally inhibited this organism. Horizontal inhibitory zones ranged from ~0.4 ± 0.1 to 1.1 ± 0.1 mm (Fig. 4.2). Films 7 and 14 produced the largest zones of similar magnitudes, ~1.1 ± 0.1 mm. When viable organisms in the inhibition zones were quantified, the films reduced the organism by similar amounts (~1 log CFU/g) (Fig. 4.5B). Statistical analysis revealed that negative interactions occurred between nisin and lysozyme and between lysozyme and PP (reducing the inhibitory activity) (Table 4.5). However, PP alone was observed to be significant and effective against *L. monocytogenes*, while WPI concentration alone negatively affected the inhibitory activity.

4.4.3. Antimicrobial effects against *S. aureus*

The mixed antimicrobial films were the least effective against *S. aureus* and some film combinations did not inhibit the organism at all (Fig. 4.3). Films 6 and 16 produced the largest inhibitory zones of similar dimensions, ~0.9 ± 0.1 mm. When viable bacteria in the clear inhibition zones produced by these films were enumerated, it was found that these films reduced the bacteria by ~1.9 and 1.7 log CFU/g, respectively (Fig. 4.5C). Nisin alone was observed to be effective against *S. aureus*, however, it had negative interactions with lysozyme, EDTA and WPI (Table 4.5), such that lysozyme,

EDTA and WPI reduced the antimicrobial activity of nisin against the organism. In contrast, a positive interaction was observed between lysozyme and PP.

4.4.4. Antimicrobial effects against *S.* Typhimurium

The mixed antimicrobial WPI films had marginal effects on *S.* Typhimurium, producing horizontal inhibition zones from ~0.4 ± 0.2 to 1.4 ± 0.1 mm (Fig. 4.4). Films 14 and 16 produced the largest inhibition zones, with similar sizes (~1.4 ± 0.1 mm). When the clear inhibition zones produced by these films were quantified, results showed that the films reduced *S.* Typhimurium by similar amounts (~0.80 log CFU/g) (Fig. 4.6). Statistical analysis revealed that nisin effectively inhibited the organism; however, negative interactions occurred between nisin and lysozyme, nisin and EDTA and between nisin and WPI, such that the inhibitory activity was reduced (Table 4.5).

4.5. Discussion

In the present study, nisin was significantly inhibitory towards all organisms tested; however, no increase in its antimicrobial activity was observed when it was used with lysozyme, EDTA or with PP against any test organism. In addition, when lysozyme was used with EDTA or with PP, the combination did not yield increased activity against the Gram- bacterium *S.* Typhimurium. However, many authors have shown that nisin (Stevens *et al.*, 1991, 1992; Schved *et al.*, 1994; Cutter and Siragusa, 1995a, 1995b; Gao *et al.*, 1999; Zhang and Mustapha, 1999; Boziaris and Adams, 1999; Helander and Mattilla-Sandholm, 2000; Cutter *et al.*, 2001; Tu and Mustapha, 2002) or lysozyme (Garibaldi *et al.*, 1969; Hughey and Johnson, 1987; Payne *et al.*, 1994;

Razavi-Rohani and Griffiths, 1996; Ibrahim *et al.*, 1997) have increased antimicrobial activity against Gram- and + organisms in the presence of agents such as EDTA.

Gao *et al.* (1999) showed that nisin alone did not inhibit *B. thermosphacta* in BHI broth but when used with EDTA the viable numbers decreased by 2 log CFU/ml, and the same agents used together completely inhibited the organism (by 6.76 log CFU/ml) on fresh beef stored at 4°C for 25d (Tu and Mustapha, 2002). The growth of *E. coli* 0157:H7 and several species of *Salmonella* in buffer was inhibited by a nisin (50 μg/ml) plus EDTA (20 mM) combination, while each agent used alone did not inhibit the organisms (Stevens *et al.*, 1991; 1992). Schved *et al.* (1994) also showed that the viable numbers of *E. coli* and *S.* Typhimurium in buffer were reduced by ~ 4 and 3 log CFU/ml, respectively when a nisin (3200 IU/ml) plus EDTA (20 mM) combination was used. A nisin (100 IU/ml) plus EDTA (10 mM) combination inhibited *E. coli* in broth (pH 6.5), much better than when EDTA was used alone (Boziaris and Adams, 1999). Beef cubes treated with 50 μg/ml of pure nisin and 50 mM EDTA caused a slight reduction (0.42 log CFU/cm^2) in *E. coli* and *S.* Typhimurium numbers (Cutter and Siragusa, 1995a), however the organisms in a buffer system were inhibited to a greater extent (~4 and 2 log CFU/ml, respectively) (Cutter and Siragusa, 1995b).

Although some work has shown that EDTA enhances the effectiveness of nisin, others, like the present study did not demonstrate this. Zhang and Mustapha (1999) did not observe increased inhibition against *L. monocytogenes* and *E. coli* on fresh beef cubes using a nisin (5000 IU/ml) plus EDTA (20 mM) combination compared to a nisin solution used alone. In another study, the same combination and concentration of agents was found to be ineffective against *S.* Typhimurium on the same product (Tu and Mustapha, 2002).

In most of the literature reviewed, EDTA has been observed to enhance the activity of lysozyme mainly against Gram+ organisms, with a few references indicating that it increased the effectiveness of lysozyme against Gram- organisms. In the present study, lysozyme and EDTA did not produce significant interactions against any of the organisms tested. Hughey and Johnson (1987) demonstrated that although lysozyme was able to decrease the growth of *L. monocytogenes* on fresh vegetables, a lysozyme plus EDTA combination had a better inhibitory effect on the organism. In addition, *L. monocytogenes* and *S. aureus* in BHI broth have been shown to be inhibited to a greater extent using a lysozyme plus EDTA solution than with either agent used alone (Razavi-Rohani and Griffiths, 1996). However, the same agents used together did not yield greater inhibition against *E. coli* 0157:H7 or *S.* Typhimurium in BHI broth (Razavi-Rohani and Griffiths, 1996). In other work, it was demonstrated that a lysozyme ($\leq$200 μg/ml) plus EDTA ($\leq$2.5 mg/ml) combination exhibited a greater bactericidal effect against *L. monocytogenes*, decreasing numbers by 6 log CFU/ml in UHT processed milk; however, the combination of agents did not inhibit *S.* Typhimurium and did not yield increased inhibition over EDTA against *E. coli* in the same food product (Payne *et al.*, 1994). However, Garibaldi *et al.* (1969) and Ibrahim *et al.* (1997) showed that lysozyme used with EDTA decreased viable *S.* Typhimurium cells in egg white and *E. coli* cells in broth, respectively.

In contrast with the present study Chung and Hancock (2000) demonstrated that nisin (500 μg/ml) and lysozyme used together had a better inhibitory effect than either agent used alone against several Gram+ bacteria, including *B. thermosphacta*, *Lactobacillus sake*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *S. aureus* or *L. monocytogenes* in broth. The addition of nisin (27.5 U/ml)

and lysozyme (690 U/ml) together to freshly squeezed orange juice (without pulp) was observed to have a greater bactericidal effect against *S.* Typhimurium than when either agent was used alone; bacterial counts were reduced by $\leq$1.4 log CFU/ml compared to reduction caused by agents used alone (Liang *et al.*, 2002). Nattress *et al.* (2001) also showed that nisin and lysozyme, used alone or in combination, inhibited the growth of *B. thermosphacta* and *Carnobacterium* species in APT broth, pork juice and on fat and lean meat. It was observed that the use of the agents in combination had increased antimicrobial activity than the agents used alone on meat samples. Nisin (12 μg/ml) and lysozyme (5 mg/ml) used in combination acted synergistically on hot dogs and reduced *L. monocytogenes* and *S. aureus* numbers by ~2 log CFU/g after 3d of storage (Proctor and Cunningham, 1993). However in the present study, there were significant negative interactions between nisin and lysozyme against all organisms tested, which reduced the overall inhibitory activity. It is possible that the antimicrobial agents used together were not effective due to the concentrations used in our study or perhaps nisin or lysozyme were not able to effectively be released from the protein films.

Thus antimicrobial agents behave differently against different organisms and in different media. In addition, nisin or lysozyme used with EDTA appears to be more effective on organisms that are suspended in buffers rather than on food products or in nutritive media. It has been suggested that organisms attached on food surfaces can become protected against the action of bacteriocins by fat components in the food product (Dean and Zottola, 1996; Mahadeo and Tatini; 1994; Cutter and Siragusa, 1996a). In addition, antimicrobials (applied as a solution) over time can lose their activity on foods such as meats (Chung *et al.*, 1989; Fang and Lin, 1995) or can become degraded or inactivated by endogenous enzymes present in food (Rose *et al.*, 1999).

Furthermore, EDTA may have reduced antimicrobial action because other ions present in meat could interfere with EDTA's ability to chelate cations from the LPS of the OM of the organisms (Cutter and Siragusa, 1995b).

Other studies have shown that antimicrobial agents incorporated in a gel structure can remain stable and their antimicrobial activities have been observed to persist longer than when not immobilized (Fang and Lin, 1995; Cutter and Siragusa, 1996b; Cutter and Siragusa, 1997). This suggested that antimicrobial agents might be more effective when used as antimicrobial films rather than as solutions. However, the agents may have altered activities when they are incorporated into films due to interactions with film components. Ozdemir and Floros, (2003) reported that increasing concentrations of WPI and beeswax in WPI films decreased the diffusivity of potassium sorbate. In the present study, WPI concentrations alone were observed to negatively affect the inhibitory activity of test agents against *L. monocytogenes* and interacted negatively with nisin against *S. aureus* and *S.* Typhimurium. Corn zein films containing nisin or nisin plus EDTA were equally effective (~5.25 log CFU/ml) in reducing *L. monocytogenes* in broth after 48h (Hoffman *et al.*, 2001). Similarly, soy protein films containing nisin (4% w/w) or a nisin (4% w/w) plus lauric acid (8% w/w) combination produced equally good inhibitory films (~1 log CFU/ml reduction) against the same organism on the surface of turkey bologna (Dawson *et al.*, 2002). However, hydroxypropyl methyl cellulose (HPMC) films containing nisin (5000 IU/ml) or those containing nisin (5000 IU/ml) plus stearic acid (15% w/w) mixtures did not produce films with the same antimicrobial effectiveness (Sebti *et al.*, 2002). HPMC films without stearic acid, produced inhibition zones against *L. monocytogenes* that were 70% larger than the ones that contained the fatty acid. The authors suggested that the fatty acid

negatively affected the activity of nisin (Coma *et al.*, 2001; Sebti and Coma, 2002; Sebti *et al.*, 2002). A lysozyme plus EDTA combination in cast corn zein films was found to be effective against *E. coli* 0157:H7, however lysozyme or nisin plus EDTA incorporated into heat-pressed corn zein or soy protein films did not inhibit the organism (Padgett *et al.*, 1998). It was suggested that the higher temperatures needed to produce heat-pressed films may have caused the antimicrobials to lose some activity. Cha *et al.* (2002) observed that Na-alginate films which only contained nisin plus EDTA or lysozyme plus EDTA were not able to inhibit *E. coli* and *S.* Enteritidis; however, films containing all three agents were effective in inhibiting the organisms. Furthermore, Na-alginate films containing lysozyme, nisin, grapefruit seed extract (GFSE) and EDTA were shown to produce larger inhibition zones against *E. coli*, *S.* Enteritidis, *S. aureus* and *L. innocua*, than κ-carrageenan films containing the same antimicrobial agents at the same concentrations (Cha *et al.*, 2002). An antimicrobial gel coating containing a lysozyme plus nisin mixture and EDTA reduced *B. thermosphacta*, *L. monocytogenes* and *S.* Typhimurium by 3-4 log CFU/cm^2 on cooked ham and bologna sausages; however, it had no effect on *E. coli* (Gill and Holley, 2000).

Furthermore, the addition of a group of natural agents to an edible film does not necessarily guarantee that the film will be more inhibitory than films containing only one antimicrobial. Nonetheless, corn zein dissolved in propylene glycol with the addition of nisin (1000 IU/g) with or without calcium propionate (1%) reduced the initial *L. monocytogenes* population of 6.8 log CFU/g on ready to eat chicken by ~5 and 3 log CFU/g, respectively, after 24d (Janes *et al.*, 2002). Soy protein films containing only 2.6% (w/w) malic acid decreased *L. monocytogenes* and *E. coli* by 2.8 and 2.1 log CFU/ml, respectively, while those containing nisin (205 IU/g protein) and malic acid

together had decreased action against both organisms (Eswaranandam *et al.*, 2004). Furthermore, Padgett *et al.* (2000) observed that corn zein films containing lauric acid (0, 4 and 8% w/w) and nisin (5 mg/g film) produced visible inhibition zones against *Lactobacillus plantarum* on agar; however, as the concentration of lauric acid increased in the presence of nisin, the inhibition zones decreased.

The results of the experiments reported here suggest that nisin has a broad range of inhibitory action and enhanced the overall antimicrobial activity of the mixed antimicrobial WPI films against all organisms tested. Furthermore, the results suggest that there are significant interactions between pairs of antimicrobial agents, and overall activity depends upon the target organisms. There were no positive interactions between nisin, lysozyme, EDTA and PP against the Gram- bacterium *S.* Typhimurium. In addition, no positive interactions were observed between nisin and EDTA, lysozyme and EDTA, nisin and PP or EDTA and PP against any organism. Strong negative interactions were observed between nisin and lysozyme against all organisms; nisin and EDTA against *S. aureus* and *S.* Typhimurium and between lysozyme and PP against *B. thermosphacta* and *L. monocytogenes*. However, interactions between lysozyme and PP resulted in increased antimicrobial activity against *S. aureus*. In addition, negative interactions between nisin and WPI were observed against *S. aureus* and *S.* Typhimurium.

The results obtained from this study suggested that nisin, lysozyme, EDTA and PP can interact negatively among themselves or with WPI, and that optimum mixed antimicrobial WPI films for each organism must be formulated to contain higher levels of nisin with lower concentrations of the other agents.

Table 4.1. Minimum inhibitory concentrations (MICs) of antimicrobial agents and WPI:GLY concentrations used in optimization experiments

Antimicrobials and WPI:GLY	**MICs[a]**		
	50% MIC	**100% MIC[b]**	**150% MIC**
Nisin (mg/g)	0.8	1.6	2.4
Lysozyme (mg/g)	21	42	63
EDTA (mM)	8	17	25
Propyl paraben (ppm)	150	300	450
WPI:GLY (w:w)[c]	60:40	65:35	70:30

[a] MICs were determined by least significant difference (LSD) tests
[b] 100% MIC values are mean MICs across genera
[c] Whey protein isolate concentration used in WPI films made with glycerol (GLY): 60:40, 65:35, 70:30 WPI:GLY (w:w)

Table 4.2. Mixed antimicrobial WPI film compositions[a]

Film combination number	Nisin (mg/g)	Lysozyme (mg/g)	EDTA (mM)	Propyl paraben (ppm)	WPI:GLY (w:w)
1	2.4	63	25	450	70:30
2	2.4	63	25	450	60:40
3	2.4	63	25	150	70:30
4	2.4	63	25	150	60:40
5	2.4	63	8	450	70:30
6	2.4	63	8	450	60:40
7	2.4	63	8	150	70:30
8	2.4	63	8	150	60:40
9	2.4	21	25	450	70:30
10	2.4	21	25	450	60:40
11	2.4	21	25	150	70:30
12	2.4	21	25	150	60:40
13	2.4	21	8	450	70:30
14	2.4	21	8	450	60:40
15	2.4	21	8	150	70:30
16	2.4	21	8	150	60:40
17	0.8	63	25	450	70:30
18	0.8	63	25	450	60:40
19	0.8	63	25	150	70:30
20	0.8	63	25	150	60:40
21	0.8	63	8	450	70:30
22	0.8	63	8	450	60:40
23	0.8	63	8	150	70:30
24	0.8	63	8	150	60:40
25	0.8	21	25	450	70:30
26	0.8	21	25	450	60:40
27	0.8	21	25	150	70:30
28	0.8	21	25	150	60:40
29	0.8	21	8	450	70:30
30	0.8	21	8	450	60:40
31	0.8	21	8	150	70:30
32	0.8	21	8	150	60:40

[a] The experiment was set up as a 2 level, 5 factor full factorial experimental design

Table 4.3. Film types used in the determination of vertical inhibitory activity and inhibition of bacterial growth caused by mixed antimicrobial WPI films against the test microorganisms[a]

Microorganisms	Film combination number
B. thermosphacta	7[b], 14, 100% MIC[c], and control[d]
L. monocytogenes	7, 14, 100% MIC, and control
S. aureus	6, 16, 100% MIC, and control
S. Typhimurium	14, 16, 100% MIC, and control

[a] *E. coli* 0157: H7 was not tested
[b] Films 6, 7, 14 and 16 are described in Table 4.2
[c] 100% MIC films contained 1.6 mg/g nisin, 42 mg/g lysozyme, 17 mM EDTA and 300 ppm PP in 65:35 WPI:GLY film (w:w)
[d] Control films [60:40, 65:35 and 70:30 WPI:GLY (w:w)] contained no antimicrobials

Table 4.4. Vertical inhibitory activity (mm) of mixed antimicrobial WPI films

	Film combination number					
Microorganisms	**6**[a]	**7**	**14**	**16**	**100% MIC**	**Control**[b]
B. thermosphacta	N/A[c]	8.83 ± 0.08	9.98 ± 0.42	N/A	8.50 ± 0.05	0
L. monocytogenes	N/A	3.08 ± 0.07	2.94 ± 0.10	N/A	2.35 ± 0.08	0
S. aureus	2.98 ± 0.04	N/A	N/A	2.57 ± 0.20	2.00 ± 0.07	0
S. Typhimurium	N/A	N/A	3.33 ± 0.06	3.21 ± 0.13	2.96 ± 0.05	0
E. coli 0157:H7	N/T[d]	N/T	N/T	N/T	N/T	N/T

[a] Antimicrobials used in film: 6 - 2.4 mg/g nisin, 63 mg/g lysozyme, 8 mM EDTA and 450 ppm PP in 60:40 WPI:GLY film
7 - 2.4 mg/g nisin, 63 mg/g lysozyme, 8 mM EDTA and 150 ppm PP in 70:30 WPI:GLY film
14 - 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 450 ppm PP in 60:40 WPI:GLY film
16 - 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 150 ppm PP in 60:40 WPI:GLY film
100% MIC - 1.6 mg/g nisin, 42 mg/g lysozyme, 17 mM EDTA and 300 ppm PP in 65:35 WPI:GLY film

[b] Control film [60:40, 65:35 and 70:30 WPI:GLY (w:w)] contained no antimicrobials

[c] N/A - not applicable

[d] N/T - not tested

Table 4.5. Significance[a] of two agent inhibitory interactions against test bacteria

Effect	*B. thermosphacta*	*L. monocytogenes*	*S. aureus*	*S.* Typhimurium
nisin	0.0099^{+}	0.5905	$< 0.0001^{+}$	$< 0.0001^{+}$
lysozyme	0.0119^{+}	0.8596	0.4828	0.2490
nisin*lysozyme	$< 0.0001^{-}$	$< 0.0001^{-}$	0.0064^{-}	$< 0.0001^{-}$
EDTA	0.9019	0.2384	0.0586	0.2043
nisin*EDTA	0.0663	0.8929	$< 0.0001^{-}$	$< 0.0001^{-}$
lysozyme*EDTA	0.4838	0.7964	0.5750	0.6505
PP	0.1325	0.0263^{+}	0.5222	0.7062
nisin*PP	0.9397	0.1066	0.1698	0.6505
lysozyme*PP	0.0425^{-}	0.0001^{-}	0.0012^{+}	0.8157
EDTA*PP	0.4143	0.3706	0.2771	0.7170
WPI	0.1613	0.0427^{-}	0.1034	0.1386
nisin*WPI	0.2232	0.4734	0.0497^{-}	0.0226^{-}
lysozyme*WPI	0.1367	0.1165	0.6010	0.8970
EDTA*WPI	0.6902	0.1443	0.1737	0.0650
PP*WPI	0.2473	0.1090	0.4538	0.6693

[a] Significance at the 95% confidence level (studied in 5 factor, 2 level tests) with $^{-}$ denoting significant factors in which the factor or its interaction had a negative effect on the inhibitory activity and with $^{+}$ denoting significant factors in which the factor or its interaction had a positive effect on the inhibitory activity.

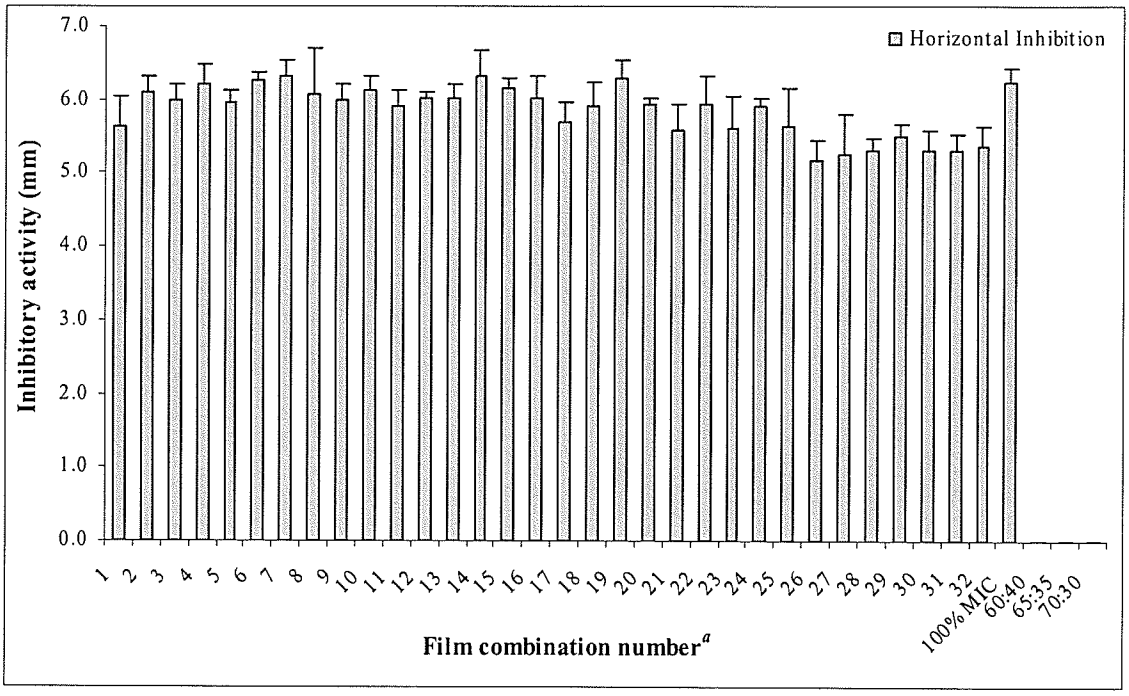

Figure 4.1. Horizontal inhibitory activity (mm) of mixed antimicrobial WPI films against *B. thermosphacta*

[a] Refer to Table 4.2 for exact film compositions and concentrations

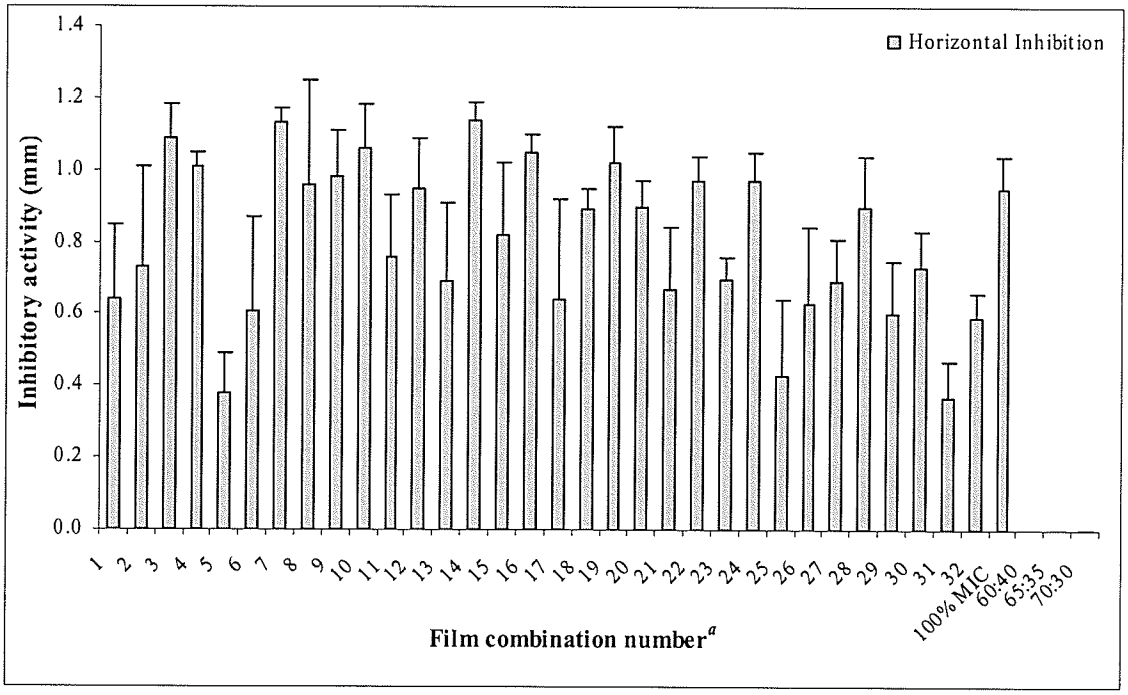

Figure 4.2. Horizontal inhibitory activity (mm) of mixed antimicrobial WPI films against *L. monocytogenes*

[a] Refer to Table 4.2 for exact film compositions and concentrations

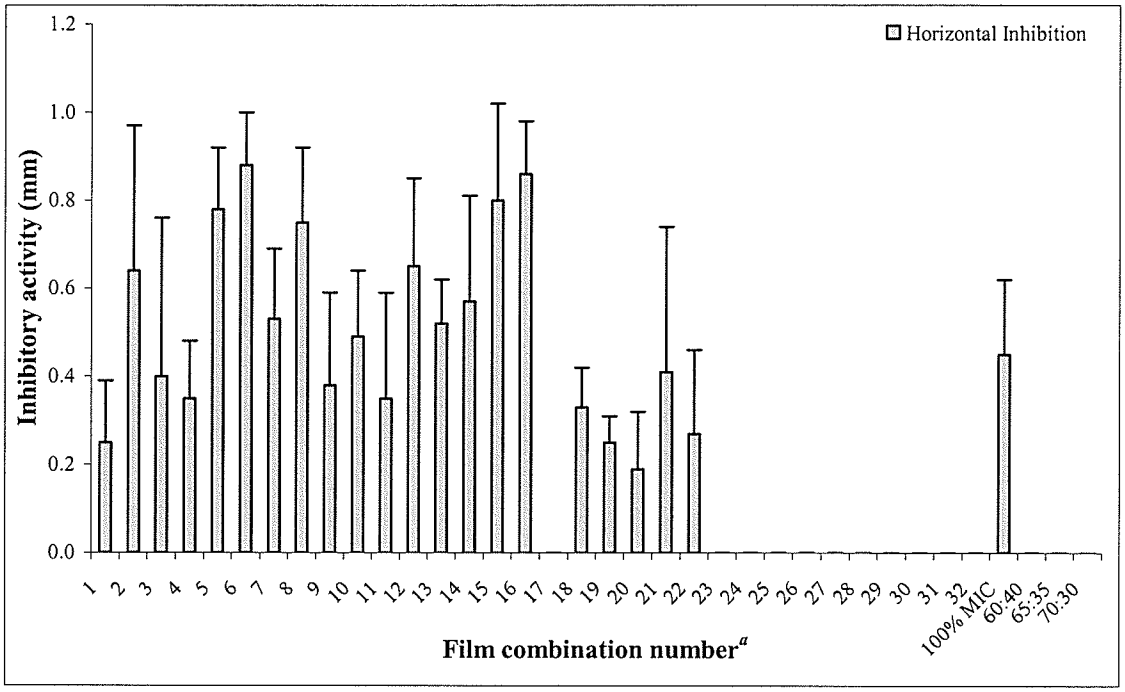

Figure 4.3. Horizontal inhibitory activity (mm) of mixed antimicrobial WPI films against *S. aureus*

[a] Refer to Table 4.2 for exact film compositions and concentrations

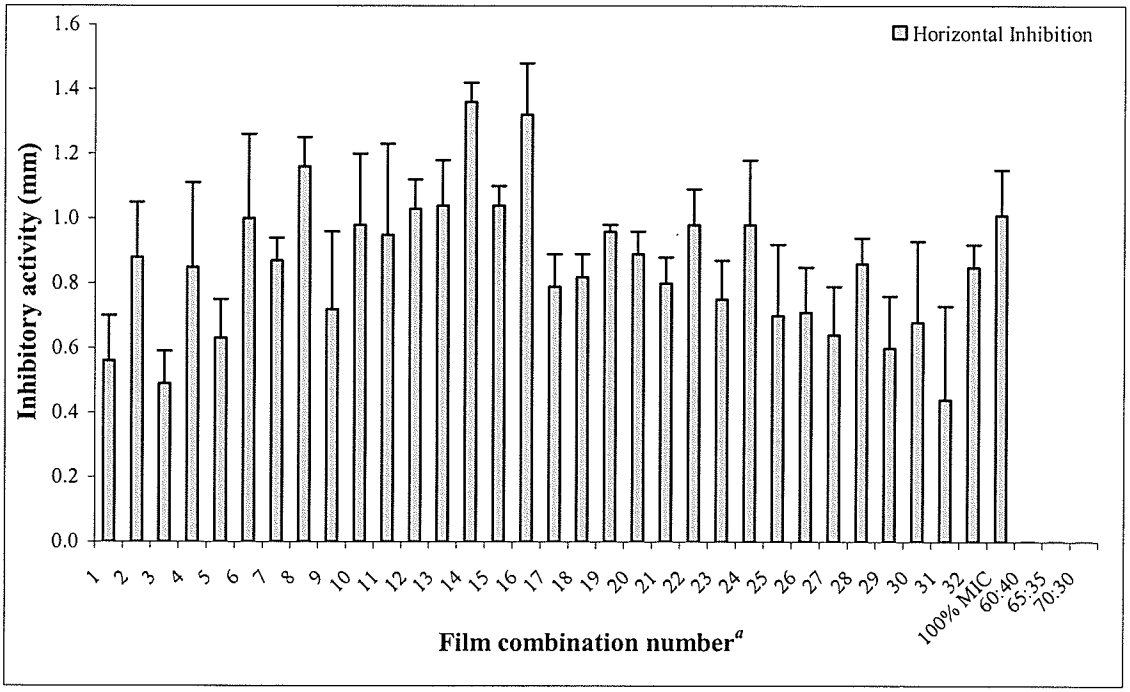

Figure 4.4. Horizontal inhibitory activity (mm) of mixed antimicrobial WPI films against *S.* Typhimurium

[a] Refer to Table 4.2 for exact film compositions and concentrations

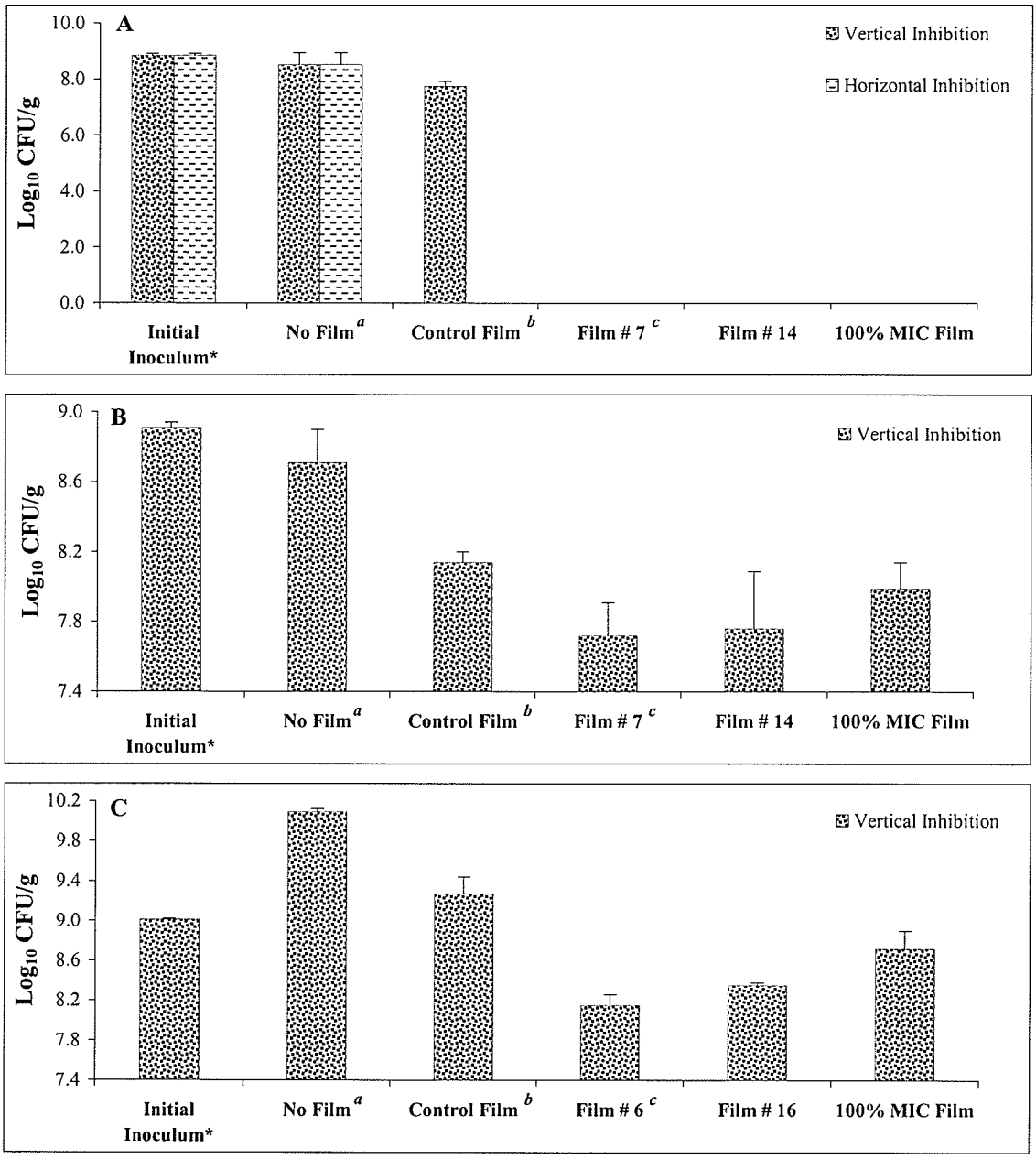

Figure 4.5. Quantification of bacterial inhibition by mixed antimicrobial WPI films against gram positive organisms: A - *B. thermosphacta*, B - *L. monocytogenes* and C - *S. aureus*

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

[b] Control film [(60:40, 65:35, 70:30 WPI:GLY (w:w)] contained no antimicrobials

[c] Antimicrobials used in film 6 - 2.4 mg/g nisin, 63 mg/g lysozyme, 8 mM EDTA and 450 ppm PP in 60:40 WPI:GLY film
7 - 2.4 mg/g nisin, 63 mg/g lysozyme, 8 mM EDTA and 150 ppm PP in 70:30 WPI:GLY film
14 - 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 450 ppm PP in 60:40 WPI:GLY film
16 - 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 150 ppm PP in 60:40 WPI:GLY film
100% MIC film - 1.6 mg/g nisin, 42 mg/g lysozyme, 17 mM EDTA and 300 PP in 65:35 WPI:GLY film

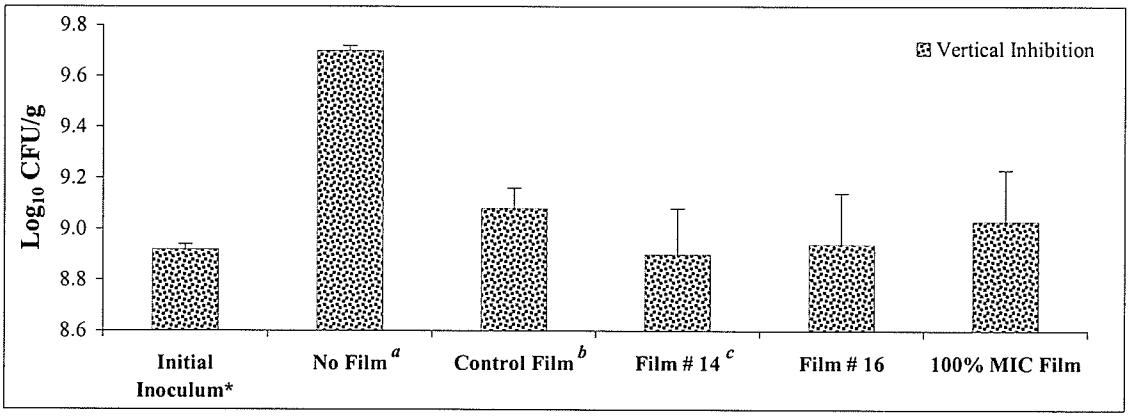

Figure 4.6. Quantification of bacterial inhibition by mixed antimicrobial WPI films against *S.* Typhimurium

* was verified by plate count on BHI agar plates

[a] No Film - control bacterial agar area not affected by a WPI film

[b] Control film [(60:40, 65:35, 70:30 WPI:GLY (w:w)] contained no antimicrobials

[c] Antimicrobials used in film 6 - 2.4 mg/g nisin, 63 mg/g lysozyme, 8 mM EDTA and 450 ppm PP in 60:40 WPI:GLY film
7 - 2.4 mg/g nisin, 63 mg/g lysozyme, 8 mM EDTA and 150 ppm PP in 70:30 WPI:GLY film
14 - 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 450 ppm PP in 60:40 WPI:GLY film
16 - 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 150 ppm PP in 60:40 WPI:GLY film
100% MIC film - 1.6 mg/g nisin, 42 mg/g lysozyme, 17 mM EDTA and 300 PP in 65:35 WPI:GLY film

CHAPTER # 5

Inhibition of *Brochothrix thermosphacta* by an Antimicrobial Whey Protein Isolate Coating on Fresh Pork

5.1. Abstract

Consumers demand packaging systems that not only protect food commodities, but are environmentally friendly and extend shelf-life. Edible antimicrobial films have the potential to improve food quality, extend product shelf-life and reduce plastic packaging waste.

A previous study determined that WPI films containing a combination of nisin, lysozyme, EDTA and propyl paraben (PP) were able to inhibit the growth of *Brochothrix* (*B.*) *thermosphacta* on BHI agar as measured by zones of inhibition and viability tests.

A WPI coating containing 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 450 ppm PP was tested for its ability to reduce the viability of *B. thermosphacta* on refrigerated fresh pork. Fresh pork loin cubes inoculated with *B. thermosphacta* were treated with a control WPI coating (without antimicrobials) or an antimicrobial WPI coating (with all four antimicrobials), vacuum packaged and stored at 4°C for 12d. Samples were analysed for *B. thermosphacta*, total bacterial survivors and lactic acid bacteria present at days 0, 3, 6, 9 and 12.

The antimicrobial WPI coating was bactericidal and decreased *B. thermosphacta* numbers by ~ 4 log CFU/cm^2 on pork loin cubes throughout the storage period.

5.2. Introduction

Edible films and coatings are increasingly being developed as new types of packaging materials to increase food quality since plastic packaging materials are not easily biodegradable and create substantial amounts of solid waste (Krochta and Mulder-Johnston, 1997).

Excess whey, a protein by-product from the cheese manufacturing industry (Gennadios *et al.*, 1994) has been converted into whey protein concentrate (WPC) and further purified to yield whey protein isolate (WPI). Both of these products have been extensively used in the production of edible films (McHugh *et al.*, 1994; McHugh and Krochta, 1994a, 1994b; Banerjee and Chen, 1995; Maté and Krochta, 1996, 1998; Shellhamer and Krochta, 1995; Pérez-Gago and Krochta, 1999; Miller *et al.*, 1997, 1998; Guilbert *et al.*, 1996; Gontard *et al.*, 1996; Ankar, 2000).

In recent years, more interest has arisen in the use of natural antimicrobials. Antimicrobials are increasingly being incorporated into film-forming solutions to form films or coatings which are used to reduce the growth of spoilage and pathogenic bacteria, yeast and mold, improving the shelf-life and safety of treated foods (Han, 2000 Padgett *et al.*, 1998). The antimicrobials in the films can directly inhibit the bacteria at the surface of the food or can diffuse through its surface layers thereby preventing further microbial development (Han, 2000). Many antimicrobials have been used to produce types of antimicrobial films which are edible (Han, 2000, 2002, 2003, 2005; Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002; Cagri *et al.*, 2004).

Brochothrix (B.) thermosphacta is the principal spoilage organism found on fresh and cured packaged meats (Tu and Mustapha, 2002) that have not been properly vacuum packaged and refrigerated. This is because the organism has the ability to tolerate high

salt concentrations and in the presence of low O_2 (>0.2%) levels can grow at low water activity and low temperature (Holley, 1999). Although the organism does not produce meat discoloration it leads to objectionable odours and quickly leads to meat spoilage. However, if meat products are properly vacuum packaged with good O_2 barrier films, such as polyvinylediene chloride (PVDC)-based packaging bags, *B. thermosphacta* should not cause problems (Holley, 1999). Nonetheless, meat products in the retail industry are packaged and distributed on trays that are overwrapped with high gas-permeable films providing *B. thermosphacta* optimal conditions to grow, if present.

Protein films such as WPI films are excellent O_2 barriers at low to moderate RH (McHugh and Krochta, 1994a) and could be used instead of PVDC to control the growth of the organism and to alleviate biodegradability concerns. However, fresh meat surfaces are moist making the use of WPI films or coatings alone less desirable. Therefore, natural antimicrobials have been incorporated into solutions to create edible antimicrobial films which have the potential of being a good adjunct packaging material that can protect foods from microbial contamination and early spoilage.

Nisin and lysozyme are natural antimicrobials that act by interfering with the stability of the cytoplasmic membrane and cell wall structure of sensitive cells (Padgett *et al.*, 1998). Nisin (a bacteriocin) is a biologically active peptide which contains 34 amino acids and is produced by *Lactococcus lactis* subspecies *lactis* (Winkowski *et al.*, 1996; Crandall and Montville, 1998; Verheul *et al.*, 1997). Due to its charge and small size it is suggested that nisin monomers aggregate to form oligomeric pores in the cytoplasmic membrane as it inserts itself in the membrane (Winkowski *et al.*, 1994, 1996; Carneiro de Melo *et al.*, 1996; Crandall and Montville, 1998; Padgett *et al.*, 1998). As a result, low molecular weight cytoplasmic components leach out and the cellular

proton motive force is disrupted (Bruno *et al.*, 1992; Davies and Adams, 1994; Verheul *et al.*, 1997) leading to termination of biosynthesis, cell inactivation and consequently cell death (Verheul *et al.*, 1997; Davies and Adams, 1994; Winkowski *et al.*, 1994).

Lysozyme is an enzyme composed of 129 amino acid residues that cleaves the β(1,4)-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglycosamine of the peptidoglycan in the cell wall of Gram+ bacteria (Lück and Jager, 1997; Payne *et al.*, 1994; Liberti *et al.*, 1996; Appendini and Hotchkiss, 1997). Cell wall hydrolysis damages the cellular structural integrity and subsequently leads to cell lysis.

Neither of these agents have any substantial action against Gram- bacteria because these possess an outer membrane (OM) which shields them from antimicrobial action.

While lysozyme and nisin are not effective against Gram- bacteria, EDTA and propyl paraben (PP) have been shown to be active (Razavi-Rohani and Griffiths, 1996; Payne *et al.*, 1994). Chelating agents such as EDTA are found to alter the OM in Gram- bacteria by complexing with magnesium and calcium (Padgett *et al.*, 1998). When these cations are chelated from the OM, the membrane becomes destabilized and bacterial inhibition results (Boziaris and Adams, 1999; Padgett *et al.*, 1998). The antimicrobial PP is effective against both Gram- and + bacteria, however it is most inhibitory against Gram+ bacteria and fungi. Parabens inhibit membrane transport of electrons, essential amino acids and nutrients (Baranowski and Nagel, 1983; Lück and Jager, 1997).

When antimicrobial agents are used in combination with each other or with EDTA they may have increased effectiveness against Gram- and + organisms. It is suggested that once EDTA destabilizes the membrane, other antimicrobials used in parallel can reach sensitive sites at cell surfaces they would not normally find accessible.

Several antimicrobials (including nisin, lysozyme and EDTA) in solutions (Cutter and Siragusa, 1994a, 1994b, 1995a, 1996a, 1996b, 1997; Fang and Lin, 1995; Murray and Richard, 1997; Shefet *et al.*, 1995; Zhang and Mustapha, 1999; Tu and Mustapha, 2002; Ariyapitipun *et al.*, 2000; Natrajan and Sheldon, 2000a; Scannell *et al.*, 2000) or in coatings or films (Ming *et al.*, 1997; Franklin *et al.*, 2004; Cagri *et al.*, 2002, 2003; Gill and Holley, 2000; Janes *et al.*, 2002; Dawson *et al.*, 2002) have been used to treat the surface of fresh or cured meat products and retard bacterial activity.

Nisin used as a spray solution reduced inoculated *L. innocua*, *B. thermosphacta* and *Carnobacterium divergens* by 3, 2.8 and 3.1 log CFU/cm^2, respectively on beef carcass surfaces (Cutter and Siragusa, 1994a). Further reduction of *L. innocua* (2.83 log CFU/cm^2) and *B. thermosphacta* (4.5 log CFU/cm^2) on beef carcass tissue was achieved when the nisin spray treatment was followed by vacuum packaging and refrigeration (Cutter and Siragusa, 1996a). Zhang and Mustapha (1999) effectively inhibited *E. coli* 0157:H7 and *L. monocytogenes* on fresh beef cubes using a 5000 IU/ml nisin (1.02 and 2.01 log CFU/cm^2, respectively) and a 5000 IU/ml nisin plus 20 mM EDTA solution (0.8 and 0.99 log CFU/cm^2, respectively) when followed by vacuum packaging and storage at 4°C. In addition, under the same conditions, *B. thermosphacta* was completely inhibited (by 6.76 log CFU/ml) by nisin and by nisin plus EDTA, however, neither solution inhibited *S.* Typhimurium (Tu and Mustapha, 2002). When beef cubes were treated with a solution of 400 IU/ml nisin alone or in combination with 2% (w/v) lactic acid or 2% (w/v) polylactic acid, vacuum packaged and stored at 4°C for 42d *L. monocytogenes* numbers were decreased from ~5.33 log CFU/cm^2 to 2.21, 0.89 or 0.84 log CFU/cm^2, respectively (Ariyapitipun *et al.*, 2000). When fresh turkey drumstick skin was dipped in a solution of 100 μg/ml nisin, 5mM EDTA, 3% w/v citric acid and 0.5%

Tween 20 for 30 min *S.* Typhimurium cells were reduced by ~5 log CFU/ml more than by a 20 ppm chlorine solution (Shefet *et al.*, 1995).

Nisin immobilized in a calcium alginate gel successfully inhibited *L. monocytogenes*, *B. thermosphacta* and *S.* Typhimurium on cooked pork tenderloins (Fang and Lin, 1995), on the surface of lean and adipose beef tissue (Cutter and Siragusa, 1996b) and on broiler samples, respectively (Natrajan and Sheldon, 2000a). Beef surfaces coated with a calcium alginate gel containing 100 μg/ml nisin and processed to form ground beef also inhibited *B. thermosphacta* (Cutter and Siragusa, 1997), and was more effective than when nisin was applied without the gel. Cellulose-based inserts containing nisin inhibited *L. innocua* and *S. aureus* when placed between cheese slices or cooked ham that were stored under modified atmosphere packaging (MAP) at 4°C (Scannell *et al.*, 2000).

Edible polysaccharide and protein coatings or films with nisin or nisin plus other agents have also been used to control the proliferation of organisms on the surface of meat products. Cellulosic casings coated with the bacteriocin, pediocin, inhibited the growth of *L. monocytogenes* ≤3 logs on fresh turkey breasts, ham and beef after 12 weeks at 4°C, compared to uncoated casings (Ming *et al.*, 1997). Hot dogs, surface inoculated with *L. monocytogenes*, vacuum packaged in methylcellulose/hydroxypropyl methylcellulose (MC/HPMC) pouches containing nisin and stored at 4°C for 60d reduced numbers of the organism to undetectable levels (≤ 2.9 log CFU/package) (Franklin *et al.*, 2004). *P*-aminobenzoic acid (PABA) or sorbic acid-containing WPI films decreased numbers of *L. monocytogenes*, *S.* Typhimurium and *E. coli* 0157:H7 by 3.4 - 4.1, 3.1 - 4.1 and 3.1 - 3.6 log CFU/g, respectively, on bologna and summer sausage slices after 21d at 4°C (Cagri *et al.*, 2002). However, on hot dogs inoculated

with *L. monocytogenes* that were vacuum packaged and stored at 4°C, PABA-containing WPI films only inhibited the growth of the organism after 42d (Cagri *et al.*, 2003). Gill and Holley (2000) used an antimicrobial gel coating containing a lysozyme plus nisin and EDTA mixture and reduced both *B. thermosphacta* and *L. monocytogenes* ≤4 log CFU/cm^2 and *S.* Typhimurium by 3-4 log CFU/cm^2 on cooked ham and bologna sausage slices; however, the coating had no effect on *E. coli* 0157:H7. Corn zein films dissolved in propylene glycol or ethanol with the addition of nisin and calcium propionate inhibited *L. monocytogenes* by 5 and 4 log CFU/g, respectively, after 24d on ready-to-eat chicken (Janes *et al.*, 2002). Soy protein films containing lauric acid and those containing nisin or nisin plus lauric acid applied on the surface of turkey bologna decreased numbers of *L. monocytogenes* by 0.5 and 1 log CFU/ml, respectively, after 21d at 4°C while control films caused minimal reductions (< 0.05 log CFU/ml) of the bacteria (Dawson *et al.*, 2002).

Previous work (Chapter 4) demonstrated that an antimicrobial WPI film containing 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 450 ppm PP (film 14) prevented the growth of *B. thermosphacta* on BHI agar plates incubated at room temperature for 48h. Although films 7 and 14 produced similar horizontal inhibitory activities against *B. thermosphacta*, film 14 produced a larger vertical inhibitory activity and therefore it was chosen for this study.

The objective of this study was to determine if the growth of *B. thermosphacta* inoculated on pork loin cubes could be inhibited by an antimicrobial WPI coating containing a mixture of nisin, lysozyme, EDTA and PP.

5.3. Materials

Boneless pork loins individually vacuum packaged were donated by Maple Leaf Pork (Warman Rd., Winnipeg, MB). Low-O_2 permeable (O_2 transmission <15 $cm^3/cm^2/day \cdot atm \cdot 23°C$) polyvinylediene chloride (PVDC) packaging bags (16.6 by 17.2 cm, Deli*1) were provided by Winpak (Winnipeg, MB). Whey protein isolate (WPI) powder was donated by Davisco Foods International (Le Sueur, MN, USA). Lysozyme (> 97% pure) and nisin (30 % pure) were donated by Canadian Inovatech Inc. (Abbotsford, BC). Ethylenediaminetetraacetic acid (EDTA), n-propyl p-hydroxy benzoate (propyl paraben, PP) and glycerol (GLY) were from Sigma Chemicals Co. (St. Louis, MO., USA). Brain Heart Infusion (BHI) agar and broth and deMan, Rogosa and Sharpe (MRS) agar were from Difco, Becton Dickinson and Company (Sparks, MD, USA). Polypeptone (peptone) was from BBL, Becton Dickinson and Company (Cockeysville, MD, USA). Streptomycin thallous acetate actidione (STAA) agar base and STAA selective supplement were from Oxoid Ltd. (Basingstoke, Hampshire, England). An Autoplate 4000 equipped with a CASBA-4 automated counting system was used for surface plating on pre-poured agar media (Spiral Biotech Inc., Bethesda, MD.). Anaerobic jars were used to enumerate lactic acid bacteria (LAB) and anaerobic conditions were maintained using the Gas Pak plus anaerobic system with a palladium catalyst (BBL). Sterile filter stomacher bags (~18 x 30.5 cm; filtra-bags) from VWR Int. (Mississauga, ON) and a stomacher (Model 400, A. J. Seward, London) were used to homogenize the meat samples.

5.4. Methods

5.4.1. Bacterial culture maintenance

B. thermosphacta B2, originally isolated from fresh pork (Dr. G. Greer, Agriculture and Agri-Food Canada, Lacombe, AB) was used. Cultures were transferred monthly and maintained on BHI agar slants at 4°C. For experimental use, cultures were streaked on BHI plates and a single colony from the BHI agar plates was inoculated in 10 ml BHI broth and incubated overnight at 24°C. From the incubated broth, fresh BHI agar plates were streaked and incubated to ensure purity of the culture.

B. thermosphacta was recovered from inoculated pork samples using the selective medium, STAA agar, and incubated aerobically at 24°C for 48 h.

The total LAB were enumerated from pork samples using MRS agar incubated in anaerobic jars at 24°C for 48 h.

5.4.2. Coating solution formation

Aqueous solutions of 10 % (w/w) WPI powder were prepared with deionized water and glycerol (GLY) as the plasticizer. The plasticizer was added to the protein and water solution to produce a 60:40 (w:w) WPI/plasticizer ratio (WPI:GLY). The solutions were stirred using a magnetic bar to ensure that the protein was properly dissolved, were heated at 90°C for 30 min in a water bath to denature the protein and cooled at 4°C.

When the solutions were cooled to about 15°C, the pH of the coating solution (7.12 ± 0.05) was adjusted to 3.02 ± 0.05 using 1M HCl monitored with a pH meter (Accumet model 910, Fisher Scientific).

The antimicrobial agents were individually dissolved in sterile deionized water to produce 4 stock solutions [nisin (2.4 mg/g WPI in the film), lysozyme (21 mg/g WPI in the film), EDTA (8 mM) and PP (450 ppm)]. The solutions were filter sterilized separately and then added into the 60:40 WPI:GLY solution, to produce the antimicrobial coating solution. A control coating solution was prepared without the antimicrobials.

5.4.3. Preparation of pork samples

Fresh pork loins were obtained from Maple Leaf Pork the day prior to the experiment and kept overnight at 4°C. On the day of the experiment the loins were held at –18°C for about 5 to 6 h to ensure that just the outer surface of the meat became frozen. The outer surface of the meat was then trimmed away with a stainless steel knife sanitized with 200 ppm aqueous chlorine. The loins were then cut into 2 cm^3 cubes, weighing approximately 10 ± 1 g and separated into 6 treatment groups [1, control samples; 2, control-coated samples; 3, antimicrobial-coated samples; 4, bacteria-inoculated samples; 5, bacteria-inoculated control-coated samples; and 6, bacteria-inoculated antimicrobial-coated samples] (Table 5.1). Three cubes per treatment were prepared and samples for all 5 subsequent analyses were prepared simultaneously.

A 50 ml BHI broth *B. thermosphacta* culture was prepared by transferring a loopful of growth from pure culture plates to the broth and incubating overnight at room temperature. Following incubation the culture was adjusted to contain a bacterial population of about 10^8 CFU/ml. This was done by taking 30 ml of the bacterial culture and centrifuging at 5500 rpm for 10 min at 15°C (Sorvall RC2-B refrigerated centrifuge,

Du Pont, Newtown, CT). The supernatant was discarded and the pellet re-suspended in 30 ml of autoclaved deionized water and re-centrifuged at 5500 rpm for 10 min at 15°C. The pellet was then re-suspended in sterile deionized water and diluted to an OD of 1.0 at 600 nm (Ultrospec 2000, Pharmacia Biotech Inc., Baie D'Urfe, QC) to yield 8 log CFU/ml. This was confirmed by plating on STAA agar plates. A dipping bath containing the bacteria was prepared by diluting 2 ml of culture in 2 L of 0.1% peptone water. Following meat treatments, there was an initial *B. thermosphacta* population of approximately 3 log CFU/cm^2 on the surface of the inoculated meat.

For each treatment, the pork cubes were pierced with a sterile threaded needle and hung on the attached sterile thick thread, to ensure that when they were dipped into the various baths and hung in air to dry, there would be reasonably even coating distribution. Prior to packaging, the thread between each pork cube was cut using sterile scissors and 3 pork cubes were placed together in packaging bags.

Control pork samples (treatment 1) did not receive any treatment and were simply vacuum packed and heat-sealed in low O_2 permeable PVDC plastic bags using a Bizerba Canada Inc., model GM-2002 vacuum packaging machine (Mississauga, ON).

Control and antimicrobial-coated samples (treatments 2 and 3) were dipped twice for 30s each time, in a 10% aqueous solution of 60:40 WPI:GLY or in a 10% aqueous solution of 60:40 WPI:GLY containing the antimicrobials, respectively, and allowed to dry in a laminar airflow hood for 15 min between each dip to set the coating and to permit even surface coverage. Samples of each treatment were then separately vacuum packaged.

For treatments 4, 5 and 6, the pork loin cubes were first inoculated by dipping them into the *B. thermosphacta* bath for 30s and then dried in a laminar airflow hood for 2 min. The pork cubes for treatment 4 were then vacuum packaged and those for treatment 5 and 6 were coated with either the control or the antimicrobial-coating, as in treatments 2 and 3, and then vacuum packaged.

Three pork cubes from each treatment group were used as zero time samples, (held at 4°C and tested within 5 h). The other vacuum packed cubes (3/bag, 4 bags/treatment) were placed at 4°C and incubated for subsequent sampling every 3d up to 12d after inoculation.

At each sampling day, the pork cubes in one bag from each treatment group were unpackaged and individually placed in a sterile stomacher filtra-bag with 90 ml of 0.1% peptone water and blended for 1.5 min to produce a 10^{-1} dilution. Each sample was serially diluted with peptone water to produce 10^{-2} to 10^{-5} dilutions.

Each dilution was plated in duplicate on BHI, STAA and MRS agar plates using the spiral plater. Both the BHI and STAA-inoculated plates were incubated at room temperature for 48 h. The MRS plates were incubated in anaerobic jars with the Gas Pak plus anaerobic system at room temperature, to enumerate total anaerobic bacteria (presumably, LAB). The bacteria growing on the MRS plates were identified by biochemical testing (oxidase and catalase) and by their morphology during phase contrast microscopic observation.

5.4.4. Statistical Analysis

Statistical Analysis System software program, version 8.2 (SAS Institute Inc., Cary, N.C.) was used to analyze all data. Analysis of variance by the General Linear Models procedure and Duncan's multiple range tests were used to determine significant differences ($P < 0.05$) among treatments at a specific time (number of days) and among different times for each treatment.

5.5. Results

Initially on uninoculated pork samples (T1-T3) there were < 2 log CFU bacteria/ cm^2 present (Table 5.2). As expected on samples inoculated with *B. thermosphacta* (T4, T5) there were 4 log CFU/cm^2 present initially. However on inoculated pork treated with the antimicrobial coating (T6) < 1 log CFU bacteria/cm^2 was recovered at day 0. Organisms naturally present became undetectable by day 3 on pork treated with WPI coatings (T3 with, or T2 without, antimicrobials) and by day 9 on untreated pork (T1). Organisms present on inoculated samples, otherwise untreated (T4) and on inoculated samples with the WPI control coating (T5) remained constant throughout storage (Table 5.2). It was of interest that there was no significant difference in numbers of colonies recovered from *B. thermosphacta* inoculated uncoated (T4) and control-coated samples (T5) when plated on BHI and STAA agars (Tables 5.2 and 5.3, respectively) throughout storage. Almost exclusively, the bacteria recovered on BHI agar from inoculated samples were *B. thermosphacta*.

When pork loin cubes were inoculated with *B. thermosphacta* and treated with WPI coating containing nisin, lysozyme, EDTA and PP (T6) *B. thermosphacta* was not recovered on selective media during meat storage, whereas the organism was found on

uncoated samples and on samples with the control coating (T4 and T5, Table 5.3). The inoculated pork cubes treated with the control coating (T5) had significantly higher numbers of *B. thermosphacta* present than the uncoated inoculated pork samples (T4) although differences were not large. Growth of *B. thermosphacta* occurred on inoculated uncoated pork during storage, and while numbers at 12d were statistically significant, they were not large enough (<0.5 log CFU/cm^2) to be microbiologically significant (Table 5.3).

The occurrence of organisms from treatments plated on MRS agar (Table 5.4) was similar to that obtained when the treatments were plated on BHI and STAA agars. Few organisms were isolated initially from uninoculated treatments and none were found after 3d storage. In inoculated treatments T4 and T5, numbers increased slightly during storage, but in the presence of the antimicrobial coating < 1 log CFU bacteria/cm^2 was found on days 0 and 6, with no organisms being detected on the remaining sample days. Viable organisms on MRS plates from T4 and T5 samples, were non-motile, catalase positive and oxidase negative cells and were characteristic of *B. thermosphacta*.

5.6. Discussion

Meat products that are properly vacuum packaged and refrigerated have increased shelf-life since these conditions discourage the growth of aerobic spoilage and pathogenic bacteria due to O_2 deprivation or inhibition due to CO_2 (Cutter and Siragusa, 1996a). However, *B. thermosphacta*, *Listeria* and *Lactobacillus* species are psychrotrophic and are capable of growing under these conditions (Siragusa *et al.*, 1996a; Tu and Mustapha, 2002).

Previous research has demonstrated that *B. thermosphacta* and *L. innocua* were reduced by 4.5 and 2.83 log CFU/cm^2 on beef carcass tissue that was subjected to a nisin spray treatment, followed by vacuum packaging and storage at 4°C (Cutter and Siragusa, 1996a). Nisin or nisin plus EDTA solutions and vacuum packaging of fresh beef cubes reduced *B. thermosphacta* (up to 6.76 log CFU/ml) during storage for 25d at 4°C (Tu and Mustapha, 2002). Nisin immobilized in calcium alginate edible gel successfully inhibited *B. thermosphacta* on the surface of lean and adipose beef tissue (Cutter and Siragusa, 1996b) as well as in ground beef (Cutter and Siragusa, 1997). Immobilized nisin was more effective in inhibiting the organism than non-immobilized nisin. In addition, the immobilized nisin was able to reduce the organism on the surface of beef tissues up to 14d at 4°C (Cutter and Siragusa, 1996b). However, in beef that was later ground, the immobilized nisin was only able to cause an immediate suppression of the organism to undetectable levels (<1.3 log CFU/g) on day zero, and by day 14 the inhibition of the organism was not significantly different from the untreated sample (Cutter and Siragusa, 1997). Gill and Holley (2000) reduced *B. thermosphacta* and *L. monocytogenes* up to 4 log CFU/cm^2 and *S.* Typhimurium by 3-4 log CFU/cm^2 on cooked ham and bologna sausage slices using an antimicrobial gel coating containing a lysozyme, nisin and EDTA mixture.

In this study we demonstrated that the antimicrobial WPI coating (T6) consisting of a mixture of nisin, lysozyme, EDTA and PP was bactericidal and rapidly decreased *B. thermosphacta* numbers by ~ 4 log CFU/cm^2 on pork loin stored at 4°C for up to 12d (Table 5.3), while their number slightly increased on the uncoated samples (T4, 0.51 log CFU/cm^2) and remained almost constant on control-coated samples (T5). Similar results but with different organisms were observed by Cagri *et al.* (2002) where they reported

that control WPI films on bologna and summer sausage slices caused numbers of *L. monocytogenes*, *E. coli* or *S.* Typhimurium to remain lower than on uncoated samples.

Pork cubes treated with WPI coatings with or without antimicrobials (T3 or T2), respectively (Table 5.2) did not have significantly different numbers of bacteria present; however, these treatments had significantly lower numbers of organisms initially than the untreated control (T1). After day 6 all three treatments (T1, T2 and T3) were not significantly different. The presence of the coating may have initially restricted bacterial growth due to O_2 deprivation or reduced access to nutrients.

The activity of the antimicrobials in the coating was sustained throughout the 12d storage period since *B. thermosphacta* on samples treated with the antimicrobial coating (T6, Tables 5.2 and 5.3) could not be recovered during this period. The action of the antimicrobial was lethal to *B. thermosphacta* since no injured cells were recovered on BHI agar (T6, Table 5.2). Gill and Holley (2000) also reported that the activity of the antimicrobials in a gel coating containing 25.5 g/l lysozyme-nisin (1:3) and 25.5 g/l EDTA remained throughout the storage period. However, Fang and Lin (1995) observed a decrease in nisin activity on cooked pork tenderloins over time at 4°C. In addition, Murray and Richard (1997) observed that when nisin (300, 1500 and 3000 IU/ml) was added to ground pork inoculated with a *Listeria* suspension and stored at 5°C, numbers of bacteria initially decreased, however, growth resumed after 2d and reached similar levels as in control samples.

No colonies were detectable on the MRS plates from the antimicrobial-coated samples (T6, Table 5.4) at 9 or 12d. Control coatings caused the bacterial populations to remain more or less unchanged (T5), while the population slightly increased on uncoated samples (T4, 0.48 log CFU/cm^2). The number of colonies recovered from uninoculated

samples, T1, T2 and T3 were small but higher than recovered on STAA or BHI agars at day 0. It is likely that organisms recovered from the uninoculated meat on MRS agar were LAB. Biochemical testing of the bacteria isolated from the MRS plates prepared from T4 and T5 samples showed that the organisms were catalase positive and oxidase negative. Observation of the organisms under the microscope showed that they were non-motile, and therefore were probably *B. thermosphacta*. On the contrary, LAB are often motile and are almost always catalase negative. It was concluded that the organisms recovered from T4 and T5 samples on MRS plates were *B. thermosphacta* and not LAB. *B. thermosphacta* is a facultative anaerobic bacteria and can grow, although more slowly on MRS agar than on STAA or BHI agars.

Thus the antimicrobial WPI coating containing a mixture of nisin, lysozyme, EDTA and PP was very effective against *B. thermosphacta* inoculated on pork loin tissue stored under vacuum at 4°C for 12d.

Table 5.1. Experimental design[a] for challenge of *B. thermosphacta*[b] on pork loin cubes by WPI coatings[c] stored at 4°C under vacuum packaging for up to 12 days

Treatment codes	Treatment description
T1	pork loin cubes
T2	pork loin cubes and control coating
T3	pork loin cubes and antimicrobial coating
T4	pork loin cubes and *B. thermosphacta*
T5	pork loin cubes, *B. thermosphacta* and control coating
T6	pork loin cubes, *B. thermosphacta* and antimicrobial coating

[a] The experiment was done once with triplicate cubes plated in duplicate
[b] Initial bacterial population of 3 log CFU/cm^2
[c] Control coating (60:40 WPI:GLY) and antimicrobial coating (60:40 WPI:GLY, 2.4 mg/g nisin, 21mg/g lysozyme, 8 mM EDTA and 450 ppm PP)

Table 5.2. Recovery of bacteria on BHI agar from pork loin cubes stored at 4°C

	Days storage[a]				
Treatments	0	3	6	9	12
T1	1.65 ± 1.33 A b[b]	0.53 ± 0.83 B b	0.27 ± 0.65 B c	0 B c	0 B c
T2	0.66 ± 1.03 A b	0 B c	0 B c	0 B c	0 B c
T3	1.04 ± 1.62 A b	0 B c	0 B c	0 B c	0 B c
T4	3.58 ± 0.22 B a	3.77 ± 0.20 B a	3.69 ± 0.12 B b	4.16 ± 0.07 A a	4.02 ± 0.17 A b
T5	4.00 ± 0.13 A a	4.02 ± 0.13 A a	4.47 ± 1.07 A a	4.02 ± 0.25 A b	4.37 ± 0.39 A a
T6	0.78 ± 1.23 A b	0 B c	0 B c	0 B c	0 B c

[a] Values represent mean log CFU bacteria/cm^2 ± standard deviation of six measurements
[b] Means with different uppercase letters in the same row and different lowercase letters in the same column are significantly different ($P < 0.05$)

Table 5.3. Recovery of *B. thermosphacta* on STAA agar from pork loin cubes stored at 4°C

	Days storage[a]				
Treatments	0	3	6	9	12
T1	0 A c[b]	0 A c	0 A c	0 A c	0 A c
T2	0 A c	0 A c	0 A c	0 A c	0 A c
T3	0 A c	0 A c	0 A c	0 A c	0 A c
T4	3.48 ± 0.18 B b	3.71 ± 0.20 B b	3.64 ± 0.23 B b	4.19 ± 0.06 A a	3.99 ± 0.21 A b
T5	3.97 ± 0.19 A a	4.05 ± 0.10 A a	4.08 ± 0.53 A a	4.02 ± 0.23 A b	4.30 ± 0.48 A a
T6	0 A c	0 A c	0 A c	0 A c	0 A c

[a] Values represent mean log CFU/cm^2 *B. thermosphacta* ± standard deviation of six measurements
[b] Means with different uppercase letters in the same row and different lowercase letters in the same column are significantly different ($P < 0.05$)

Table 5.4. Recovery of lactic acid bacteria on MRS agar from pork loin cubes stored at 4°C

	Days storage[a]				
Treatments	0	3	6	9	12
T1	2.22 ± 0.86 A b[b]	0 B b	0 B b	0 B c	0 B c
T2	1.13 ± 1.28 A c	0 B b	0 B b	0 B c	0 B c
T3	0.70 ± 1.13 A c	0.43 ± 1.06 A b	0 A b	0 A c	0 A c
T4	3.50 ± 0.20 B a	3.69 ± 0.19 B a	3.62 ± 0.15 B a	4.16 ± 0.08 A a	3.98 ± 0.16 A b
T5	3.96 ± 0.10 A a	4.02 ± 0.13 A a	4.09 ± 0.51 A a	3.96 ± 0.33 A b	4.34 ± 0.46 A a
T6	0.38 ± 0.94 A c	0 A b	0.49 ± 1.19 A b	0 A c	0 A c

[a] Values represent mean log CFU bacteria/cm^2 ± standard deviation of six measurements
[b] Means with different uppercase letters in the same row and different lowercase letters in the same column are significantly different ($P < 0.05$)

CHAPTER # 6

CONCLUSIONS AND RECOMMENDATIONS

The research in this thesis examined the antimicrobial effects of WPI films containing nisin, lysozyme, EDTA and propyl paraben alone or in combination against *B. thermosphacta*, *L. monocytogenes*, *S. aureus*, *S.* Typhimurium and *E. coli* 0157:H7 in BHI agar plates. In addition, a mixed antimicrobial WPI coating was tested for its ability to eliminate *B. thermosphacta* from pork loin tissue. Several conclusions can be drawn and recommendations made.

- Inhibition zones generated on inoculated agar surfaces by films do not always accurately reflect the antimicrobial potency of films containing antimicrobials. This was verified by reduced recovery of challenged organisms from agar where inhibition was not well defined by cleared zones in the agar.
- Addition of nisin, lysozyme, EDTA and PP together in WPI films did not yield increased inhibition against all organisms tested. While nisin showed broad antimicrobial activity, its effectiveness was not always improved by higher concentrations of the other test antimicrobials.
- Incorporation of nisin alone into acidic WPI films was successful in inhibiting *B. thermosphacta*, *L. monocytogenes* and *S. aureus*. Unfortunately, nisin and lysozyme and lysozyme and PP had reduced activity against *L. monocytogenes*, while nisin and lysozyme or EDTA also interacted negatively against *S. aureus*. Interestingly, when nisin alone or lysozyme alone were formulated into neutral films each effectively inhibited *S.* Typhimurium, whereas combined antimicrobials were less effective.

- Factorial experiments indicated that interactions between nisin and EDTA, lysozyme and EDTA, nisin and PP or EDTA and PP did not increase inhibitory activity against test organisms and strong negative interactions occurred between nisin and lysozyme against all organisms and between nisin and WPI against *S. aureus* and *S.* Typhimurium.
- A WPI coating containing 2.4 mg/g nisin, 21 mg/g lysozyme, 8 mM EDTA and 450 ppm PP significantly reduced the viability of *B. thermosphacta* on refrigerated fresh pork tissue and extended product shelf life.
- In future work the diffusion or release of antimicrobials from films in which they are incorporated should be evaluated and this characteristic should be studied with others in determining the overall effectiveness of antimicrobial films.
- There is still a need to develop a broad spectrum antimicrobial film capable of inhibiting both pathogenic and spoilage organisms in perishable food products. The search for effective natural antimicrobials should continue and focus on inhibition of the psychrotrophs which are problematic in ready-to-eat foods.

CHAPTER # 7

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