1-1-2017

Incorporating Fermented By-Products of Lactobacillus Diolivorans Sp. in Food Grade Coatings Designed for Inhibition of Tyrophagus Putrescentiae on Dry-Cured Hams

Hector Asis Portillo

Incorporating fermented by-products of Lactobacillus diolivorans sp. in food grade coatings designed for inhibition of Tyrophagus putrescentiae on dry-cured hams

By

Hector Asis Portillo

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Food Science and Technology
in the Department of Food Science, Nutrition, and Health Promotion

Mississippi State, Mississippi

December 2017

Incorporating fermented by-products of Lactobacillus diolivorans sp. in food grade coatings designed for inhibition of Tyrophagus putrescentiae on dry-cured hams

By

Hector Asis Portillo

Approved:

_____

M. Wes Schilling
(Major Professor)

_____

Todd French
(Committee Member)

_____

Jerome Goddard
(Committee Member)

_____

Taejo Kim
(Committee Member)

_____

Marion W. Evans, Jr.
(Graduate Coordinator)

_____

George M. Hopper
Dean
College of Agriculture and Life Sciences

Name: Hector Asis Portillo

Date of Degree: December 8, 2017

Institution: Mississippi State University

Major Field: Food Science and Technology

Major Professor: Dr. Wes Schilling

Title of Study: Incorporating fermented by-products of Lactobacillus diolivorans sp. in food grade coatings designed for inhibition of Tyrophagus putrescentiae on dry-cured hams

Pages in Study 54

Candidate for Degree of Master of Science

Distillate solutions that were derived from concentrated ferment were incorporated into either a carrageenan (CG) and propylene glycol alginate (PGA) gum blend or into a CG, PGA, and xanthan gum (XG) blend. Distillate treatments were compared against a 10% propylene glycol treatment, a gum only control, and negative control using three hams that were cut into 2.5 cm thick slices and then cut into 2.5×2.5×2.5cm cubes (n=50) that were dipped into solutions prior to conducting mite bioassays. Coated and control ham cubes were inoculated with 20 adult mites from the species *T. putrescentiae (Schrank),* and incubated for 2 and 3 weeks. The distilled treatments with CG + PGA + XG had a greater reduction ($P<0.05$) in mite populations than all other treatments with the exception of the 10% PG coated treatments. In addition, there were no differences between treatments with respect to sensory texture, flavor, and moistness.

DEDICATION

I want to dedicate the completion of this research to those individuals who have guided and supported me on every step of the way. I have been fortunate enough to work with people of all walks and beliefs, and their impact on my life as a researcher and an individual will forever be held in my heart. To my beautiful fiancé Katie and children, Elijah and Laila, thank you for your love and support throughout this process. The love I have for you is what drives me to continually push myself. I want to thank my parents Samira Khaled and Hector Portillo for never giving up on me, and imparting their work ethic and wisdom that have guided me to this point in my life. God has been good to me, and I've been blessed with amazing opportunities. Going forward in my career, I hope to give back to all those who have given me so much, and shine a light on the field of Food Science.

ACKNOWLEDGEMENTS

There are not too many individuals that I have met in my life that have had as much of an impact on me than Dr. Wes Schilling. Sincerely, I thank you for your guidance and encouragement in my research and life. Thank you to my committee members Dr. Jerome Goddard, Dr. Taejo Kim, and Dr. Todd French for your ongoing support and resources that have made this research possible. I want to acknowledge Dr. Hassan Barbary and his PhD student Islam Elsayed for their patience and assistance with completing my analytical measurements; your time and expertise were invaluable to the completion of this research. Thank you to Shamim Howlander for your countless hours of assistance with my bioreactor trials. Your friendship is dear too me, and I wish you nothing but success in all your future endeavors. To the awesome lab: Yan Campbell, Xue Zhang, Jasmine Hendrix, Michael Byron, Morgan Von Staden, Wenje Shao, Kavitha Krishna, Marquisha Pittman, and Joseph Brummett thank you for your assistance in all parts of this research; I am deeply grateful to have such an awesome team by my side.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Curing meat products has provided a means for both early and modern civilizations to preserve meats prior to the advent of modern refrigeration. Salt and sodium nitrate have been used for dry cured ham production in various countries, with the most notable styles including Italian Prosciutto-style ham, Spanish Iberian ham, Chinese Jinhua ham, German Westphalia ham, and United States country-cured ham (Rentfrow et al., 2012). The production of dry-cured ham in the United States has deep roots within the country's history, with the oldest documented preparations dating back to the settlement of Jamestown during the 1600's (Stradley, 2004). Though the curing process is important in ham preservation and sensory characteristics, it is during the 3 months to 3 years of aging that the hams develop their distinct flavor from the lipolysis and proteolysis that occurs during ripening (Toldrá, 1998). During the aging process, ham mites, red-legged beetles, cheese skippers, and larder beetles are potential pests (Abbar, 2016). *Tyrophagus putrescentiae (Schrank)*, also known as the ham, mold, or cheese mite, infests grains and stored food products, and has an affinity towards high fat and protein containing food items. Dry-cured ham is most susceptible to mite infestation after 4-6 months of aging due to its high fat and protein content, mold growth, and water activity (Rentfrow et al., 2008). Currently, methyl bromide fumigation is the principal means of pest control in ham houses. With the signing of the Montreal Protocol in 1987, the United States joined

196 other countries in an effort towards gradually eliminating the use of ozone depleting substances, thus limiting access to methyl bromide for use in ham houses (US Environmental Protection Agency, 2016). Controlling mite infestations poses both food safety and economic concerns for ham producers in the United States. The USDA considers the presence of one or more ham mites on a dry cured ham an adulterant, and mandates that producers make corrective actions before the sale of affected products (United States Department of Agriculture, 2014; United States Department of Agriculture, 2011b; United States Department of Agriculture, 2011b). With the potential for either a lack of or limited supply of methyl bromide, it is necessary to research and develop viable alternatives that are both cost effective and efficient at controlling mites on dry-cured ham. Other than methyl bromide, there are no current commercial methods that are used as a direct replacement. Studies have been performed on the efficacy of a variety of alternative gases including carbon dioxide, ozone, sulfuryl fluoride, and phosphine (Riudavets et al., 2010; Zhao et al., 2015). Using phosphine gas in place of methyl bromide controlled mite growth under laboratory conditions, yet was ultimately ineffective due to the corrosion it caused on copper fittings and wiring (Zhao et al., 2015). Research has also been conducted on the application of food safe compounds to the surface of hams. Both 1,3 propanediol and 1,2 propanediol, most commonly known as propylene glycol, controlled mites with 99% inhibition when used at concentrations of 50 and 100% in benchtop studies (Abbar et al., 2016; Zhao et al., 2016). Propylene glycol was also effective at inhibiting the growth and reproduction of the ham mite *Tyrophagus putrescentiae* on dry-cured hams during trials performed on the benchtop when incorporated into food grade coatings that contained carrageenan and propylene

glycol alginate. Use of propylene glycol, though effective at inhibiting mite reproduction, may not be feasible on a commercial scale, due to its relatively high cost of $0.82 or greater per ham when implemented in coating formulations (Campbell et al., 2017). Though commonly synthesized through the chemical alteration of acrolein or ethylene oxide, several species of bacteria demonstrate the ability to commercially produce 1,3 propanediol through the metabolism of glycerol (Ainala et al., 2013; Lee et al., 2015; Pflügl et al., 2012). Microbial biomass that is created from fermentation may also have further applications, as species of bacteria with high lipid contents in the cell walls may be used in the production of biofuels (Howlader et al., 2017). The use of lactic acid bacteria in the genera *Lactobacillaceae*, specifically heterofermentative strains such as *L. reutteri, L. buchneri, L. Brevis,* and *L. diolivorans* can produce 1,3 propanediol for use in products that are intended for human consumption (Pflügl et al., 2014; Schütz & Radler, 1984; Stevens et al., 2011). *L. diolivorans* produces a greater concentration of 1,3 propanediol than other species of lactobacilli. Pflügl et al., (2012) reported that *L. diolivorans* produced 41.7g/L of 1,3 propanediol in MRS broth when supplemented with a 7% (w/v) concentration of glycerol using a batch cultivation method in a stirred tank bioreactor. Anaerobic fermentation using *L. diolivorans* also produces lactic acid, acetic acid, and ethanol as primary by-products. Incorporating fermented by-products of lactic acid bacteria may provide benefits beyond mite inhibition, as production of antifungal metabolites in tandem with acetic acid may contribute to mold inhibition. Ham mites prefer high fat and protein food sources, but also feed on various mold species. *Penicillium nordicum*, a strain of mold commonly found in fermented meat products, is a potential food source for *T. putrescentiae*. In contrast, several aspergillus species can

produce aflatoxins that are detrimental to mite reproduction (Rodriguez et al., 1980; Smrž & Čatská, 1987). Previous research indicated that antifungal metabolites that were produced by *Lactobacillus planterum* in the presence of acetic acid, with a pH of 4.0 were effective at preventing the growth of *P. nordicum* (Schillinger & Villarreal, 2010). Similar inhibitory effects on mold growth may potentially be exhibited from acetic acid and anti-fungal metabolites that are produced by *L. diolivorans*, which in turn would prevent mites from utilizing mold growth as a food source during aging. Integrated Pest Management Systems (IPM) as described by Abbar et al. (2016) can be used as a means to control or minimize mite infestations. Such an approach uses multiple hurdles including physical, chemical, and biological means of pest control in ham houses. The objective of this study was to determine if fermentation by-products created by *L. diolivorans*, primarily 1,3 propanediol, lactic acid, and acetic acid are effective at controlling *T. putrescentiae* growth when incorporated into food grade coatings that contained xanthan gum, propylene glycol alginate and carrageenan.

CHAPTER II

LITERATURE REVIEW

2.1 Dry Cured Ham Production and Properties

According to title 9 Section 319.106 of the United States Code of Federal Regulations (CFR), dry cured ham is defined as an uncooked, cured, dried, smoked or unsmoked meat food product made from the hind leg of a pork carcass that is prepared by the application of salt (NaCl) and one or more optional ingredients. The standard amount of time necessary to allow for curing, salt equilibration, and drying cannot be less than 55 days, and the ham must achieve a final water activity (A_W) of 0.92 or less. Abiding by these practices inhibits the growth of pathogenic bacteria and allows for storage at room temperature (Reynolds et al., 2001). The production of dry cured hams in North America is thought to have initially began in the 1600's with the settlement of Jamestown. The act of curing meat by application of salt was a skill most settlers brought with them from Europe. However, smoking meat was taught to the settlers by Native Americans, who were using the technique on venison (Stradley, 2004). Many other variations of dry cured ham are produced around the world. Among these, Italian Prosciutto-style ham, Spanish Iberian hams, Chinese Jinhua hams, and German Westphalia hams are the most notable (Rentfrow et al., 2012). Today, dry cured ham production is primarily based in the southeastern United States, specifically in Kentucky, North Carolina, Tennessee, Virginia, and Missouri. Historically, hogs were slaughtered in the colder winter months of December, January, and February. Processing hogs in colder climates helped prevent spoilage, and provided an ideal ambient environment for the curing process (Hanson et al., 2014). With the advent of modern refrigeration, curing and aging can now be done

throughout the year in an artificial environment. The process of developing a dry cured ham begins with the application of a curing mixture. Typically, such mixtures consist of 8 pounds of salt, 3 pounds of cane sugar, 2 ounces of nitrate (saltpeter), and 1 ounce of sodium or potassium nitrite. For every pound of pork, 1 ounce of curing mixture is used (Romans et al., 1985). According to the USDA, hams should be cured at temperatures above 1.7˚C but should not exceed 7.2˚C for the first 3 days. Regulations mandate that hams are cured for a minimum of 40 days, and remain at a temperature of 15.6˚C or below following the initial 3 day curing period (United States Department of Agriculture, 2012a). The next step in the process is salt equalization, which is done by removing the excess cure mixture from the ham and hanging it into a sock. This is the period in which the salt penetrates the meat and is absorbed into the inner portions of the ham (United States Department of Agriculture, 2012a). The following step of smoking the hams is optional. Other than imparting flavor, producers implement this step to eliminate the parasite *Trichinella spiralis* if they have not implemented a prior kill step (United States Department of Agriculture, 2012b). Finally, the ham is left to age for a period of 3 months to 2-3 years. It is in this step that the hams develop flavor from the lipolysis and proteolysis that occurs during ripening (Toldrá, 1998). Hams may legally be labeled as dry cured or country ham after it has lost 18% of its green weight and contains a minimum of 4% salt (United States Department of Agriculture, 2012a).

2.2 *Tyrophagus putrescentiae*

2.2.1 Introduction

Tyrophagus putrescentiae (Schrank) is a pest of dried hams, cheeses, grains, and various other stored products, with a preference for foods that have high contents of fat and protein. *Tyrophagus putrescentiae* belongs to the Acaridae family, which is defined by Stedman's medical dictionary as "exceptionally small mites, usually 0.5 millimeter or less in length, abundant in dried fruits and meats, grain, meal, and flour that frequently causes severe dermatitis in persons that are hyper-sensitized by the frequent handling of infested products" (Kilgour, 1953). The main factors that influence the survival and development of ham mites include relative humidity, temperature, and available food sources in its micro-environment (Farrell, 2017). If subjected to ideal conditions, such as temperatures above 30˚C and 85% RH, mites can complete their life cycle in 3 weeks (Barker, 1967). Though commonly found in stored food products, *T. putrescentiae* is also known to benefit from the consumption of fungi and bacteria, with the ability to produce bacteriolytic enzymes that hydrolyze the cell walls of gram positive bacteria (Erban et al., 2016a; Erban & Hubert, 2008). When reared on *F. Graminearum* at temperatures of 25℃ and a 60% RH, *T. putrescentiae* spends approximately 2 days in the egg stage, 3 days in larval stages, and 5 days as a nymph (Bahrami et al., 2007). Not all fungal species are suitable for growth. However, certain species produce mycotoxins that are inhibitory to growth and reproduction. Sterigmatocystin, T_2 toxin, and zearalenone are mycotoxins that when present at 1, 10, or 100 ppm are lethal to *T. putrescentiae*. Aflatoxin B1, citrinin, ochratoxin A, and penicillic acid were less toxic, and permitted development in the 1st generation but caused 100% mortality to the 2nd generation of mites (Rodriguez et

al., 1980). As with fungus, bacteria can also be inhibitory. *Bacillus cereus* has a strong symbiotic association with *T. putrescentiae*, and when present generally does not influence mite mortality. However, excessive concentrations of *Bacillus cereus* can significantly reduce population growth (Erban et al., 2008; Erban et al., 2016b).

2.2.2 Allergenic Components

Infestations of *T. putrescentiae* pose a food quality and safety risk. *T. putrescentiae* is the host to a variety of allergenic components. Case studies performed on ham workers in Parma, Italy and various other production establishments have shown that *T. putrescentiae* is a contributor to human cases of cutaneous and respiratory allergies (Mullen & O'Connor, 2009; Choi et al., 2009; Tafuro et al., 2015; Nuñez et al., 2016; Jeong et al., 2005). Among these cases, a link between cross reactivity of shellfish and mites has been widely established. For those individuals with a sensitivity to these allergenic components, the potential for systematic anaphylaxis has been demonstrated (Arlian et al., 2009; Matsumoto et al., 1996). Twenty different allergenic components have been identified from *T. putrescentiae*. The 16 kDa component is most commonly associated with allergic reactions, with 52% of allergic patients showing IgE (Immunoglobulin E) antibodies for this specific allergen (Green & Woolcock, 1978; Park et al., 1999).

2.2.3 Interventions for Mite Infestations

The fumigant methyl bromide is commonly used to deter mite infestations. Due to methyl bromide's ozone depleting potential, its use has been phased out by the Montreal protocol, a global initiative that was adopted by the United States Department

of State that focuses on the reduction and elimination of ozone depleting substances (US Environmental Protection Agency, 2016). Issues arising from methyl bromide use has led to research on potential alternative methods to control *T. putrescentiae* infestations on dry cured ham. Studies have been performed on the efficacy of a variety of alternative gases including carbon dioxide, ozone, sulfuryl fluoride, and phosphine (Nayak, 2006; Riudavets et al., 2010; Zhao et al., 2015). Among these alternative gases, phosphine and sulfuryl fluoride have been the most effective. Phosphine was effective at controlling *T. putrescentiae* in stored animal feed when using 1mg/L of phosphine over a six day period and dry cured hams at 1000-2000 ppm phosphine for 48h (Nayak, 2006; Zhao et al., 2016). However, phosphine implementation in ham houses has limitations since it causes the corrosion of copper piping and fittings (Zhao et al., 2016). Sulfuryl fluoride (SF) also shows promise as an alternative, and is currently used to control pest infestations, such as the drywood termite and bedbugs. Sulfuryl fluoride caused high mortality of mites in the mobile stages at 23℃ but failed to completely kill mites in the egg stage, possibly due to low permeability of the outer surface of the egg (Abbar et al., 2016). Application of food safe compounds with miticidal properties has potential as a supplementary means to control infestations. A study performed on the efficacy of selected food safe compounds to control ham mites indicated that 1,3 propanediol and1,2 propanediol, commonly known as propylene glycol, was effective at controlling mites with concentrations of 50 and 100% (Abbar et al., 2016; Zhao et al., 2016). Though effective, its implementation in food grade coatings would result in a cost of approximately \$0.82 or greater ~~70~~ per ham (Campbell et al., 2017). In addition, 100% lard and 10% butylated hydroxytoluene (BHT) were effective alternatives when applied to the surface of 1 cm ham cubes. Use of these

substances presents challenges. For example, the concentrations of BHT surpass the regulatory limitations of 0.02% for use in consumable products (United States Department of Agriculture, 2011a). Though the process of dipping hams in lard and vegetable oil is practiced in Spain, the hydrophobicity of lard may affect moisture retention during the aging process (Abbar et al., 2016; García, 2004; Zhao et al., 2016). Environmental temperature and relative humidity also influence mite mortality. Research performed on the survival of *T. putrescentiae* at 7 constant temperatures indicated that at a relative humidity of 90 ± 5% and temperatures of 10℃ and 34℃, mite mortality occurred at 93.6 and 54% respectively (Sánchez-Ramos & Castañera, 2001). Relative humidity has an indirect effect on mite proliferation, since certain relative humidity's promote fungus growth that is an additional food source for the mite colonies (Rivard, 1961). Implementation of integrated pest management (IPM) programs that are designed for ham processors could be used in the future to ensure that there are multiple tools that are available to help control mite populations (Abbar et al., 2016). Control strategies that subject mites to physical, chemical, and biological hurdles will aid processors in mitigating the occurrence of mite infestations.

2.3 Food Grade Coatings

2.3.1 Food Grade Coating Properties and Applications

Edible coatings are applied to a variety of products. An edible coating is defined as a material that enrobes and fortifies naturally existing layers to help control moisture loss and facilitate the exchange of gases such as oxygen and carbon dioxide (Pavlath & Ortis, 2009). Though this is a general definition, the application of food grade coatings also presents the opportunity to introduce various compounds of different

functionalities into the polymer matrix such as lipids, proteins, carbohydrates, active ingredients, and polymerizing agents. When using these individual components, it is generally understood that fats are used to reduce water transmission, polysaccharides are used to control oxygen and other gas transmission, and protein films provide mechanical stability (Kokoszka & Lenart, 2007). Polymers, much like protein, aid in the mechanical properties of the coating. Common polymerizing agents are used to create plasticity in edible coatings such as glycerin, propylene glycol, sorbitol, mannitol, and xylitol. When incorporating plasticizers, mechanical strength, barrier properties, and elasticity decrease with higher concentrations. Varying mixtures of these components create unique characteristics that can be tailored to specific product needs (Vieira et al., 2011). Fruits and vegetables greatly benefit from the utilization of food grade coatings due to their high enzymatic activity which leads to a shorter shelf life. The use of wax based coatings prevents excessive moisture loss by creating a hydrophobic barrier that also limits enzymatic browning due to oxidation (Falguera et al., 2011). Other products such as meat can also benefit from the ability to directly apply antimicrobial compounds such as lactic acid, acetic acid, and chitosan in a stable manner.

2.3.2 Polysaccharides in Food Grade Coatings

Polysaccharide films and coatings may contain starch, alginate, dextrins, pectin, chitosan and carrageenan's (Nieto, 2009). Films consisting primarily of these components usually act as a barrier to gasses, but not water, which is due to their hydrophilic nature (Oms-Oliu et al., 2008). Alginates are a family of linear copolymers which consist of several blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. The ratios of M and G differ depending on the natural source of the

alginate, and it is speculated that only the G blocks interact in the intermolecular cross-linking that is necessary for the formation of hydrogels, making them important for the overall physical properties that alginates contribute (Lee & Mooney, 2012). Alginic acid, the active component in alginates is derived through acid extraction, and is a naturally occurring hydrophilic colloidal polysaccharide that is obtained from various species of brown seaweed (Wüstenberg, 2014). Films that are made from alginates tend to have good tensile strength. However, alginate film strength can be further enhanced with the addition of modified starches, oligosaccharides or simple sugars (Ustunol, 2009). Carrageenan's differ from alginate in that they are primarily derived from red seaweeds. Much like alginates, carrageenan's are also linear polysaccharides. Their half-ester sulfate moieties make them a strong anionic polymer, as opposed to alginates which are considered mostly nonionic (Stanley, 1987). The structure is predominately made up of repeating galactose units and 3,6 anhydrogalactose (3,6-AG), both sulfated and non-sulfated. The units are joined by alternating alpha 1-3 and beta 1-4 glycosidic linkages (Kariduraganavar et al., 2014). The variation in composition and conformation of the structure allows for carrageenan's with different levels of functionality. Iota, Kappa, and Lambda are the three varieties of carrageenan, and are differentiated by their degree of sulfation. The addition or elimination of sulfate groups influences the formation of 3,6 anhydro-bridges which are crucial for gelation in Iota and Kappa carrageenan (Van de Velde & De Ruiter, 2005).

2.3.3 Proteins in Food Grade Coatings

Proteins used in the formation of films and coatings can be derived from animal sources such as collagen, gelatin, and whey proteins or from plant based sources such as

corn, wheat, or soy (Krochta, 2002). Intrinsic properties of proteins that influence coating functionality include amino acid composition, crystallinity (of the protein and/or plasticizer), hydrophobicity/hydrophilicity, surface charge, pI, molecular size, and three-dimensional shape (Dangaran et al., 2009). The polar R groups of proteins are highly influential in dictating barrier properties. Because of this, coatings that contain proteins have a high permeability to polar substances like water, but a low permeability to oxygen, aromas, and oils (Krochta, 2002).

2.3.4 Lipids in Food Grade Coatings

Incorporating lipids into coatings provides hydrophobic properties that can help with the diffusion of oxygen and other aromatic compounds. An issue with using lipids, especially unsaturated fatty acids, is their proneness to oxidation. To address this issue, use of saturated fatty acids with long carbon chains such as lauric acid may be beneficial in delaying oxidation (Leyton et al., 1987). A study performed on the barrier properties of starch based coatings with the addition of lipids, assessed the effects of gas and water permeability when corn starch containing films were supplemented with sunflower oil. The results indicated that when sunflower oil was incorporated at concentrations of 2g/L and above, the water vapor permeability (WVP) decreased while oxygen permeability increased when compared to starch only controls (García et al., 2000).

2.3.5 Tensile Strength and Permeability of Films

When assessing the mechanical properties of food grade coatings and films, measurements such as tensile strength (TS), elongation (E), and elastic modulus (EM) are generally analyzed. Tensile strength is the measurement of the pulling force applied to

the coating before it reaches its break point. Elongation is the degree of stretching the film can withstand before breaking, and elastic modulus is the overall stiffness of the film as characterized by the ratio of pulling force/area to degree-of-film-stretch. Measuring oxygen permeability and water vapor permeability are also important, as both dictate if the barrier properties are suitable for the intended use. For dry-cured hams, a film that provides high water and oxygen permeability is ideal. Allowing for adequate gas diffusion and water vapor permeability will aid in proteolysis and lipolysis during aging, as well as discourage moisture retention, leading to a finished product with a shelf-stable water activity as well as suitable flavor development (Olmo et al., 2013).

2.4 Lactic Acid Bacteria

2.4.1 Classification and Taxonomy

Lactic acid bacteria are classified as gram positive, non-spore forming bacteria, whose primary by-product of carbohydrate fermentation is lactic acid. They are characterized by their rod or cone shaped appearance, and generally prefer anaerobic environments, but are aerotolerant (Barrangou et al., 2012). Lactic acid bacteria can be either homofermentative, producing primarily lactic acid, or heterofermentative, producing lactic acid, carbon dioxide, ethanol, and acetic acid. Lactic acid bacterium belong to the phylum *Fermicutes*, in the class *Bacilli*, and the order *Lactobacillales.* The generas include *Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus*, and *Vagococcus* (Barrangou et al., 2012).

2.4.2 Lactobacillus sp.

Lactobacillus are a sub-classification of lactic acid bacteria that are a highly diverse gram positive, micro-aerophilic bacteria and are generally recognized as safe (GRAS) species for human consumption. This genera of LAB is commonly used as a probiotic and starter culture, and is present in a wide array of fermented products including fermented dairy and wine (Soomro & Masud, 2002). The two classifications of Lactobacillus include homofermentative and heterofermentative. Homofermentative species are generally associated with lactic acid production and contribute to the acidity and tartness of fermented foods. Heterofermentative strains produce lactic acid, as well as acetic acid, ethanol, and carbon dioxide as by-products, and are used in biotechnology for the production of metabolites (Barrangou et al., 2012). The pathway responsible for metabolism of hexoses into lactic acid is the glycolytic-pathway (Embden-Meyerhof pathway) and is primarily active in homofermentative strains whereas the phosphoketolase pathway influences heterofermentative metabolism. Though these pathways are currently understood, the bio-synthetic pathways of *Lactobacillus* contain orthologs that require further research. Genome sizes of *Lactobacillus* strains are relatively large (1.8 to 3.4 Mb) when compared to those of symbiotic bacteria, such as *Mycoplasma* strains (0.6 to 1.4 Mb), and potentially have alternative primary metabolic pathways that can be further explored (Kuratsu et al., 2010). The smallest genome in these genera belongs to *Lactobacillus Sanfrancis- censis* (1.23 Mb) and the largest in *Lactobacillus parakefiri* (4.91 Mb) (Sauer et al., 2017). Though wild type strains are commonly used, genetically modified varieties of *Lactobacillus* have been developed to increase the production capacity of primary or secondary metabolites during

fermentation. Metabolic engineering can also promote expression of new pathways, which allows for the production of metabolites that would otherwise not occur naturally. *L. plantarum* and *L. casei* are examples of strains that are relatively easy to engineer (Nguyen et al., 2011; Welker et al., 2015). However, strains such as *L. diolivorans* are more difficult to metabolically engineer, and can only be modified slightly with low efficiency (Pflügl et al., 2013). The diversity of genomic expression has in part made *Lactobacillus sp.* preferential for use in fermentative processes both in food and industrial applications. Because LAB are fastidious bacteria, there is a high requirement for nutrient dense media. However, its production efficiency has made its use economical for the production of metabolites with high utility in pharmaceutical, food, and industrial applications.

2.4.3 Industrial Application of LAB Metabolites

Lactic acid bacteria are frequently used in fermentative processes in food manufacturing settings, specifically as a starter culture in fermented dairy, meat, and vegetable products. Its use for production of primary and secondary metabolites is also common, and can be used in food, pharmaceutical, and polymer industries. Chemicals that are naturally produced by LAB include lactic acid, mannitol, 1,3-propanediol, 3-hydroxypropionaldehyde, 3-hydroxypropionic acid, and g-amino butyric acid (Florou-Paneri et al., 2013). With advancements in genetic engineering, metabolic pathways have also been modified and developed to produce exopolysaccharides and vitamins (Kleerebezem & Hugenholtz, 2003; Sybesma et al., 2004). LAB convert hexoses using pyruvate, which can subsequently be transformed into a variety of the above-mentioned metabolites. Production of these metabolites is influenced by environmental conditions,

availability of nutrients, and gene expression (Papagianni, 2012). In regards to food safety, LAB produce a variety of organic acids and bacteriocins that serve as antimicrobial compounds. Fermented food products can contain benzoic, lactic, acetic, and propionic acid that are naturally produced from strains including *L. acidophilus*, *L. casei*, *L. plantarum*, and *L. streptococcus*. Use of organic acids can enhance safety and lengthen the shelf life of products through acidification (Zacharof & Lovitt, 2012).

2.5 Bacterial Fermentation of 1,3 Propanediol

Klebsiella, *Citrobacter*, *Clostridium*, and *Lactobacilli* can convert glycerol into 1,3-propanediol (Da Silva et al., 2015; Ainala et al., 2013; Johnson & Rehmann, 2016; Pflügl et al., 2013). Lactic acid bacteria, specifically heterofermentative strains in the genera lactobacillus, are a primary choice to incorporate into coatings, as they are non-pathogenic and safe for human consumption. In the absence of oxygen, these strains of lactic acid bacteria can utilize organic compounds such as glycerol as an electron acceptor. These heterofermenters, unlike their homoferementative counterparts, have the ability to not only produce lactic acid, but also acetic acid, ethanol, carbon dioxide, and a variety of other compounds. Research performed on the production of 1,3 propanediol from glycerol and *L. diolivorans* yielded 41.7g/L of 1,3 propanediol using a batch cultivation method. This quantity is greater than other strains of lactobacillus when using the same processing technique. In order for *L. diolivorans* to produce optimal amounts, the addition of glucose is utilized as a co-substrate with glycerol, which helps to increase growth rate and biomass (Pflügl et al., 2012). When glucose metabolism occurs in the presence of glycerol, the glycerol re-oxidizes a NADH that is equivalent to the product that is formed during the catabolism of sugar via the 6-phosphogluconate pathway. This

is also observed in other heterofermentative strains such as *L. buchneri* (B190) and *L. brevis* (B22) (Veiga & Foster, 1992). The accumulation of NADH is important in the conversion of 3-hydroxypropanaldehyde to 1,3 propanediol since the expression of enzymes necessary for the conversion process is positively correlated to a raised intracellular NAD/NADH ratio (Wright & Axelsson, 2012; Veiga & Foster, 1992). Two enzymes are crucial in the conversion process of glycerol to 1,3 propanediol. Coenzyme B12-dependent dehydratase (EC 4.2.1.30, GDHt) is used to convert glycerol into 3-hydroxypropionaldehyde (3-HPA). Addition of yeast extracts, which are rich in B vitamins can supplement the necessary B12 for proper enzyme activity. The second enzyme in the process is 1,3-propanediol dehydrogenase (EC 1.1.1.202, PDOR). This enzyme is used to reduce 3-HPA into 1,3-propanediol by utilizing NADH as a cofactor. A study performed on the effects of pH and temperature on the enzymatic activity of PDOR indicated that it functions best at 37˚C with a neutral pH. However, it is much more stable in an acidic environment than an alkaline environment (Qi et al., 2014). When glucose is metabolized as the sole carbon source, the by-products of fermentation are primarily lactate, ethanol, and acetate. The consumption of NADH is required to generate ethanol, which inhibits the cell from accumulating NADH for the conversion of 3-HPA to 1,3 propanediol. When glycerol is introduced as a co-substrate, the metabolic pathway shifts towards primarily producing acetate, which generates ATP for cellular energy but does not require the utilization of NADH. With the accumulation of excess NADH, 3-HPA is formed from the dehydration of glycerol, and 1,3 propanediol can be produced when 3-HPA is reduced by 1,3 propanediol dehydrogenase by utilizing free intracellular NADH (Stevens et al., 2011; Veiga & Foster, 1992; Pflügl et al., 2012).

2.6 Fermentation and Biotechnology

2.6.1 Fermentation Processes

Fermentation is the chemical breakdown of compounds by bacteria, yeast, or other microorganisms. The process of fermentation has been used for thousands of years, with early civilizations using it to produce bread, cheese, and wine (Battcock & Azam-Ali, 1998). The industrial application of fermentation processes dates back prior to the 1900's with the creation of potable alcohol and vinegar using copper vessels. It was not until the 1940's however that the use of true fermenters, also known as bioreactors, were utilized to produce antibiotics (Stanbury et al., 2013). Bioreactors are used in cell line cultivation as well as bacterial production of primary and secondary metabolites. Fermentation is used in industrial applications to produce a range of products including microbial biomass, enzymes, metabolites, and recombinant products. Unlike chemical synthesis, by-products from biological reactions such as cell mass can be further processed for lipid or protein content, and used for external applications such as biofuel production (Howlader et al., 2017). Biotechnology can be described as the application of scientific and engineering principles to the processing of materials, for the provision of goods and services, using biological systems and agents (Battcock & Azam-Ali, 1998). The design of vessels that could be sterilized in the late 1940's has since allowed for various designs and configurations of fermentation systems, as the risk of culture contamination has been greatly reduced. Bioreactor systems allow for enhanced control of environmental conditions such as pH, temperature, and dissolved oxygen and have a significant role in manipulating the metabolic production of primary and secondary by-products. Primary by-products are produced as part of the natural metabolism of an organism, whereas

secondary metabolic products are produced as a response to the survival mechanism of the organism as it reaches the lag phase of its growth cycle. Primary metabolites include alcohol, lactic acid, and certain amino acids, whereas secondary metabolites generally include chemicals of pharmaceutical importance such as anti-biotics and atropine (Papagianni, 2012).

2.6.2 Bioreactor Design and Classification

Bioreactors are vessels that allow for the manipulation of environmental conditions, including dissolved oxygen, pH, temperature, and agitation. The four classifications of fermenters that are commonly used in food and pharmaceutical industries include: 1) Batch fermentors, 2) Continuous stirred tank fermenters, 3) Tubular fermentors, and 4) Fluidized bed fermentors. Fluid mechanics differentiate between models, which impacts oxygen transfer and growth kinetics (García-Ochoa & Gomez, 2008; McNeil & Harvey, 2008). Aerated stirred tank batch fermenters are frequently used due to their versatility. Batch and continuous fermenters essentially function in the same manner, with the primary difference being in the way the spent media is harvested. Continuous fermenters have a perpetual flow of spent and fresh media, allowing producers to harvest by-products in a manner that does not affect microbial density (Okafor, 2007). Continuous fermentation, though less labor intensive, provides more opportunities for contamination. Fermentative processes utilizing yeast as the primary organism are thus optimal for this method, as contamination is not a primary issue (Stanbury, 2002). The applications of batch and continuous bioreactor designs are diverse and are used in biofuel, food, and pharmaceutical industries. Tubular fermenters differentiate from stirred tank models in that they do not use mechanical agitation, and have a continuous flow through piping.

Advantages to this design include less foam formation because of no mechanical agitation, and comparatively easy scale-up. On a small scale, tubular fermenters work similarly to stir tanked reactors, with both designs influencing changes in the cells macro- and microenvironments in a similar manner (Russell et al., 1974). Fluidized bed fermenters are also inherently a continuous reactor design, and have an advantage over traditional mechanically agitated systems in that it is less abrasive, and therefore ideal for more delicate cells. Fluidized beds are generally more difficult than other systems, primarily because biocatalysts such as cells or enzymes need to be immobilized into or onto a solid support, and the hydrodynamic characterization of the system can present challenges, especially when incorporating multiple phases (gas, liquid, solid) (Godia & Sola, 1995).

CHAPTER III

MATERIALS AND METHODS

3.1 Culture Preparation

A stock culture of *L. diolivorans* (DSM 14421) (DSMZ, Braunschweig, Germany) was rehydrated using 0.5 ml of MRS medium (Sigma-Aldrich. Milwaukee, WI) for 30 min at ambient temperatures under a vacuum hood (Labconco, Kansas City, MO). An aliquot of 0.25 ml hydrated inoculant was transferred to 5 ml of MRS medium while the remaining 0.25 ml of solution was streaked onto a plate containing MRS agar per the manufacturer's instructions for reconstitution. The plate and tube were subsequently placed in a Gas-Pak™ 150 anaerobic chamber (Becton Dickinson and Co. East Rutherford, NJ) with added AnaeroPack-Anaero sachets (Mitsubishi Gas Chemical Co., Inc. Tokyo, Japan) and incubated at 30˚C for 48 h. Cells were supplemented with a 50% (w/v) glycerol solution and then stored at -80 ℃.

3.2 Preparation of Growth Media

A de Man, Rogosa, Sharpe (MRS) broth (Sigma-Aldrich. Milwaukee, WI) and MRS agar (Remel, Hampshire, United Kingdom) were prepared for the enumeration of Lactobacilli according to the manufacturer's instructions and autoclaved at 121° C for 15 min in a Sterilmatic STM-E (Market-Forge Industries, Everett, MA). For Bioreactor trials, a modified MRS media was prepared. The composition of modified MRS (per liter deionized water) contained 10 g of casein peptone (pancreatic digest) (Becton, Dickinson and Co., Franklin Lakes, NJ), 10 g meat extract (Sigma-Aldrich. Milwaukee, WI), 5 g yeast extract (Himedia, West Chester, PA), 33 g glucose × H_2O (Modernist Pantry, Eliot,

ME), 5 g Na-acetate (Eisen-Golden Laboratories, CA), 1 g polysorbate 80 (Modernist Pantry, Eliot, ME), 2 g K_2HPO_4 (Fischer Scientific, Waltham, MA) 2.6 g Na3-citrate x 2H2O (Sigma-Aldrich. Milwaukee, WI), 1.17 g $(NH_4)_2HPO_4$ (Sigma-Aldrich. Milwaukee, WI), 0.2 g $MgSO_4 \times 7H_2O$ (Eisen-Golden Laboratories, CA), and 0.05 g $MnSO_4 \times H_2O$ (Eisen-Golden Laboratories, CA). Media was supplemented with 7% (w/v) glycerol (Bulk Apothecary, Aurora, OH) for all cultivations. When preparing media for bioreactor trials, proteins and minerals were prepared separately from glucose and glycerin to prevent the caramelization of carbohydrates during autoclave cycles. Twenty g of casein peptone (pancreatic digest), 20 g meat extract, 10 g yeast extract, 10 g Na-acetate, 2 g Tween 80, 4 g K_2HPO_4, 5.2 g Na_3-citrate $\times 2H_2O$, 2.34 g $(NH_4)_2HPO_4$, 0.4 g $MgSO_4 \times 7H_2O$ and 0.10g $MnSO_4 \times H_2O$ were combined into 1 L of distilled water, and autoclaved for 15 min at 121°C inside the vessel compartment of the bioreactor system. Sixty-six g glucose $\times H_2O$ and 140g USP grade vegetable glycerin were combined in 960 ml of distilled water and prepared using a 500 ml bottle top sterile filtration unit with a 0.45 um membrane (Nalgene, Rochester, NY) and incorporated into the protein and mineral solution using a feed pump 24 h prior to inoculation

3.3 Preparation of Inoculant

A stock culture of *L. diolivorans* (DSM 14421) (DSMZ, Braunschweig, Germany) was removed from storage at -80 ℃ and thawed for 1h under a sterilized fume hood (Labconco, Kansas City, MO). One ml of inoculant was pipetted into 40 ml of sterile MRS media in a 100 ml conical flask. Conical flasks were sealed using parafilm, and the inoculant was subsequently placed in a Gas-Pak™ 150 anaerobic chamber (Becton Dickinson and Co. East Rutherford, NJ) with AnaeroPack-Anaero sachets (Mitsubishi Gas Chemical Co., Inc.

Tokyo, Japan) and incubated at 35℃ for 28 h. Cultured media was removed under a fume hood and aseptically transferred into a sterile 60 ml syringe (Becton, Dickinson and Co., Franklin Lakes, NJ). The rubber injection ports that are located on top of the bioreactors head plate were sterilized using a 70% (v/v) alcohol solution, and inoculant was subsequently injected into the modified MRS media inside the pre-sterilized vessel. MRS media was sparged to create an anoxic environment and stabilized to a temperature of 35℃ prior to injecting the inoculant. The injected inoculant amounted to 2% of the total working volume.

3.4 Vessel Preparation

A Celligen/BioFlo 310 bioreactor system (Eppendorf, Hamburg, Germany) was utilized to conduct batch fermentation studies. In all studies, a 2 L working volume was used for fermentation trials. Media components were combined and then stabilized to 35 °C and sparged using 10 standard L per minute (SLPM) of industrial grade nitrogen (Airgas, Radnor, PA). Dissolved oxygen concentration was monitored using an internal probe, with media maintaining a 3% ± 2 oxygen concentration throughout the duration of the experiment. pH probes were calibrated prior to autoclaving using standardized pH solutions of 4 and 7, and checked against an Acumet Portable pH meter (Model AP61 Fisher Scientific Pittsburgh, PA) before and after autoclave cycles. Initial pH of the media was 6.2 ± 0.1 and was maintained at 5.5 using a 5M KOH solution as a buffer. Stir rate was maintained at 150 rpm for the duration of the study. A 10% (w/v) antifoam solution was prepared using antifoam 204 solution (Sigma-Aldrich. Milwaukee, WI) and distilled water as a diluent. Temperature, pH, dissolved oxygen concentrations, and antifoam inputs

were automated using a PID controller that was programmed to maintain the above stated conditions.

3.5 Broth Harvesting and Refinement

3.5.1 Centrifugation

Ten ml of sample was harvested at 24 and 72 h and subsequently centrifuged to remove cell mass using an Eppendorf Centrifuge 5415 C (Eppendorf, Hamburg, Germany). Samples were centrifuged for 25 min at 10,000 rpm, and the supernatant was extracted for further analysis. Upon completion of fermentation trials at 144 h, the remaining broth was transferred into a sterilized 2L PYREX media storage bottle. At 1 h post-harvest, the supernatants were transferred into 250 ml Nalgene centrifugation bottles (Nalgene, Rochester, NY) and centrifuged using a Sorvall Rc-5b Refrigerated Superspeed Centrifuge. Parameters were the same as the 24 and 72 h centrifugation steps. Supernatant was extracted and stored for 24 h at 4°C prior to charcoal treatment.

3.5.2 Charcoal Treatment

Centrifuged supernatant was combined with 30g/L activated charcoal (Sigma-Aldrich. Milwaukee, WI) in 500 ml batches. Contents were combined in a 1 L Pyrex Erlenmeyer flask, and placed on a stir plate to incubate for 30 min at ambient temperature (20 °C) prior to filtration using a cellulose acetate bottle top filter with a 0.22 um membrane (Corning Inc. Corning, NY).

3.5.3 Vacuum Distillation

Charcoal treated samples were distilled using a vacuum distillation unit (Roto vapor R-210, Buchi) that was set at 100 mbar and a water bath temperature of 60 ± 2 °C. Five

hundred ml of charcoal treated samples were placed in a 1 L distillation flask and distilled until samples were concentrated to ten times that of their initial concentration. Samples were stored at 4 °C for 24 h and subsequently centrifuged at 3,000 x g for 15 min. Supernatant was separated from crystallizing salts and stored at 4 °C until further analysis.

3.6 HPLC Analysis

3.6.1 Sample Preparation

Ten ml of broth was harvested after 24, 72, and 144 h of fermentation and subsequently centrifuged at 10,000 × g for 20 min. Nine hundred ul of supernatant and one hundred ul of 0.04 M H_2SO_4 were combined into a syringe with a 0.45 um filter (Nalgene, Rochester, NY). Solutions were injected into 1.5 ml HPLC vials for analysis. The 144 h treatments were further processed using activated charcoal and vacuum distillation, with charcoal treatments prepared using a 9:1 dilution, and distilled treatments prepared as a 1/10 dilution using 900 ul 0.04 M H_2SO_4 as the diluent in combination with 100 ul of the distillate.

3.6.2 Fermentation by-product Analysis

The concentrations of 1,3 propanediol, acetic acid, and lactic acid (Sigma-Aldrich, Bellefonte, PA) that were present in the supernatant were quantified using a high performance liquid chromatograph (HPLC) (Agilent Technologies, Santa Clara, CA) that was equipped with an organic acids column (Aminex® HPX-87H Ion Exlusion Column) and a refractive index detector. Column temperature was maintained at 60˚C temperature, with a 0.6 ml/min flow rate and .004 M H_2SO_4 as the mobile phase (Pflügl et al., 2012).

Standard curves were generated using chemstation (Agilent Technologies, Santa Clara, CA) and analytical grade solutions of lactic acid, acetic acid, and 1,3 propanediol that were dissolved in MRS media at 5, 10, 15, 20, and 25mg/ml concentrations as external reference standards (Fischer Scientific, Waltham, MA). Standard curves were calibrated until an R^2 value of 0.99 or greater was obtained.

3.7 Mite Study Preparation

3.7.1 Coating Materials

Xanthan gum (TIC GUMS, White March, MD), carrageenan (TIC GUMS, White March, MD), propylene glycol alginate (TIC GUMS, White March, MD), and propylene glycol (Essential Depot. Sebring, FL) were utilized as coating materials.

3.7.2 Coating Preparation

Two hundred ml of each coating solution (n=4) was prepared in 500 ml glass beakers (PYREX®, Corning, NY). Xanthan gum was solubilized at room temperature, and other gums were solubilized by boiling in either MRS distillate (98% distillate, 1% PGA, 1% CG), MRS distillate with additional xanthan gum (97% distillate, 1% PGA, 1% CG, 1% XG), distilled water (98% dH_2O, 1% CG, 1% PGA), or propylene glycol (88% dH_2O, 10% propylene glycol, 1% CG, 1% PGA) on heated stirring plates until homogenous. To better incorporate gums in treatments that did not contain propylene glycol, an immersion blender (Hamilton Beach, Glenn Allen, VA) was used. Propylene glycol treatments were prepared by dissolving gums into PG and subsequently adding distilled water.

3.7.3 Coating pH

Prior to application on ham cubes, instrumental pH measurements were taken (n=4) for each coating. The pH was measured using an Acumet Portable pH meter (Model AP61 Fisher Scientific Pittsburgh, PA) by inserting a penetrating pH probe (model 05998-20, Cole Palmer, Vernon Hills, IL) into coating solutions, and allowing readings to stabilize prior to recording measurements.

3.7.4 Ham Preparation and Coating Percent Pick-up

Hams were cut into 2.5×2.5×2.5 cm cubes for mite bioassays. Cubes were dipped in coating solution treatments for 1 min and allowed to drip for 1h prior to inoculation with *T. putrescentiae*. Raw weights of ham cubes (n=10 per treatment) were measured for each coated treatment using a top loading balance (Mettler Toledo ML802T. Columbus, OH). Ham cubes were dipped in respective solutions for 1 min and allowed to drip for 1h prior to taking coated weights. Percent pick-up was measured as the difference between cubes before and after coating.

% Pickup = [(Coated Weight – Raw Weight)/(Raw Weight)] × 100

3.8 Mite Infestation Study

Twenty adult mites from the species *T. putrescentiae (Schrank)* with 10 or more females per group were transferred onto each cube from a laboratory colony, and the cube was placed in a ventilated, mite-proof 130-ml glass canning jar and incubated at 23 ± 2 °C and 70% RH. Mites were incubated for 14 and 21 d to allow for reproduction. Resulting mite populations on ham cubes were counted at the end of the 2 and 3-week incubation periods using a dissecting stereo-microscope (Olympus Model SZX10, Olympus Surgical &

Industrial America INC.). Only adult or immature mobile stages of mites were counted to estimate the level of reproduction from the initial 20 mites at the beginning of the trial. This method was adapted from Zhao et al. (2016).

3.9 Sensory Analysis

3.9.1 Ham Preparation

Ham slices (3.8 cm thick) were used for both coating and control treatments. Slices were suspended using a meat hook (100 mm × 5 mm, Rebel Butcher Supply, Flowood, MS) above the leg bone, submerged in coating solutions for 1 min and allowed to drip for 1 h. Slices were horizontally suspended from steel chains across two retort stands using burette clamps to hold the chains in place. After dripping, slices were placed into gallon freezer bags (S. C. Johnson & Son, Racine, WI) and allowed to age for 2 weeks at 4 °C prior to use in sensory evaluation tests.

3.9.2 Coating Preparation

Two L of each individual coating solution was prepared in 3.79 L stainless steel cooking pots. All raw materials that were used for sensory evaluation were certified USP grade. Distilled treatments were recreated using a blend of 2.5g/L acetic acid, 20.0 g/L lactic acid, 0.5g/L 1,3 propanediol, and glycerin constituting as the base. Xanthan gum was solubilized at room temperature, and other gums were solubilized by boiling in either a distillate blend (97% distillate, 1% PGA, 1% CG, 1% XG), distilled water (98% dH_2O, 1% CG, 1% PGA), or a propylene glycol solution (88% dH_2O, 10% propelyne glycol, 1% CG, 1% PGA) on stirring plates and heated until homogenous. To better incorporate gums in treatments that did not contain propylene glycol, an immersion blender (Oster Hand Blender w Blending

Cup Rancine, WI) was used. Propylene glycol treatments were prepared by dissolving gums into PG and subsequently adding additional components.

3.9.3 Panel Preparation

Coatings on ham slices were washed off with tap water at room temperature prior to cooking. The leg bone was removed, and ham slices were cut into 2 cm thick halves prior to wrapping in aluminum foil bags and baked at 177 °C to an internal temperature of 71 °C. Upon serving, ham slices were cut into 2.5 cm × 2.5 cm square pieces and placed into 29.5 ml clear plastic containers that were coded with 3-digit random codes. Samples were presented in a randomized order to panelists (6–8), with greater than 30 h of experience in tasting dry cured ham. Water, apple juice, unsalted crackers, and expectorant cups were provided to panelists who were seated in separate booths during each panel. A negative control was applied to setup the baseline to determine whether differences existed. The scale for the difference from control test was: 1 = no difference, 2 = slight difference, 3 = moderate difference, 4 = large difference, 5 = very large difference (Zhao et al., 2016).

3.10 Statistical Analysis

A completely randomized design with 2 replications and 5 sub-samples was used to test the treatment effects of incorporating fermented distillate into PGA+CG and PGA+CG+XG in comparison to 10% propylene glycol coatings, a gum control, and negative control on mite mortality (SAS version 9.4, SAS Institute, Cary, NC). When significant differences occurred ($P<0.05$), Duncan's multiple range test was utilized to separate the treatment means. A randomized complete block design with three replications was used to determine if trained panelists (n= 6-8) could detect a difference

between coated and non-coated hams ($P<0.05$). Duncan's multiple range test was utilized to separate the treatment means of samples in comparison to the blind control.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 HPLC Analysis

Lactic acid concentration did not differ (P>0.05) between the 24, 72, and 144 h sampling times (Table 4.1). The charcoal treatment did not impact (P>0.05) lactic acid concentration. However, the distilled samples had greater concentrations (P<0.05) than the other treatments, due to a 10 fold concentration of the by-products. Acetic acid concentrations were greater (P<0.05) after 144 h of fermentation than the 24 h and 72 h samples, and the 72 h charcoal treated samples had greater (P<0.05) acetic acid concentrations than the treatment that was fermented for 24 h. The distilled sample had a greater concentration (P<0.05) of acetic acid than all other treatments. The remaining constituents in solution were composed of residual glucose, glycerol, inorganic salts, and biomass. Refinement techniques were incorporated to remove cell mass and proteins from solution which could potentially serve as a food source. *T. putrescentiae* prefers high fat and protein foods, and can digest gram positive bacteria as a nutrient source. Therefore, removing these constituents from solutions would potentially enhance the efficacy of distilled treatments. Research on novel refinement techniques of fermentation broth containing 1,3 PD indicated that activated charcoal at 30 g/L led to a 96% reduction of protein in solution. Charcoal treatments using quantities of 30 g/L also reported the loss of 1,3-propanediol at (15.2%) (Anand et al., 2011). Charcoal (30 g/L) purification resulted in 12.5% reduction in 1,3 propanediol with a final concentration of 0.7 g/L in comparison to the initial 0.8g/L from the untreated 144 h samples. Use of activated charcoal also reduced odor and clarified broth, which initially had a yellow hue. The

distilled sample contained 6.0 g/L of 1,3 propanediol due to concentration of the sample, which caused this sample to have a greater concentration ($P<0.05$) than all other treatments. This is a concentration of 0.6 %, which is much less than the 10 % or greater concentrations used in testing by Abbar et al., (2016) and Zhao et al., (2016). Production of 1,3 propanediol was significantly lower in comparison to quantities reported by Pflügl et al., (2012), which reported 41.7 g/L 1,3 PD when using the same nutrient media and fermentation conditions. Strains of Lactobacilli including *L. Brevis*, *L. buchneri*, and *L. reuteri*, though not as efficient in 1,3 PD production, have reported upwards of 16.8 g/L of 1,3 PD under anaerobic conditions, and should be explored as alternatives, or added in combination with *L. diolivorans* (Lee et al., 2015). In future trials, a longer fermentation time may be necessary to allow bacteria to fully utilize the glycerol that is present in the media. Ensuring anoxic conditions is another important aspect of proper metabolism that will need to be monitored further. In addition, these ingredients can potentially be included inside an anaerobic food grade coating that could continue to produce lactic acid, acetic acid, and 1,3 propanediol during the 3 month or longer aging period for dry-cured hams.

4.2 Coating pH and Pick-up %

The distilled treatment with carrageenan (CG), propylene glycol alginate (PGA) and xanthan gum (XG) (distilled treatment 2) had the lowest pick-up percentage at 3.7% as compared to the distilled treatment 1 that was the same as distilled treatment 2, without xanthan gum (distilled treatment 1) which had a pick-up of 9.6% (Table 4.2). The addition of xanthan gum was used as a supplementary hydrocolloid to CG and PGA in distilled treatment 2. Functionality of the gums used in this study may have been affected

by variables including pH and lack of free water in distillates due to the concentration of media. The viscosity of carrageenan is stable over a wide pH range, but is optimal between a 4.5 and 5.5 pH. pH values above or below this range leads to hydrolysis over time. Distillates for treatments 1 and 2 had pH values of 6.02 and 5.77 prior to application to gums, and a 5.89 and 5.68 pH after incorporating gums, respectively. pH above the optimal functionality range may have been a contributing factor to the varying viscosity between treatments. In respect to gum functionality, residual potassium hydroxide that was used as a buffer during fermentation trials was expected to assist with gelation of carageenans in solution, as potassium and calcium ions promote the mechanism of gelation (Wüstenberg, 2014). This can be observed with the high pick-up % of distilled treatment 1 (9.0%), but was not consistent for distilled treatment 2 (3.7%). Propylene glycol alginate is also stable over a wide range of pH's, but tends to have a higher viscosity at a pH above 5. Alginates require full hydration to achieve optimal functionality. Therefore, the limited amount of free water in the distillate that was used may have had contributed to the limited functionality in distilled treatment 2, despite having an optimal pH. Considering the effectiveness of distilled treatment 2 in comparison to distilled treatment 1, the limited pick-up % did not influence mite inhibition, and with refinement could prove to be a more economical treatment because of the lower amount of coating necessary for adequate inhibition.

4.3 Mite Mortality Studies

4.3.1 Mite Mortality 2 Weeks Incubation

On average, the control samples had a greater ($P<0.05$) number of mites than the coated samples (Table 4.3). Gum controls were more effective at controlling mite reproduction

(P<0.05) than negative controls, but had a greater number of mites (P<0.05) than the 10% PG and distilled treatments. Mite counts for 10% PG samples were less than the initial inoculation amount, and had fewer (P<0.05) mites in comparison to the control and gum control samples. The two distilled treatments did not differ (P>0.05) from the 10 % PG treatments and were both effective at controlling mites. Zhao et al. (2016) previously demonstrated that food-grade coatings that were made from carrageenan and propylene glycol alginate or xanthan gum with propylene glycol as the active ingredient inhibited mite reproduction over a 2 week incubation period without impacting the sensory quality of the ham. This suggests that the hydrocolloids themselves have mite inhibiting properties, acting either as a chemical deterrent, or simply as a physical barrier to the hams themselves. Zhao et al. (2016) also reported on the effect of 10% PG when used in (1% PGA + 1% CG) as coatings, and found an average of 2 mites after 2 weeks incubation, as compared to the average of 16.5 found in this study. Since mite populations had not reproduced as much as expected in control treatments after 2 weeks of incubation, samples were incubated for an additional week (3 weeks total) and mites were counted again.

4.3.2 Mite Mortality 3 Weeks Incubation

On average, control treatments had greater mite counts (P<0.05) than all other treatments (Table 4.4). Gum control samples had fewer mites (P<0.05) than the control, but were less effective than the 10% PG and distilled treatments. The 10% PG treatment was more effective (P<0.05) in reducing mite populations than the control and gum control, but was equally as effective in mite control as both the distilled treatment 1 (98% MRS + 1% CG + 1% PGA) and distilled treatment 2 (98% MRS + 1% CG + 1% PGA + 1% XG). The

distilled treatment 2 had fewer mites ($P<0.05$) than the distilled 1 treatment after 3 weeks of incubation and had fewer mites ($P<0.05$) than all other treatments with the exception of the 10 % PG treatment, which was equally as effective ($P>0.05$). Proliferation of mold was observed on controls, gum controls, and distilled treatments after both incubation periods, but was absent on 10% PG treatments. Mold growth on distilled treatments was more pronounced than the control and gum control treatment samples. A combination of Aw, pH, and residual nutrients within the distilled media may have contributed to higher proliferation of mold in the distilled treatments and gum controls, whereas the lack of mold on 10% PG treatments can be attributed to the inherent antifungal and antimicrobial properties of propylene glycol (Kinnunen & Koskela, 1991; Nevarez et al., 2009; Rivard, 1961). Despite the presence of mold on the surface of the distilled treatments, which is a potential food source for *T. putrescentiae*, there was still a significant reduction in mite populations for both distilled treatments. The production of lactic acid during fermentation may have contributed to the lower mite populations on distilled treatments. Research conducted on the orientation of *T. putrescentiae (Schrank)* towards olfactory stimuli showed that concentrations of 1-5% lactic acid acted as an attractant to mites. However, at concentrations at or above 15%, lactic acid acted as a strong repellent (Žďárková, 1998). Distilled treatments were concentrated up to ten times from raw weight, and had an average of 16.4% lactic acid when incorporated into coatings, and in combination with the 1,3 PD present from fermentation, could have acted synergistically in mite inhibition. Another compound found to repel mites in higher concentrations, cinnamic aldehyde, has also been correlated with the inhibition of mold and aflatoxin production when incorporated in a yeast-extract sucrose broth at 150 ppm (Bullerman et

al., 1977; Žďárková, 1998). Lactic acid bacteria are known to produce fungistatic metabolites as well. Heterofermentative LAB produce propionic acid in trace amounts, and at a pH of 4.5 or below reduces fungal growth (Reis et al., 2012). The relatively high pH in distilled treatments (<5.60) may have impacted the efficacy of antifungal compounds that may have been produced during fermentation. Use of mold inhibiting substances in future coating formulations may assist with delaying or mitigating the presence of mold growth, thus limiting the viable food sources for *T. putrescentiae*. Mite counts increased ($P<0.05$) for control, gum control, and distilled treatment 1 between the 2nd and 3rd week counts. However, the distilled treatment 2 and 10 % PG treatment did not differ in counts between 2 and 3 weeks of incubation time, which indicates that these two treatments were still effective at controlling mites after distillation treatment 1 was no longer effective.

It is hypothesized that lactic acid was the major inhibiting component in distilled treatments. Due to the minimal sensory effects and mite inhibition of distilled treatments, which contained a relatively high concentration of lactic acid, it would be beneficial to conduct future coating trials to evaluate the effects of incorporating 15% lactic acid in varying concentrations of propylene glycol. This combination can potentially provide optimal mite inhibition with lower amounts of PG, thus reducing the cost of coating formulations. Additional studies are needed to determine if lactic acid is effective at controlling mites without the presence of other ingredients.

4.4 Sensory Evaluation

No differences ($P>0.05$) existed between control and coated treatments with respect to flavor, texture, or moistness of samples (Table 4.5). On average, all attributes were rated

between slightly and moderately different in comparison to the control for both treatment samples and the blind control. Zhao et al. (2016) previously reported that 100% propylene glycol dipped coatings and propylene glycol within food grade coatings did not differ in comparison to blind controls, and were rated as slightly different from blind controls. Prior to preparation of samples, distilled treatments had a noticeable acidic aroma, yet when washed off and cooked, were not differentiable. This suggests that the acids that were used remained in gum suspensions, since there was no detectable sensory difference between the controls, when compared to the blind control. Variability from piece to piece dependence due to differences in muscle cuts was taken into consideration, and panelists were served samples from the same anatomical muscle for different treatments. Despite this consistency, a high standard error was observed for all attributes, with texture having the highest overall SEM. This is likely due to natural variability in products that are aged for months and has been previously seen in many studies on dry cured ham and fresh pork sausage.

Table 4.1 Mean concentration of primary metabolites in MRS media at 24, 72, and 144 h that were subsequently refined using activated charcoal and vacuum distillation and quantified using HPLC-RID.

Sample (h)	Lactic acid (mg/ml)	Acetic acid (mg/ml)	1,3 Propanediol (mg/ml)
24	23.1[b]	2.8[d]	0.7[c]
72	23.8[b]	3.2[c]	0.8[bc]
144	26.4[b]	3.6[b]	0.8[b]
144 (CT)	23.4[b]	3.1[c]	0.7[c]
144 (DT)	164[a]	24[a]	6.0[a]
SEM	3.51	0.029	0.002

[1]CT: Charcoal treatment, DT: Distilled treatment
[2]Means with same letter within each row are not significantly different (P>0.05)

Table 4.2 Coating pH and average pick-up percentage of solutions on (n=40) ham cubes prepared for 2 and 3 week mite bioassay studies.

Treatment	Substrate pH	Coating pH	% Pick-up
Gum Control	(dH_2O) 7.00	3.69	4.7
10% PG	(PG) 6.00	3.75	8.0
Distilled Trt 1	(MRS) 6.02	5.89	9.6
Distilled Trt 2	(MRS) 5.77	5.68	3.7

[1]PG: propylene glycol, dH_2O: distilled water

Table 4.3 Mean number of mites on ham cubes that have been coated with different polysaccharides and base ingredient combinations and inoculated with (20 mites/cube) after 2 weeks incubation

Treatment	Polysaccharides	Base	Mean
Control	n/a	n/a	66.0[a]
Gum Control	PGA (1%) + CG (1%)	dH_2O (98%)	38.4[b]
10% PG	PGA (1%) + CG (1%)	PG (10%) + dH_2O (88%)	16.5[c]
Distilled Trt 1	PGA (1%) +CG (1%)	Distilled MRS (98%)	3.3[c]
Distilled Trt 2	PGA (1%) + CG (1%) + XG (1%)	Distilled MRS (97%)	0.8[c]
SEM			6.5

[1]PGA: propylene glycol alginate, CG: carrageenan, XG: xanthan gum, PG: propylene glycol MRS: De man, Rogosa, and Sharpe Broth

[2]Means with same letter within each row are not significantly different ($p>0.05$)

Table 4.4 Mean number of mites on ham cubes that have been coated with different polysaccharides and base ingredient combinations and inoculated with (20 mites/cube) after 3 weeks incubation.

Treatment	Polysaccharides	Base	Mean
Control	n/a	n/a	236.2[a]
Gum Control	PGA (1%) + CG (1%)	dH_2O (98%)	164.9[b]
10% PG	PGA (1%) + CG (1%)	PG(10%) + dH_2O (88%)	15.0[cd]
Distilled Trt 1	PGA (1%) + CG (1%)	Distilled MRS (98%)	45.8[c]
Distilled Trt 2	PGA (1%) +CG (1%) + XG (1%)	Distilled MRS (97%)	0.3[d]
SEM			11.5

[1]PGA: propylene glycol alginate, CG: carrageenan, XG: xanthan gum, PG: propylene glycol, MRS: De man, Rogosa, and Sharpe Broth

[2]Means with same letter within each row are not significantly different ($p>0.05$)

Table 4.5 Mean scores for sensory differences for flavor, texture, and moisture of coated ham samples using a difference from control test with trained panelists (n=6-8).

Treatment	Composition	Flavor	Texture	Moisture
Blind Control	N/A	2.0	2.2	2.3
Gum Control	dH_2O (98%), PGA (1%), CG (1%)	2.0	2.2	2.4
10% PG	dH_2O (88%), PG (10%), PGA (1%), CG (1%)	2.1	2.5	2.4
Distilled Trt	DB (97%), CG (1%), PGA (1%), XG (1%)	2.0	2.5	2.1
SEM		0.71	1.18	1.16

[1]PGA: propylene glycol alginate, CG: carrageenan, XG: xanthan gum, PG: propylene glycol, DB: distillate blend

[2]Means with same letter within each row are not significantly different (P>0.05)

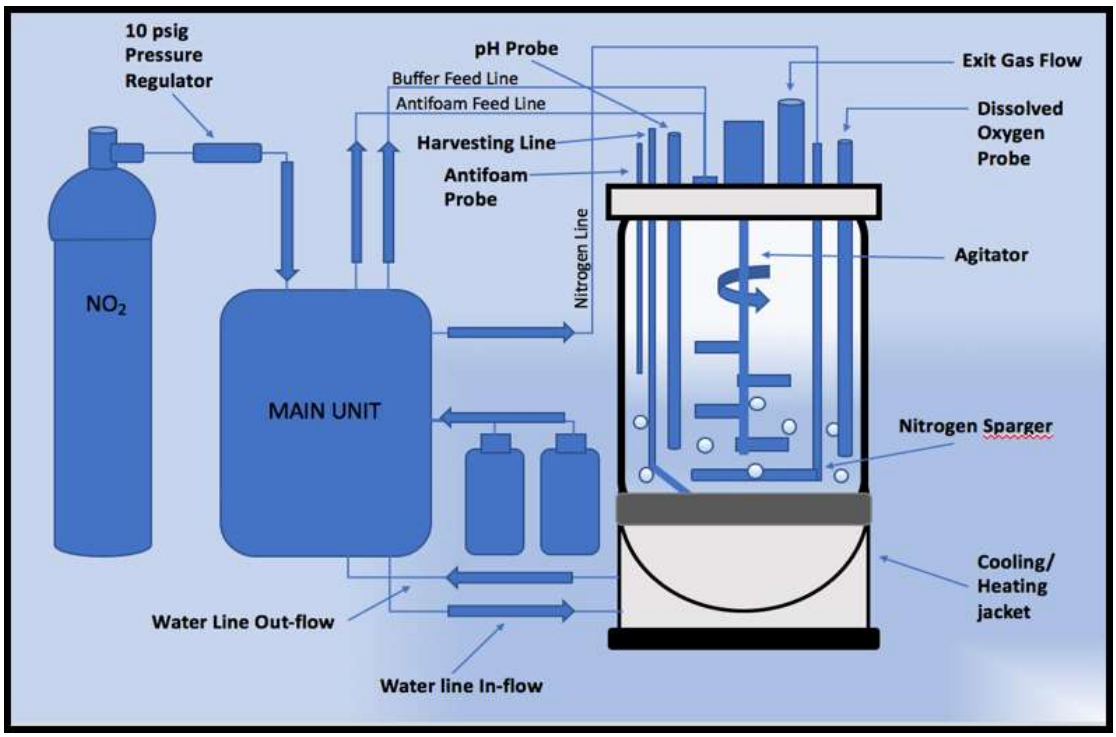

Figure 4.1 Schematics of a Bioflo 310 bioreactor system designed for anaerobic fermentation.

CHAPTER V

CONCLUSION

Coating formulations that incorporated fermented by-products of *L. diolivorans* had a significant reduction in mite populations as compared to the control and gum control treatments. In addition, these coating formulations were equally as effective as the positive control (10% PG) which was effective at controlling mite reproduction in previous studies. In future studies, optimizing gum functionality and incorporating mold inhibitors will help produce more effective and consistent coating solutions when incorporating viscous liquids. Optimizing both bioreactor design and operation, as well as refinement procedures will also be necessary to increase metabolic production and harvest rates of 1,3 propanediol. Higher 1,3 PD producing strains should be explored, as *L.diolivorans* DSM 19668, a genetically modified version of the strain used in this experiment, has shown a 20% increase of 1,3 PD in batch cultures (Pflügl et al., 2013). Despite the low production of 1,3 propanediol in fermented and distilled treatments, a significant reduction in mite populations was observed. Because of this result, more research should be conducted on fermentation constituents to determine which compounds are specifically causing mite inhibition. Lactic acid, the leading by-product in distilled treatments, is hypothesized to have aided in inhibition, and should be further tested in combination with other effective compounds to assist with cost reduction in the development of coating formulations designed for inhibition of *T. putrescentiae*.

REFERENCES

Abbar, S. 2016. Methods for management of *Tyrophagus putrescentiae* (Schrank) (Sarcoptiformes: Acaridae) in dry-cured ham facilities. Ph.D. diss., Kansas State University., Manhattan. (https://krex.kstate.edu/dspace/bitstream/handle/2097/34466/SaleheAbbar2016.pdf?sequence=1).

Abbar, S., B. Amoah, M.W. Schilling, and T. W. Phillips. 2016. Efficacy of selected food-safe compounds to prevent infestation of the ham mite, *Tyrophagus putrescentiae* (Schrank) (Acarina: Acaridae), on southern dry-cured hams. Pest Management Science, 72(8): 1604-12.

Ainala, S., S. Ashok, Y. Ko, and S. Park. 2013. Glycerol assimilation and production of 1,3-propanediol by *Citrobacter amalonaticus* Y19. Applied Microbiology and Biotechnology, 97(11): 5001-5011.

Anand, P., R. K. Saxena, and R. G. Marwah. 2011. A novel downstream process for 1,3-propanediol from glycerol-based fermentation. Applied Microbiology and Biotechnology, 90(4): 1267–1276.

Arlian, L.G., M. S. Morgan, D. L. Vyszenski-Moher, and D. Sharra. 2009. Cross-reactivity between storage and dust mites and between mites and shrimp. Experimental and Applied Acarology, 47(2):159-72.

Bahrami, F., K. Kamali, and Y. Fathipour. 2007. Life history and population growth parameters of *Tyrophagus putrescentiae* (Acari: Acaridae) on Fusarium Graminearum in laboratory conditions. Journal of Entomological Society of Iran, 26(2): 7-18.

Barrangou R., S. J. Lahtinen, F. Ibrahim, and A. C. Ouwehand. 2012. Genus *Lactobacillus*. In: Lahtinen, S., A. C. Ouwehand, S. Salminen, A. V. Wright, editors, Lactic acid bacteria: microbiological and functional aspects. 4. (pp. 77-85): CRC Press. Boca Raton, FL.

Barker, P. S. 1967. The effects of high humidity and different temperature on the biology of *Tyrophagus putrescentiae* (Schrank) (Acarina: Tyrogliphidae). Canadian Journal of Zoology, 45(1): 91–96.

Battcock, M. and S. Azam-Ali. 1998. Fermented foods: an ancient tradition. In Fermented Fruits and Vegetables: A Global Perspective (pp. 1-6): Food and Agriculture Organization of the United Nations. Rome, Italy.

Bullerman, L. B., F. Y. Lieu, and S. A. Seier. 1977. Inhibition of growth and aflatoxin production by cinnamon and clove oils, cinnamic aldehyde and eugenol. Journal of Food Science, 42(4): 1107–1109.

Campbell, Y. L., Y. Zhao, S. Abbar, T. W. Phillips, and M. W. Schilling. 2017. Mite control and sensory evaluations of dry cured hams with food grade coatings. Meat and Muscle Biology, 1(1): 100-108.

Choi, B., Y. Lee, T. Song, K. Kim, M. Sohn, and J. Baek. 2009. Sensitization to *Tyrophagus putrescentiae* in Korean Children with Allergic Diseases. Journal of Allergy and Clinical Immunology, 125(2): 1.

Da Silva, G. P., C. J. B. Lima, and J. Contiero. 2015. Production and productivity of 1,3-propanediol from glycerol by *Klebsiella pneumoniae* GLC29. Catalysis Today, 257: 259–266.

Dangaran, K., P. M. Tomasula, and P. Qi. 2009. Structure and function of protein-based edible films and coatings. In Embuscado, M. E. and Huber, K.C., editors, Edible Films and Coatings for Food Applications (pp. 25–56): Springer. New York, NY.

Erban, T. and J. Hubert. 2008. Digestive function of lysozyme in synanthropic acaridid mites enables utilization of bacteria as a food source. Experimental and Applied Acarology, 44(3):199–212.

Erban, T., P.B. J. Klimo. T. Smrz, W. Phillips, M. Nesvorna, J. Kopecky, and J. Hubert. 2016a. Populations of stored product mite *Tyrophagus putrescentiae* differ in their bacterial communities. Frontiers in Microbiology, 7: 1046.

Erban, T., D. Rybanska, K. Harant, B. Hortova, and J. Hubert. 2016b. Feces derived allergens of *Tyrophagus putrescentiae* reared on dried dog food and evidence of the strong nutritional interaction between the mite and *Bacillus cereus* producing protease bacillolysins and exo-chitinases. Frontiers in Physiology, 7: 53.

Falguera, V., J. P. Quintero, A. Jiménez, J. A. Muñoz, and A. Ibarz. 2011. Edible films and coatings: structures, active functions and trends in their use. Trends in Food Science & Technology, 22(6): 292–303.

Farrell J. 2017. Diagnostic methods for mould mite *Tyrophagus putrescentiae*. Australia Department of Agriculture. pbt.padil.gov.au/index.php?q=node/193&pbtID=184. (Accessed on 22 October 2017).

Florou-Paneri, P., E. Christaki, and E. Bonos. 2013. Lactic acid bacteria as source of functional ingredients. In Marcelino, K., editor, Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes (pp. 589–614): InTech. London, UK.

García, M.A., M. N. Martino, and N. E. Zaritzky. 2000. Lipid addition to improve barrier properties of edible starch-based films and coatings. Journal of Food Science, 65: 941–944.

García, N. 2004. Efforts to control mites on Iberian ham by physical methods. Experimental and Applied Acarology, 32(1/2): 41–50.

García-Ochoa, F. and E. Gomez. 2008. Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. Biotechnology Advances, 27: 153–176.

Godia, F. and C. Sola. 1995. Fluidized-bed bioreactors. Biotechnology Progress, 11(5): 479–497.

Green W. F. and A. J. Woolcock. 1978. *Tyrophagus putrescentiae*: an allergenically important mite. Clinical Allergy, 8: 135–144.

Hanson D. J., G. Rentrow, M. W. Schilling, W. B. Mikel, K. J. Stadler, and N. L. Berry. 2014. US products-dry-cured hams. In: Toldrá, F., Y. H. Hui, I. Astiasarán, J. G. Sebranek, R. Talon, editors, Handbook of fermented meat and poultry. 2. (pp. 347-350): Blackwell Publications. Ames, IA.

Howlader, M. S., W. T. French, S. A. Shields-Menard, M. Amirsadeghi, M. Green, and N. Rai. 2017. Microbial cell disruption for improving lipid recovery using pressurized CO_2 : role of CO_2 solubility in cell suspension, sugar broth, and spent media. Biotechnology Progress, 33(3): 737–748.

Jeong, K. Y., W. K. Kim, J. S. Lee, L. Lee, I. Lee, K. Kim, J. W. Park, C. Hong, H. Ree, and T. Yong. 2005. Immunoglobulin E reactivity of recombinant allergen Tyr P 13 from *Tyrophagus putrescentiae* homologous to fatty acid binding protein. Clinical and Diagnostic Laboratory Immunology, 12(5): 581–585.

Johnson, E. and L. Rehmann. 2016. The role of 1,3-propanediol production in fermentation of glycerol by *Clostridium pasteurianum*. Bioresource Technology, 209:1-7.

Kariduraganavar, M. Y., A. A. Kittur, and R. R. Kamble. 2014. Polymer synthesis and processing. In: Kumbar, S. G., C. T. Laurencin, M. Deng, editors, Natural and Synthetic Biomedical Polymers. (pp. 15-17): Elsevier. Burlington, MA.

Kilgour, F. G. 1953. Stedman's medical dictionary. The Yale Journal of Biology and Medicine, 26(2): 175.

Kinnunen, T., and M. Koskela. 1991. Antibacterial and antifungal properties of propylene glycol, hexylene glycol, and 1,3-butylene glycol in vitro. Acta Dermato-Venereologica, 71(2): 148–50.

Kleerebezem, M., and J. Hugenholtz. 2003. Metabolic pathway engineering in lactic acid bacteria. Current Opinion in Biotechnology, 14(2): 232–7.

Kokoszka S., and A. Lenart. 2007. Edible coatings – formation, characteristics and use – a Review. Polish Journal of Food and Nutritional Science, 57(4): 399–404.

Krochta, J. 2002. Proteins as raw materials for films and coatings: definitions, current status, and opportunities. In: Gennadios, A., editor, Protein-based Films and Coatings. (pp. 1-10): CRC Press. Boca Raton, FL.

Kuratsu, M., Y. Hamano, and T. Dairi. 2010. Analysis of the Lactobacillus metabolic pathway. Applied and Environmental Microbiology, 76(21): 7299–301.

Lee C. S., M. K. Aroua, W. M. A. W. Daud, P. Cognet, Y. Pérès-Lucchese, P. L. Fabre, O. Reynes and L. Latapie. 2015. A review: conversion of bioglycerol into 1,3-propanediol via biological and chemical method. Renewable and Sustainable Energy Reviews, 42: 963–972.

Lee, K. Y., and D. J. Mooney. 2012. Alginate: properties and biomedical applications. Progress in Polymer Science, 37(1): 106–126.

Leyton, J., P. J. Drury, and M. A. Crawford. 1987. Differential oxidation of saturated and unsaturated fatty acids in vivo in the rat. British Journal of Nutrition, 57: 383-393.

Matsumoto, T., T. Hisano, M. Hamaguchi, and T. Miike. 1996. Systemic anaphylaxis after eating storage-mite-contaminated food. International Archives of Allergy and Immunology, 109(2): 197–200.

McNeil, B., and L. M. Harvey. 2008. Fermentation: an art from the past, a skill for the future. In: McNeil, B., and L. M. Harvey, editors, Practical Fermentation Technology. (pp. 1–2): John Wiley & Sons, Ltd. Chichester, UK.

Mullen G. R., and B. M. OConnor. 2009. Mites. In Mullen G. R. and L. Durden, editors, Medical and Veterinary Entomology. 2. (pp. 423–482): Academic Press. Cambridge, MA

Nayak, M. K. 2006. Management of mould mite *Tyrophagus putrescentiae* (Schrank) (Acarina: Acaridae): a case study in stored animal feed. International Pest Control, 48(3): 128–130.

Nevarez, L., V. Vasseur, A. Le Madec, M. A. Le Bras, L. Coroller, I. Legurinel, and G. Barbier. 2009. Physiological traits of *Penicillium glabrum* strain LCP 08.5568, a filamentous fungus isolated from bottled aromatised mineral water. International Journal of Food Microbiology, 130(3): 166–171.

Nguyen, T., G. Mathiesen, L. Fredriksen, R. Kittl, T. H. Nguyen, V. G. H. Eijsink, C. K. Peterbauer. 2011. A food-grade system for inducible gene expression in *lactobacillus plantarum* using an alanine racemase-encoding selection marker. Journal of Agricultural and Food Chemistry, 59(10): 5617–5624.

Nieto, M. B. 2009. Structure and function of polysaccharide gum-based edible films and coatings. In: Embuscado, M. E. and Huber, K.C., editors, Edible Films and Coatings for Food Applications (pp. 57–112): Springer. New York, NY.

Nuñez, N. K., A. A. da Cunha, M. dos Santos Dutra, G. L. Barbosa, A. L. Morassutti, R. G. de Souza and P. M. Pitrez. 2016. Acute and chronic exposure to *Tyrophagus putrescentiae* induces allergic pulmonary response in a murine model. Asia Pacific Allergy, 6(1): 48–55.

Okafor, N. 2007. Fermenters and fermenter operation. In: Okafor, N., editor, Modern Industrial Microbiology and Biotechnology (pp. 196-202): Taylor & Francis Group. Boca Raton, FL.

Olmo, A., J. Calzada, and M. Nuñez. 2013. Lipolysis, lipid peroxidation, and color characteristics of serrano hams from duroc and large white pigs during dry-curing. Journal of Food Science, 78(11): C1659–C1664.

Oms-Oliu, G., R. Soliva-Fortuny, and O. Martín-Belloso. 2008. Using polysaccharide-based edible coatings to enhance quality and antioxidant properties of fresh-cut melon. LWT–Food Science and Technology, 41: 1862–1870.

Papagianni, M. 2012. Metabolic engineering of lactic acid bacteria for the production of industrially important compounds. Computational and Structural Biotechnology Journal, 3(4). doi: https://doi.org/10.5936/csbj.201210003.

Park, J. W., S. H. Ko, T. S. Yong, H. I. Ree, B. J. Jeoung, and C. S. Hong. 1999. Cross-reactivity of *Tyrophagus putrescentiae* with *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* in urban areas. Annals of Allergy, Asthma, and Immunology, 83(6): 533-539.

Pavlath, A. E., & W. Orts. 2009. Edible films and coatings: why, what, and how? In: Embuscado, M. E. and Huber K. C., editors, Edible Films and Coatings for Food Applications (pp. 1–23): Springer. New York, NY.

Pflügl, S., H. Marx, D. Mattanovich, and M. Sauer. 2012. 1,3-Propanediol production from glycerol with *Lactobacillus diolivorans*. Bioresource Technology, 119: 133–140.

Pflügl, S., H. Marx, D. Mattanovich, and M. Sauer. 2013. Genetic engineering of *Lactobacillus diolivorans*. FEMS Microbiology Letters, 344(2): 152–158.

Pflügl, S., Marx, H., Mattanovich, D., & Sauer, M. 2014. Heading for an economic industrial upgrading of crude glycerol from biodiesel production to 1,3-propanediol by *Lactobacillus diolivorans.* Bioresource Technology, 152: 499–504.

Qi, X., J. Yun, Y. Qi, H. Zhang, F. Wang, Q. Guo, and Z. Cao. 2014. Expression and characterization of a novel 1,3-propanediol dehydrogenase from *Lactobacillus brevis.* Applied Biochemistry and Biotechnology, 179(6): 959-72.

Reis, J. A., A. T. Paula, S. N. Casarotti, and A. L. B. Penna. 2012. Lactic acid bacteria antimicrobial compounds: Characteristics and Applications. Food Engineering Reviews, 4(2): 124–140.

Rentfrow G., D. J. Hanson, M. W. Schilling, and W. B. Mikel. 2008. The use of methyl bromide to control insects in country hams in the Southeastern United States. Extension Publication. Lexington KT: University of Kentucky Extension/National Country Ham Association. Publication # ASC-171, pp. 1-2.

Rentfrow, G., R. Chaplin, and S.P. Suman. 2012. Technology of dry-cured ham production: science enhancing art. Animal Frontiers, 2.4: 26-31.

Reynolds, A. E., M. A. Harrison, R. Rose-Morrow, and C. E. Lyon. 2001. Validation of dry cured ham process for control of pathogens. Journal of Food Science, 66(9): 1373–1379.

Riudavets, J., C. Castañé, O. Alomar, M. J. Pons, and R. Gabarra. 2010. The use of carbon dioxide at high pressure to control nine stored-product pests. Journal of Stored Products Research, 46(4): 228–233.

Rivard, I. 1961. Influence of temperature and humidity on mortality and rate of development of immature stages of the mite *Tyrophagus pustrescentiae* (Schrank) (Acarina: Acaridae) reared on mold cultures. Canadian Journal of Zoology, 39(4): 419–426.

Rodriguez, J. G., M. F. Potts, and L. D. Rodriguez. 1980. Mycotoxin Toxicity to *Tyrophagus putrescentiae*. Journal of Economic Entomology, 73(2): 282–284.

Romans J. R., K. W. Jones, W. J. Costello, C. W. Carlson, and P. T. Ziegler. 1985. The meat we eat. 12th ed. Interstate Printers. Danville, IL.

Russell, T. W. F., I. J. Dunn, and H. W. Blanch. 1974. The tubular loop batch fermenter: basic concepts. Biotechnology and Bioengineering, 16(9): 1261–1272.

Sánchez-Ramos, I. & P. Castañera, P. 2001. Development and survival of *Tyrophagus putrescentiae* (Acari: Acaridae) at Constant Temperatures. Environmental Entomology, 30(6): 1082–1089.

Sauer, M., H. Russmayer, R. Grabherr, C. K. Peterbauer, and H. Marx. 2017. The efficient clade: lactic acid bacteria for industrial chemical production. Trends in Biotechnology, 35(8): 756-769.

Schillinger, U., and J. V. Villarreal. 2010. Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods. Food Control, 21(2): 107–111.

Schütz, H., and F. Radler. 1984. Anaerobic reduction of glycerol to propanediol-1.3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. Systematic and Applied Microbiology, 5(2): 169–178.

Smrž, J., and V. Čatská. 1987. Food selection of the field population of *Tyrophagus putrescentiae* (Schrank) (Acari, Acarida). Journal of Applied Entomology, 104(1–5): 329–335.

Soomro A. H., and T. K. A. Masud. 2002. Role of lactic acid bacteria (LAB) in food preservation and human health – a review. Pakistan Journal of Nutrition, 1(1): 20–24.

Stanbury, P. F., A. Whitaker, and S. J. Hall. 2013. An introduction to fermentation processes. In: Stanbury, P. F., A. Whitaker, and S. J. Hall, editors, Principles of Fermentation Technology. (pp. 1-9): Elsevier Science Inc. Tarrytown, NY.

Stanbury, P. F. 2002. Fermentation technology: the mode of operation of fermentation. In: Walker, J. M., R. Rapley, editors, Molecular Biology and Biotechnology. 4. (pp. 15-17): Royal Society of Chemistry. Cambridge, UK.

Stanley, N. 1987. Production, properties, and uses of carrageenan. In: McHugh, D. J., editor, Production and Utilization of Products from Commercial Seaweeds. Rome, Italy. Food and Agriculture Organization of the United Nations.

Stevens, M. J. A., S. Vollenweider, L. Meile, and C. Lacroix. 2011. 1,3-Propanediol dehydrogenases in *Lactobacillus reuteri*: impact on central metabolism and 3-

hydroxypropionaldehyde production. Microbial Cell Factories, 10: 61. doi:10.1186/1475-2859-10-61.

Stradley, L. 2004. Country ham and red eye gravy history. Accessed February 12, 2017. (www.whatscookingamerica.net/History/CountryHamHistory.html.).

Sybesma, W., C. Burgess, M. Starrenburg, D. van Sinderen, and J. Hugenholtz. 2004. Multivitamin production in *Lactococcus lactis* using metabolic engineering. Metabolic Engineering, 6(2): 109–115.

Tafuro, F., E. Ridolo, M. Goldoni, M. Montagni, A. Mutti, and M. Corradi. 2015. Work-related allergies to storage mites in Parma (Italy) ham workers. BMJ Open, 5(5): 1-8.

Toldrá, F. 1998. Proteolysis and lipolysis in flavor development of dry-cured meat products. Meat Science, 49(1): S101-S110.

United States Department of Agriculture. 2011a. BHT, 21 CFR 172.115. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.115. (Accessed 21 October 2017).

United States Department of Agriculture. 2011b. Regulatory requirements under the federal meat Inspection act and the poultry products inspection act, 9 CFR 416.2. https://www.gpo.gov/fdsys/pkg/CFR-2002-title9-vol2/pdf/CFR-2002-title9-vol2-sec416-2.pdf. (Accessed 21 October 2017).

United States Department of Agriculture. 2012a. Country ham, country style ham, dry cured ham, country pork shoulder, country style pork shoulder, and dry cured pork shoulder, 9 CFR 319.106. https://www.gpo.gov/fdsys/pkg/CFR-2012-title9-vol2/pdf/CFR-2012-title9-vol2-sec319-106.pdf. (Accessed 21 October 2017).

United States Department of Agriculture. 2012b. Prescribed treatment of pork and products containing pork to destroy trichinae, 9 CFR 318.10. https://www.gpo.gov/fdsys/pkg/CFR-2012-title9-vol2/pdf/CFR-2012-title9-vol2-sec318-10.pdf. (Accessed 21 October 2017).

United States Department of Agriculture. 2014. Regulatory requirements under the federal meat inspection act and the poultry products inspection act, 9 CFR 301.2. https://www.gpo.gov/fdsys/pkg/CFR-2011-title9-vol2/pdf/CFR-2011-title9-vol2-sec301-2.pdf. (Accessed 21 October 21 2017).

US Environmental Protection Agency. 2016. International actions - the montreal protocol on substances that deplete the ozone layer. https://www.epa.gov/ozone-layer-protection/international-actions-montreal-protocol-substances-deplete-ozone-layer (Accessed 11 October 2017).

Ustunol, Z. 2009. Edible Films and Coatings for Meat and Poultry. In: Embuscado, M. E. and Huber, K.C., editors, Edible Films and Coatings for Food Applications. (pp. 245-268): Springer. New York, NY.

Van de Velde, F., and G. A. De Ruiter. 2005. Polysaccharides II: polysaccharide from eukaryotes. In: Steinbüchel A., R. H. Marchessault, editors, Biopolymers. 6. (pp. 245-274): Wiley-VCH. Weinheim, Germany.

Veiga da Cunha, M., and M. A. Foster. 1992. Sugar-glycerol cofermentations in *Lactobacilli*: The Fate of Lactate. Journal of Bacteriology, 174(3): 1013–1019.

Vieira, M.G.A., M. A. Silva, L. O. dos Santos, M. M. Beppu. 2011. Natural-based plasticizers and biopolymer films: a review. European Polymer Journal, 47(3): 254–26.

Welker, D. L., J. E. Hughes, J. L. Steele, and J. R. Broadbent. 2015. High efficiency electrotransformation of *Lactobacillus casei*. FEMS Microbiology Letters, 362(2): 1–6.

Wright, A. T. and L. Axelsson. 2012. Lactic acid bacteria: an introduction. In: Lahtinen, S., A. C. Ouwehand, S. Salminen, A. V. Wright, editors, Lactic acid bacteria: microbiological and functional aspects. 4. (pp. 2-11): CRC Press. Boca Raton, FL.

Wüstenberg, T. 2014. General overview of food hydrocolloids: alginates and PGA. In: Wüstenberg, T., editor, Cellulose and Cellulose Derivatives In the Food Industry: Fundamentals and Applications. (pp. 6-10): Wiley-VCH. Weinheim, Germany.

Zacharof, M. P. and R. W. Lovitt. 2012. Bacteriocins produced by lactic acid bacteria a review article. APCBEE Procedia, 2: 50–56. doi: https://doi.org/10.1016/j.apcbee.2012.06.010.

Zd'árková, E. 1998. Biological control of storage mites by Cheyletus eruditus. Integrated Pest Management Reviews, *3*(2): 111-116.

Zhao Y., S. Abbar, T. W. Phillips, M. W. Schilling. 2015. Phosphine fumigation and residues in dry-cured ham in commercial applications. Meat Science, 107: 57-63.

Zhao, Y., S. Abbar, T. W. Phillips, J. B. Williams, B. S. Smith, and M. W. Schilling. 2016. Developing food-grade coatings for dry-cured hams to protect against ham mite infestation. Meat Science, 113: 73–79.