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Application of Food-grade Ingredient Treated Nets to Control Tyrophagus Putrescentiae (Schrank) (Sarcoptiformes: Acaridae) Infestations on Dry Cured Hams

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Application of food-grade ingredient treated nets to control Tyrophagus putrescentiae

(Schrank) (Sarcoptiformes: Acaridae) infestations on dry cured hams

By

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Application of food-grade ingredient treated nets to control Tyrophagus putrescentiae

(Schrank) (Sarcoptiformes: Acaridae) infestations on dry cured hams

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Tyrophagus putrescentiae (Schrank) (Sarcoptiformes: Acaridae), also known as the ham mite, may infest dry cured hams during the aging process. The fumigant methyl bromide is currently used to control mite infestations, but eventually will not be available for use since it contributes to the depletion of the ozone layer. The use of ham nets treated with xanthan gum, carrageenan, propylene glycol alginate, propylene glycol (PG), and lard were evaluated for their impact on mite orientation to or oviposition on treated or untreated ham cubes, mite reproduction and population growth over a 10-week period.

When nets were infused with gum and PG, behavioral tests indicated that greater than 95% of the mites oriented to the ham cubes that were wrapped in untreated nets when compared to treated nets and no eggs were laid on the latter. The reproduction assays indicated that there were fewer ($P < 0.05$) *T. putrescentiae* produced over a two-week period on ham cubes covered with both gum and PG treated nets when compared to the untreated or gum-only treated nets over the 10-week storage period of the experiment. Medium and high concentrations of PG treatments had the lowest mite reproduction

rates. No more than four mites could be found on each of these treatments in comparison to 200-300 mites that were on the untreated ham cubes.

When nets were infused with gum, PG, and lard, behavioral tests indicated that fewer mites oriented to the ham cubes that were wrapped with gum, lard, and medium PG than those with untreated nets. The oviposition assays revealed that on average less than three eggs were laid on the ham cubes with treated nets in comparison to 69-165 eggs on the untreated ham cubes. Reproduction assays demonstrated that fewer *T. putrescentiae* ($P < 0.05$) were on ham cubes with treated nets containing PG when compared to the number of mites on ham cubes with untreated nets over 10 weeks of storage. Lard infused nets without PG did not decrease the mite population ($P > 0.05$). The net without coating slowed the growth and reproduction of *T. putrescentiae* since net controls had fewer mites ($P < 0.05$) than controls without nets. With a few exceptions, fungi were not present on ham cubes that were treated with PG-containing nets over 10 weeks of storage.

This research demonstrated the efficacy of using nets treated with food-grade ingredients during ham aging to control mite infestations on a laboratory scale. Further research will be conducted to determine the effectiveness of the same treated nets on whole hams in commercial aging rooms.

Keywords: *Tyrophagus putrescentiae*, mite reproduction, propylene glycol, lard, gum, dry cured ham

DEDICATION

I would like to dedicate this dissertation to my lovely family: my parents,

Lianrong Zhang and Xiuyun Duan, my parents-in-law, Qingyu Zhang and Xiuyun Fu, my

husband, Li Zhang, and my daughters, Jiatong Zhang and Anna Zhang.

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CHAPTER I

INTRODUCTION

American dry cured ham, also known as country ham, is the uncooked, cured, dried, smoked, or unsmoked food product from a single piece of meat from the hind leg of a hog (USDA FSIS 9 CFR 319.106). A typical American country ham is cured with salt, nitrate/nitrite, sugar, and/or seasoning at 2-4 °C and aged for 3 to 24 months at 25-30 °C in aerobic conditions, which allows the ham to lose greater than 18% of its original weight (USDA FSIS 9 CFR 319.106). The prolonged curing and aging process as well as the presence of oxygen gives dry cured ham a salty, rich, meaty flavor, and a firm texture, but also facilitates the growth of molds as well as attracts pests (Hughes, 1976; Lee, Sung, & Lee, 2006; Macchioni et al., 2002; Sánchez-Molinero, García-Regueiro, & Arnau, 2010). Commonly found pests of dry cured hams are mites, cheese skippers, larder beetles, red-legged ham beetles, rats, and mice (Hoy, 2011; Zhao, Abbar, Amoah, Phillips, & Schilling, 2016a).

Tyrophagus putrescentiae (Schrank) (Sarcoptiformes: Acaridae) is an ubiquitous mite species, also known as the ham mite, mold mite, cheese mite, or copra mite. It is a major non-predatory mite species in stored food products and the house dust mite complex (Cui, 2014; Cui et al., 2016). *T. putrescentiae* frequently infests foods with relatively high fat, high protein and low moisture content (10-45%), such as dry cured ham, grains, aged cheese, spices, dried fruit, nuts, and other stored foods (Hughes, 1976;

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Rentfrow, Hanson, Schilling, & Mikel, 2006). This mite may enter the curing and aging plants by humans, insects or by floating on air currents (Garcia, 2004). Mites locate their prey mostly through their tactile and chemical senses (Qu, Li, Ma, & Song, 2016a; Qu, Ma, Li, Song, & Hong, 2016b). In the ham plant, mites feed directly on the meat, fungi, or dead bodies of mites with the help of pedipalps and chelicerae located in the front of their body (Edde, Eaton, Kells, & Phillips, 2012).

T. putrescentiae mites are tiny and difficult to detect at the early stage of infestations. They grow and multiply rapidly. Under ideal conditions (25 °C and 90% relative humidity), 100 mites can render approximately 100 g of dog foods to dust in less than four weeks (Sánchez-Ramos, Alvarez-Alfageme, & Castañera, 2007). Severely infested dry cured ham has brown dust on the surface and in the cracks and crevices as well as emits a putrid smell (Garcia, 2004; Jeong et al., 2005; Zhao et al., 2016a), which results in product rejection and economic losses. In addition, T. putrescentiae and its feces may cause allergic reactions including sensitization (Boquete et al., 2000; Garcia, 2004), dermatitis (Quiñones Estévez, 2006; Vidal & Rial, 1998), and occupational asthma in people who work with mite-infested products (Jeong et al., 2005).

Mites are controlled by multiple strategies including physical, chemical, cultural or biological methods. Among them, fumigation with methyl bromide is currently the only known effective control method for T. putrescentiae by the dry cured ham industry in the United States. Methyl bromide is an odorless, colorless gas that damages the nerve cell membranes in pests (Fields & White, 2002). The use of methyl bromide is being phased out internationally since it was listed as one of several substances responsible for the depletion of ozone in the atmosphere in the international Montreal Protocol (UNEP,

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1992). Although the United States dry-cured ham industry can use existing stocks of methyl bromide, no additional methyl bromide can be produced at the current time (EPA, 2017b). In response to the phase-out of methyl bromide, researchers from USDA, U.S. EPA, companies, and universities are working to develop effective alternatives by examining a variety of potential methyl bromide alternatives, including fumigants, natural acaricides, temperatures control, traps, and food grade coatings (Abbar, Amoah, Schilling, & Phillips, 2016a; Amoah, Schilling, & Phillips, 2016; Phillips & Throne, 2010; Zhao, Abbar, Phillips, Smith, & Schilling, 2016b).

Sulfuryl fluoride, commercially produced as ProFume, was effective at controlling the adult stage of mites but was ineffective at killing eggs (Phillips, Hasan, Aikins, & Schilling, 2008). Phosphine was effective at controlling mites in all stages of development under laboratory conditions, but it was not as effective in the commercial plants and caused corrosion of copper fittings (Zhao, Abbar, Phillips, & Schilling, 2015). Most natural acaricides including plant extracts and essential oils lessened mite infestations but did not achieve 100% mite mortality or had unacceptable flavor (Collins, 2006; Song, Kim, Lee, Yang, & Lee, 2016). Physical control methods including cold, heat and low relative humidity were effective at killing mite cultures (Abbar, Schilling, & Phillips, 2016b; Eaton & Kells, 2009). However, practical applications of these methods need to be evaluated at commercial plants to determine if they can be scaled up to production.

Application of food-grade ingredients on the ham surface has been tested for their potential to control mites. In Spain, coating with lard or vegetable oil is commonly done to manage mite infestations (Garcia, 2004), but no technical details were available. It was

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reported that applying a thin layer of lard to the dry cured ham cubes prevented *T.*
putrescentiae reproduction completely after inoculation with 20 adult mites on each ham
cube and two to three weeks' incubation (Abbar, Schilling, & Phillips, 2012; Zhao et al.,
2016b). One concern with this method is that lard is hydrophobic and therefore may slow
the moisture loss of dry cured ham and prevent the product from losing 18% of its
original weight during aging. Propylene glycol was also effective at controlling mite
infestations on dry cured ham (Abbar et al., 2016a; Zhao et al., 2016b). Studies indicated
that coatings that were composed of food grade gums and propylene glycol effectively
controlled mite infestations on dry cured ham cubes and slices without affecting sensory
quality. Typically, dry cured hams are aged in ham nets and hung from racks. Using ham
nets that are infused with coatings would be an easier and more practical option for ham
producers since spraying or dipping hams with coating requires an additional processing
step and labor. The initial test indicated that ham nets infused with food grade coating
were effective at controlling mite infestations in a bench-top study.

  Therefore, the objective of this study was: 1) to develop additional food-grade
coating formulations with gum, propylene glycol, and/or lard; 2) to determine the most
effective formulations of food-grade ingredients for use in ham nets with respect to
inhibiting mite contact and controlling mite reproduction over a 10-week storage period;
3) to evaluate the effect of food-grade ingredient infused nets on moisture content, water
activity, and weight loss of hams.

CHAPTER II

LITERATURE REVIEW

2.1   Dry cured ham

Dry-curing or salting meat was a necessity prior to the extensive use of refrigeration. Dry cured hams are primarily produced in a distinct climate zone, called the 'ham belt,' which includes most of lower Europe, the Mediterranean, China, and the Southeastern United States. The most popular hams from around the world include Spanish Iberian, French Corsican, Italian Parma, Germany Westphalian, Chinese Jinhua, and American country ham (Toldrá & Aristoy, 2010). American dry cured ham, also known as country ham, is the uncooked, cured, and dried product that results from a single piece of meat from the hind leg of a hog (USDA FSIS 9 CFR 319.106) that undergoes curing, salt equalization, ripening and drying, and may or may not be smoked. Country hams are mainly produced in Virginia, Tennessee, Kentucky, North Carolina, Georgia, and Missouri (Rentfrow, Chaplin, & Suman, 2012). Ham production starts from the green ham, which weighs approximately 9.5 kg, and should not be dark, firm, and dry (DFD) or pale, soft, and exudative (PSE) (Toldrá, 2002). The curing mix, consists of salt, nitrate/nitrite, sugar, and seasoning, and is rubbed onto all lean surfaces of the ham. The required period for curing (2-7 °C) and salt equalization (around 12.8 °C) is 45 days or longer (USDA FSIS 9 CFR 318.10 and 319.106), which allows salt to penetrate to the central part of the ham through osmotic pressure. The curing time for the ham is

approximately two days for every 0.45 kg of the green weight of the ham. Smoking is an optional process that follows the salt equalization step. If dry cured hams are smoked, the smoking temperature must be 32.2 °C or less to maintain the enzymatic activity of the proteins that are responsible for the development of the flavor and aroma of dry cured hams (FSIS, 1995). The final step of the aging process, also known as ripening or summer sweat, is a process that is used to develop the typical ham flavor, aroma, and texture (Rentfrow et al., 2012). Dry cured hams are hung on racks using hooks and/or ham nets and are aged for six weeks to two years (Graham, 2011), which contributes to the intense ham flavor. The aging environment is variable depending on the season and location. USDA requires that the internal temperature during aging should not exceed 35 °C (USDA FSIS 9 CFR 319.106). American country-style hams are normally aged at 25-30 °C and a relative humidity (RH) of 65-75% (Marriott, Graham, & Claus, 1992). In comparison, European dry cured hams are aged at a lower temperature range of 16-25 °C and 65-80% RH (Toldrá & Aristoy, 2010). The finished dry cured ham must have lost at least 18% of its original weight and contain at least 4% salt to be a legal product (USDA FSIS 9 CFR 319.106). On average, American dry cured ham contains 6.5% salt and has a water activity of 0.88 (Mikel & Newman, 2003).

2.2   *T. putrescentiae* infestations of dry cured ham

Dry cured ham pests include mites, cheese skippers, larder beetles, red-legged ham beetles, vertebrates (rats and mice), and fungi (Hoy, 2011; Zhao et al., 2016a). Among these pests, mites are the most difficult to detect and control. This is due to their small size, high fecundity, short life cycle, rapid dispersion, and strong adaptability to various ecological conditions. *T. putrescentiae* (Schrank) (Sarcoptiformes: Acaridae),

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also referred to as the ham mite, mold mite, cheese mite, or copra mite, is the most common non-predatory mite species that is isolated from dry cured ham (Mueller, Kelley, & VanRyckeghem, 2006). *T. putrescentiae* frequently infests stored products with relatively high fat, high protein and low moisture content, such as grains, aged cheese, spices, dried fruit, nuts, and other stored foods (Hughes, 1976; Rentfrow et al., 2006). According to a survey conducted in 2008, 22 out of 35 ham plants reported that their hams become infested with ham mites, especially when hams were aged more than five months (Rentfrow, Hanson, Schilling, & Mikel, 2008). Mite infestations occur on the surface of dry cured ham. However, mites can also penetrate into cracks and crevices (Garcia, 2004). Sometimes, the infestation spreads to the adjacent zone of the cracks or even the whole ham (Garcia, 2004). There are visible moving mites and brown dust on the surface of heavily infested hams that are made of biomass from live and dead mites, mite eggs, frass, and food articles, which leads to product rejection and economic losses (Jeong et al., 2005; Zhao et al., 2016a). In addition, mites are notorious for producing volatile chemicals which are responsible for sweet, minty, or putrid smells that are associated with mite-infested grain and dry cured hams (Garcia, 2004; Townsend, 2007; Tuma, Sinha, Muir, & Abramson, 1990). Tridecane is a major volatile odor compound produced by storage mites (Stejskal & Hubert, 2006).

T. putrescentiae is an allergen producer (Arlian, Geis, Vyszenski-Moher, Bernstein, & Gallagher, 1984; Cui et al., 2016; Liao, Ho, Yin, & Tsai, 2013; Liao, Lin, Chiu, Lin, & Tsai, 2013; Wakefield, 2006; Yu, Liao, & Tsai, 2014). Both mites and their feces are allergenic. The faecal pellets break up into small pieces, combine with dust particles and form allergenic dust. These allergens affect human skin and breathing. *T.*

putrescentiae is also a member of the house mite dust complex. Moreover, *T. putrescentiae* mites are known as vectors of mycotoxin-producing fungi (Hubert et al., 2003). Therefore, mite contamination in the food processing environment poses a health risk both in food products and plant workers (Hubert, Pekar, Nesvorna, & Sustr, 2010).

2.3 Taxonomy, morphology and biology of *T. putrescentiae*

Having a thorough knowledge of the biology and behavior of *T. putrescentiae* is necessary for ham producers, and other manufacturers to utilize integrated pest management (IPM) to control mite infestations.

2.3.1 Taxonomy and morphology

T. putrescentiae belongs to the phylum Arthropoda, the class Arachnida, the subclass Acari, the order Sarcoptiformes, the suborder Astigmatina, and the family Acaridae. It is a major pest of stored food products and houses. *T. putrescentiae* is a small and slender mite. Females and males are 320-420 µm and 280-350 µm long, respectively. The body surface of *T. putrescentiae* mites is translucent and smooth. Their body's appendages and cuticle are also colorless (Edde et al., 2012; Zhang, 2003).

The adult Acari mites have two major body regions (or tagmata), which include the gnathosoma and the idiosoma and are usually fused together (Walter & Proctor, 1999). Mites have no defined head. The gnathosoma consists of a pair of pedipalps and a pair of chelicerae (mouthparts). The pedipalps and chelicerae are used for sensory recognition and sometimes for capturing, tasting and ingesting food (Edde et al., 2012), locomotion, defense, and/or reproductive functions.

The idiosoma is the region with legs. Most adult mites have eight legs, but some may have six or even four legs. The exoskeleton of the typical mite includes an epidermis, Schmidt layer, endocuticle, and epicuticle. The epidermis produces hair-like setae (Hoy, 2011). The outermost layer of the cuticle, called the cerotegument, is a layer of secretions of waxes and proteins. The cerotegument is mainly responsible for water retention.

2.3.2   Life stages

The life stages of *T. putrescentiae* include the egg, prelarva, larva, protonymph, deutonymph, tritonymph, and adult. Eggs are oval or flattened with strong elasticity and has an exochorion layer that provides a barrier to desiccation (Witaliñski, 1993). The prelarva usually has three pairs of segmented legs but does not have mouth parts or setae (Klompen, 2000). Therefore, they can move but do not feed on external resources. The larva is the first active instar and is able to feed because it develops an open mouth. However, some larvae may not feed but can still develop into a nymph. The larva develops into the nymph, which includes the protonymph, deutonymph, and tritonymph stages. The pronymph is the first eight-legged nymphal instar; it is free living and active, and may or may not feed. The deutonymph is the second nymphal stage and closely resembles the adult but is smaller and does not have its external sexual parts. At this stage, most Astigmatans have an unusual deutonymph that is modified for dispersal, called a hypopus. However, *T. putrescentiae* is an exception since it does not develop a hypopus stage. The trionymph is the third nymphal stage and does not commonly occur in *T. Putrescentiae* (Vacante, 2016).

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2.3.3   Longevity

The longevity of mites depends on sex, temperature, relative humidity, and food availability. *T. putrescentiae* males live longer than females (Sánchez-Ramos & Castañera, 2005), which might be due to the higher physiological consumption required by females to continually produce eggs. The death of females reduces the metabolic cost that males need for gametogenesis and mating (Parkinson, Barron, Barker, Thomas, & Armitage, 1991), which also allows males to live longer. Unmated females of *T. putrescentiae* live longer than mated females (Boczek, 1991; Sánchez-Ramos & Castañera, 2005). Within the thermal threshold of growth, mite longevity decreases with increasing temperature. Adult mites live 115 days at 9.3 °C, and only 42.8 days at 31 °C. The greatest longevity for males occurred at 20 °C, with decreasing longevities above and below 20 °C (Sánchez-Ramos & Castañera, 2005). The longevity of mites also depends on the interaction effect of sex and temperature. The longevity difference between males and females is much greater at intermediate temperatures than extreme temperatures. Longevity decreases as the relative humidity decreases from 85% to 75%. In addition, mites live for a shorter period of time at 85% RH or greater. Suitable food sources help mites to live longer. Females and males can live for 99 days and 120 days on wheat germ, but can only live for 80, 75, and 25 days on pumpkin seeds, powdered milk, and rolled oats, respectively (Boczek, 1991). Larvae and nymphs of *T. putrescentiae* mites are more susceptible to starvation than adult mites. *T. putrescentiae* adults can live without food for at least two months at 24 °C and saturated conditions. They can live for up to five months at 10-12 °C.

2.3.4   Vision

Most mite species, including *Tyrophagus*, lack the complex compound eyes, but are still able to perceive day length and tend to move away from intense light. *T. putrescentiae* orients positively to violet or ultraviolet light, shows no preference to blue, and avoids orientation to green, yellow, red and white lights (Amoah, 2016).

It is likely that light is perceived through the exoskeleton directly by the brain (Hoy, 2011). Little is known about the vision of *T. putrescentiae*. The simple eyes of other mites (ex. *Tetranychus urticae*) can detect light through receptors that are present in their eyes. The anterior pair of eyes acts as a scanning point detector, but no image is formed (McEnroe, 1969; McEnroe & Dronka, 1969). Receptors for green and ultraviolet light are present in the anterior eye. The posterior pair of eyes, with simple convex lenses, has a non-directional receptor for near-ultraviolet light (Hoy, 2011).

2.3.5   Respiration

Respiration involves the intake of oxygen and elimination of carbon dioxide. Astigmatic mite *T. putrescentiae* does not have a stigma, the opening to the tracheal system. Little is known about the respiration mechanism of *T. putrescentiae*, but there is a positive correlation between the respiration rate of *T. putrescentiae* and its development at different temperatures (Hubert et al., 2010). Mite respiration almost increased linearly from 10 to 30 °C and decreased from 30 to 50 °C. The respiration optimum temperature was 31.5 °C, which was close the optimal growth temperature of 30 to 32 °C (Hubert et al., 2010; Sánchez-Ramos & Castañera, 2001, 2005) for *T. putrescentiae* development. The thermal preference range of *T. putrescentiae* was 29-35 °C, which was at least two times narrower than other species, including *Acarus siro* (L. 1758) (13-31 °C),

Dermatophagoides farina (Hughes, 1961) (19-36 °C), and *Lepidoglyphus destructor* (Schrank, 1871) (21-33 °C). For respiration, however, the lower thresholds were lesser and the upper thresholds were greater than for development. The respiration rate of *T. putrescentiae* was greater than the eurythermic species mentioned above at 30-45 °C (Hubert et al., 2010). This may partially explain why *T. putrescentiae* can survive for a long enough period in unfavorable temperature or moisture conditions to escape to suitable microhabitats (Eaton & Kells, 2009). In addition, the rate of respiration increases as acclimation temperature increases, which promotes the population growth of thermophilous mites in favorable conditions (Hubert et al., 2010).

2.3.6   Sensory system

Setae are the main sensing organ by which mites interact with their environment and are present on their body, pedipalps, and legs. Setae are tactile, and are sensitive to CO_2, vibrations, heat, light, relative humidity, pheromones (chemical cues perceived within the species), and kairomones (chemical cues perceived between species). The location and structure of setae can be observed under a phase-contrast microscope and are used to identify mite species (Hoy, 2011; Walter & Proctor, 1999).

T. putrescentiae mites have undeveloped eyes and no antennae, and therefore explore their environment mainly through their tactile setae and chemical senses (Qu et al., 2016a). Chemosensory genes are responsible for host location or sex pheromone detection in *T. putrescentiae* (Qu et al., 2016b). Qu et al. (2016) identified two putative chemosensory proteins (CSPs) in *T. putrescentiae*, which have been labeled TputCSP1 and TputCSP2. TputCSP1 mediates host recognition. TputCSP2's function is still unknown.

Mites also use their olfactory systems to recognize semiochemicals in the surrounding environment that guide their behavior, such as the detection of target hosts, mating, laying eggs and others (Qu et al., 2016b). Seven odorant receptors (ORs), one gustatory receptor (GR) and five ionotropic receptors (IRs) were identified from the transcriptome of *T. putrescentiae* (Qu et al., 2016a).

2.3.7   Water balance

Astigmatid mites are susceptible to water loss due to their small size, large surface-to-volume ratio, weakly sclerotized cuticles, and water activity (0.99) of their body fluids (Arlian, 1992). Water is lost by simple diffusion from the body surface, secretion of digestive fluids, defecation, excretion, production of pheromones or defensive fluids, or the production of reproductive products. Water is conserved by the epicuticle, which consists of several distinct sublayers and a wax covering. If the water-protective waxy coverings are damaged or lost due to the contact with solvents, detergents, pesticides, or abrasive dust, mites lose their water reservoir and may die from desiccation (Hoy, 2011).

Astigmatid mites have an active mechanism that allows them to extract water vapor from unsaturated air, moist foods and the environment. The supracoxal gland secretes a hygroscopic fluid that absorbs moisture and maintains the water and ionic balance (Arlian, 1992). The functionality of the supracoxal gland is highly dependent on relative humidity. Each mite species has a specific value at which the supracoxal gland loses its ability to absorb water. This specific value is known as the critical equilibrium humidity (CEH). At 25 °C, the CEH for *T. putrescentiae* is 70-80%. If the relative

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humidity is lower than the CEH value, organisms are not able to maintain their water balance (Sánchez-Ramos et al., 2007).

2.3.8   Mating and egg laying (oviposition)

Male and female mites repeatedly mate throughout their life spans. Through olfactory stimuli, one male can mate with as many as 450 female mites. Under favorable rearing conditions, a female may lay 4-8 eggs or 1-2 times of its body mass every day. In a mite community, the fecundity is attributed to but not limited to the ratio of males to females, available food sources, temperature, and relative humidity. Fecundity was reduced by 50% when the ratio of males to females was 4:1 in comparison to a 1:1 ratio. Females produce different amounts of eggs on different foods. For example, one female can lay 500 eggs on wheat germ or yeast, but only 85 eggs on dried milk or 12 eggs on dried plums (Boczek, 1991). Under ideal conditions, the amount of food consumed by an adult mite can be as great as its body weight (6-8 µg) each day (ŽĎárková & Reška, 1976). Females mainly use the energy that is acquired from consuming food for egg production. Females can mate but seldom lay eggs if they do not have access to a sufficient food source (Boczek, 1991). Egg laying is also dependent on the temperature and relative humidity of the environment. Females lay eggs at a temperature range of 8-36 °C with an optimal temperature of 22-26 °C. Sánchez-Ramos & Castañera (2005) found that fertile mating was almost 100% at 15-34 °C but only 69% at 10 °C. The preoviposition period was longer when the temperature was above or below the optimal temperature with the shortest being 1.2 days at 30 °C. As relative humidity increased from 80% to 90%, the preoviposition period of *T. putrescentiae* decreased from 2.1 to 1.5 days, and the fecundity increased from 259 to 488 eggs/female (Sánchez-Ramos et al.,

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2007). In addition, there was no egg production below 62% RH in the environment and/or 13.5-14% moisture content in foods.

2.3.9   Reproduction and development

Mite reproduction and development is influenced by abiotic and biotic factors (Rybanska, Hubert, Markovic, & Erban, 2016). Abiotic factors consist mainly of temperature and relative humidity (Aspaly, Stejskal, Pekar, & Hubert, 2007; Sánchez-Ramos et al., 2007; Sánchez-Ramos & Castañera, 2001). Biotic factors include the sex ratio of males to females, age, population density, the amount of sperm transferred to the female, nutrient composition, and diseases (Collins, 2012; Vacante, 2016).

The temperature and relative humidity effects are better known than other factors regarding mite growth and reproduction. In general, the rate of increase in mite population decreases when the temperature is below 18 °C or above 27 °C, or the relative humidity is lower than 70% (Vacante, 2016). Under ideal conditions (25 °C and 90% RH), 100 mites can render about 100 g of dog food to dust in less than four weeks (Sánchez-Ramos et al., 2007).

The life cycle of this species takes approximately one to three weeks, and up to 118 days depending on temperature, relative humidity, and the food sources on which the mite is reared (Kheradmand, Kamali, Fathipour, Goltapeh, & Ueckermann, 2007b). *T. putrescentiae* develops between 10 and 35 °C, and the optimum temperature for growth is around 30 °C, at which the life cycle is 8.5 days when feeding on brewer's yeast flake (Sánchez-Ramos & Castañera, 2001). Fecundity was adversely affected by extreme low and high temperatures. A female produced the highest number of eggs (555 eggs) at 20 °C and the lowest number of eggs at 10 °C and 35 °C (Sánchez-Ramos & Castañera,

2005). The life cycle was 53 days at 11 °C and only nine days at 25 °C and 90 ± 5% RH (Al-Safadi, 1991). *T. putrescentiae* prefers 85-95% RH and spends a shorter time to develop from eggs to adults when compared to lesser RHs. When reared on yeast, various fungi or wheat germ, mites grew fast and completed a generation in 2-3 weeks at 20-25 °C under near-saturation conditions (Rivard, 1961). Light exposure reduces fecundity and egg viability and increases the duration of the life cycle (Zhang, 2003).

The optimal growth and development temperature for *T. putrescentiae* is variable (25, 30, or 32 °C), which is highly dependent on the thermal history (Aspaly et al., 2007; Barker, 1967; Sánchez-Ramos & Castañera, 2001). Acclimation is the main factor that changes the developmental temperature thresholds (Sánchez-Ramos & Castañera, 2001), which is particularly important when considering the use of heat or cold treatments to control mite infestations (Hubert et al., 2010).

2.3.10   Food source

T. putrescentiae mites are fungivorous. Mites feed on fungi, and this benefits both mites and fungi. Mites carry fungal spores on their body surface or in the digestive tract to new substrates where fungi can thrive by using new food resources (Hubert, Jarošík, Mourek, Kubátová, & Žďárková, 2004). Also, the feeding behavior of mites on fungi induces a fast compensatory growth of the mycelium (Hedlund, Boddy, & Preston, 1991). Furthermore, mite eggs and dead bodies provide nitrogen sources for the growth of fungi (Klironomos & Hart, 2001).

Fungal preference of mites depends on fungal mycotoxin and secondary metabolite content of the digestive capabilities of mites and the availability of alternative foods (Parkinson et al., 1991). As a result, Hubert et al. (2004) divided fungi into four

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groups according to mite preferences, fungal attractiveness, and fungal suitability for mite development: 1) preferred and suitable, 2) preferred but unsuitable, 3) avoided but suitable, and 4) avoided and unsuitable. The fungal preference difference of mites is likely due to their fungal digestibility. The cell content of fungal mycelium that is digested by mites is rich in trehalose (Hubert et al., 2001). Therefore, trehalase, the digestive enzyme for trehalose, plays an important role in digestion of fungi by mites (Siepel & Maaskamp, 1994). The *Tyrophagus* species have high levels of trehalase, which explains why *Tyrophagus* mites can digest fungi. *T. putrescentiae* prefers more fungal species than *A. siro* or *L. destructor* (Hubert et al., 2004).

Other than fungi, *T. putrescentiae* can grow on a wide range of foods, including stored food products, semi-moist and dry dog foods, mushrooms, laboratory fungal cultures, and bacteria (Qu et al., 2015). Type of food affects the enzyme physiology of mites, which results in differences in mite population growth (Collins, 2012; Erban, Rybanska, Harant, Hortova, & Hubert, 2016; Nesvorná, Gabrielová, & Hubert, 2012).

The diet that was enriched with starch accelerated the growth of *T. putrescentiae* because of the presence of alpha-amylase in the gut of mites. However, the inhibitor acarbose suppressed starch hydrolysis and the population growth of *T. putrescentiae*. Compared to *T. putrescentiae*, *Tyroborus lini* showed higher alpha-amylase activity. Mite species with greater starch hydrolytic activity are more tolerant to acarbose than species with lesser starch hydrolytic activity (Erban, Erbanova, Nesvorna, & Hubert, 2009). Therefore, alpha-amylase of mites can be used as the targets for designing inhibitor based strategies to control mites.

2.3.11   Dispersal

Dispersal refers to the behavior of leaving one area or habitat for another, including migratory and phoresy dispersals. Migratory behavior happens when mites suspend normal feeding, development, and reproduction and make an effort to move from one place to another. *Tyrophagus* species are not dispersed by phoresy. *T. putrescentiae* are tiny (280-420 µm) and wingless, and therefore transport from place to place such as hooks, racks, or shelves in the ham aging room.

The distribution and dispersion of mites are affected by many factors including mite size, temperature, moisture, microflora, mite waste products and food quality. The mechanism involved in the dispersal process of mites is not fully understood. Mites tend to disperse rapidly under unfavorable conditions from overcrowding, food depletion, or degradation. Dispersal is triggered by the pheromone, neryl formate and the movement of contaminated foodstuffs, equipment, plant, and animal material to new locations (Edde et al., 2012; Kuwahara, Ishii, & Fukami, 1975).

2.4   Mite control

Key points in the development of IPM programs are the ability to detect and monitor, identify species, understand their biology and behavior, and determine the most appropriate control tactic or tactics. Although the control methods mentioned below focus on *T. putrescentiae* mites and dry cured ham infestations, most strategies are compatible with other pests in stored products.

2.4.1 Mite detection

Mites are tiny (280-420 μm) and usually undetectable until the infestation becomes severe, which results in product exclusion and economic losses. Mites are more difficult to control as mite density increases. It is important to determine where mite growth is likely to occur so that infestations can be detected and controlled before they spread throughout the plant. Therefore, early detection and continuous monitoring are critical for dry cured ham producers to be able to take effective measures to control mite infestations during early stages of infestation. Many detection and trapping systems have been employed, including baited traps, sticky tapes, food grade mineral oil, and alarm pheromones.

2.4.1.1 Semiochemicals

Mites discriminate between different olfactory stimuli and are attracted or repelled by different odors (Žďárková, 1971). By definition, a semiochemical is a chemical substance or mixture that carries a message for the purpose of communication. Pest semiochemicals include pheromones, kairomones, allomones, attractants, and repellents.

T. putrescentiae contains semiochemicals, including pheromones (neryl formate, neral, geranial, beta-acaridial) and attractants (methyl palmitate, stearic acid, methyl stearate, oleic acid, methyl oleate, linoleic acid, methyl linoleate, linolenic acid, methyl linoleate, myristic acid, pentadecanoic acid, and margaric acid) (Kuwahara, Fukami, Ishii, Matsumoto, & Yoshitake, 1979; Kuwahara et al., 1975; Leal, Kuwahara, & Suzuki, 1989; My-Yen, Matsumoto, Wada, & Kuwahara, 1980; Sato, Kuwahara, Matsuyama, & Suzuki, 1993). Mites are sensitive to the pheromones that they produce. Therefore, mite

infestations can be detected at very low levels when other strategies are not successful (Bell, 2014). Žďárková (1971) determined that 1% and 5% of lactic acid and low concentrations of cinnamaldehyde and anisaldehyde attracted *T. putrescentiae*. However, 15% and 45% of lactic acid, and high concentrations of cinnamaldehyde and anisaldehyde were strong repellents. If the pheromones or attractants listed above are placed in the ham aging environment, it is likely that mites would recognize these signals and move towards them.

2.4.1.2   Traps

Pest traps are used to detect, monitor or directly reduce pest populations. The first referenced mite trap was the BT trap. It is a small black disc that contains moist food bait. The laboratory and field tests indicated that BT traps were effective at capturing mites even after professional sanitation (Thind & Ford, 2004; Wakefield, 2006). The PC pitfall cone trap was originally used to detect insects, but was also proven effective at detecting mites in stored grain and oilseed (Clarke et al., 2002). Both BT and PC traps were effective at detecting a small population of storage mites, including *T. longior* (Gervais), *Acarus siro* (L.), and *Lepidoglyphus destructor* (Schrank) (Dunn, Prickett, & Thind, 2005). Recently, one type of food-baited trap composed of a disposable petri dish and a dog food-based bait was utilized to detect and monitor mites in dry cured ham aging rooms where methyl bromide (MB) fumigation was applied (Amoah et al., 2016). Food-baited traps detected changes in mite numbers before and after fumigation, and identified potentially heavily infested locations before they were visually detected. Therefore, food-baited traps could help ham producers in three ways: 1) detection and monitoring mite activity over time and space, 2) determination of when corrective actions should be taken,

and 3) and evaluation of the effects of corrective actions on controlling mite infestations on dry cured hams and in processing facilities.

2.4.2 Chemical control

2.4.2.1 Fumigants

Chemical control of mites is currently the most widely used method by industries. Fumigants are the most effective and thorough control measures for stored product pests. Methyl bromide has been the most widely used fumigant for pest control since the 1960s. In the American dry cured ham industry, MB is currently the only known fumigant that is effective at controlling mite infestations. It is effective against all life stages of mites and some other pests within 24 h of exposure due to damage of nerve cell membranes through an attempt to respire in the presence of MB (Fields & White, 2002). The egg stage is more resistant to MB than other life stages since larvae, nymphs, and adults are more physically active than eggs and therefore breathe in more toxic MB gas (Barker, 1967).

Methyl bromide was listed as an ozone-depleting compound under the international Montreal Protocol (UNEP, 1992) and U.S. Clean Air legislation. In January 2005, MB was phased out from developed countries, with the exceptions of critical use exemptions (CUEs). The United States dry cured ham industry applied and was awarded CUEs from 2005-2016 since there were no registered effective MB alternatives available for use to control mites and other pests. In 2015 and 2016, the nominated quantity of MB for American dry cured pork industry was 3,240 kg per year (EPA, 2013). As of 2017, it was determined that the dry cured pork industry has access to remaining stockpiles of MB to meet the needs of the ham industry for the immediate future (EPA, 2017b).

Phosphine is another fumigant registered for dry-cured hams due to its low cost, ease of application, and effectiveness at controlling pests (Fields & White, 2002; Zuryn, Kuang, & Ebert, 2007). Phosphine kills pests by reducing the mitochondrial electron transport chain of the aerobically respiring organism (Zhao et al., 2015). Phosphine fumigation at 1000 ppm caused 100% mortality of all life stages of *T. putrescentiae* within 48 h in a laboratory scale controlled chamber (Sekhon et al., 2010). Phosphine fumigation may not be applicable in dry-cured ham plants due to the need for a tightly sealed enclosure, corrosion of copper wiring, equipment damage, and increased pest resistance (Zhao et al., 2015). Other fumigants, such as ozone and carbon dioxide, may not be practical for use in the ham industry due to their limited effectiveness at controlling mites in treatment times of less than or equal to 48 h (Sekhon et al., 2010).

2.4.2.2   Natural acaricides

Most natural acaricides are botanical compounds and essential oils that contain monoterpenoids as the active ingredients (Gulati & Mathur, 1995). *T. putrescentiae* can be inhibited or killed by some of these natural plant extracts, including Azadirachtin (Neem) (Collins, 2006), caraway, fenugreek, lupin extracts (Afifi & Hafez, 1988), Eucalyptus and menthe powders (Gulati & Mathur, 1995), benzyl benzoate (Acarosan spray or powder), nutmeg, organo, thyme red and white oils (Kim, Kim, & Ahn, 2003), cinnamyl alcohol, black pepper, menthone and pulegone (Sánchez-Ramos & Castanera, 2000), garlic juice (Preisser III, 2016) and essential oils extracted from *Anethum graveolens*, *Achillea millefolium*, *Eucalyptus dives*, *Leptospermum pertersonii*, *Melaleuca leucadendron*, and *Neroli birgard* (Song et al., 2016). The natural monoterpenes including pulegone, menthone, linalool and fenchone are promising for

possible use against *T. putrescentiae* due to the low doses required to produce a high mortality in the immature and adult stages. It has been reported that treating stored products with these compounds did not negatively impact product quality (Sánchez-Ramos & Castanera, 2000). These monoterpenes or their natural derivatives can potentially be used on dry cured ham to control mite infestations. However, the limitation of using natural acaricides would be a relatively low mortality in most cases, expense, and unacceptable flavor and smell that they impart to food products.

2.4.3   Physical control

The main physical factors affecting mite metabolism, fecundity, development, and survivability are temperature and relative humidity (Aspaly et al., 2007; Sánchez-Ramos & Castañera, 2001). Therefore, the knowledge of critical temperatures, relative humidities and exposure times is important in designing an effective IPM program in dry cured ham facilities and the production of finished products that are not infested with mites.

2.4.3.1   Heat

T. putrescentiae is stenothermal and therefore more tolerant of high temperatures (35-37.5 °C limits) than low temperatures (7-10 °C limits) but is also dependent on relative humidity (Collins, 2012; Hubert et al., 2010). Eggs are more tolerant of heat or cold treatments than mobile mites (Abbar et al., 2016b; Cunnington, 1976). However, the temperature preference of mites depends on thermal acclimation (Hubert et al., 2010), relative humidity, the experimental environment, and food source. Abbar et al. (2016) reported that mites and their eggs could not be completely controlled at temperatures of -

3 to 38 °C at 72 h of storage. Control (100% mortality) of mobile *T. putrescentiae* mites was achieved at 40 °C in 48 h, 42 °C in 36 h, and 45 °C in 4 h at 90% RH. Times required to kill eggs were 96 h at 40 °C, 48 h at 42 °C, and 21 h at 45 °C. This indicates that eggs are more tolerant to heat than adults and large nymphs, which is likely due to the thickness of the egg membrane, which contributes to a lower respiration requirement for the eggs. Heat control of all *T. putrescentiae* life stages can be realized at 42-45 °C in less than two days (Abbar et al., 2016b; Eaton & Kells, 2009). Heating up the ham aging house to 42-45 °C has been applied to food-processing facilities (Fields, Subramanyam, & Hulasare, 2012). Realistically, temperatures greater than 45 ºC and/or longer heating times might be necessary to kill mites that are present in ham cracks and crevices. However, the high temperature (40 ºC for 2 days) may affect ham quality. In addition, heat acclimation may happen when the aging house is gradually heated up. Acclimation may widen the temperature threshold of mites, and therefore it should be taken into consideration if high temperature is used to control mite infestations.

2.4.3.2   Cold

According to Eaton and Kells (2011), *T. putrescentiae* eggs were the most cold-susceptible life stage at -5 and -10 °C. However, Abbar et al. (2016) reported that *T. putrescentiae* eggs were more tolerant to extremely low temperatures than mobile stages, with 100% mortality of mobile *T. putrescentiae* mites achieved at -5 °C in 21 h, -10 °C in 6 h, and -20 °C in 2 h at 30% RH. To achieve 100% mortality of eggs, time and temperature combinations of -5 °C for 48 h, -7 °C for 40 h, -10 °C for 100 h, or -20 °C for 12 h were necessary. *T. putrescentiae* adults were killed after 30 min exposure at -15 °C, but only 10% of mites were killed at -5 or 0 °C after 1 h of exposure (Žďárková &

Voráček, 1993). Eaton & Kells (2011) reported that freezing packaged products or commodities and maintaining the food products at -18 °C for 5 h could kill 90 % of all life stages of *T. putrescentiae*.

The lower lethal temperature is called the super-cooling point (SCP), the highest temperature in which organisms will freeze and not survive. It is measured by placing a copper-constantan thermocouple that was attached with individual mite or egg in the center of polystyrene cubes with a starting temperature of 0 °C and cooling the system at a rate of -1.0 °C/min. The super-cooling point of *T. putrescentiae* ranged from -24 to -26 °C for adults and nymphs and was -35.6 °C for eggs, indicating the strong cold tolerance of eggs (Eaton & Kells, 2011). The LLT90 (lower lethal temperature that kills 90% of organisms) values were -22.5, -28.7 and -48.1 °C for *T. putrescentiae* adults, nymphs and eggs, respectively. Based on the difference between SCP and LLT90, it was concluded that eggs are freeze tolerant since the LLT90 value was greater than 10 °C lower than the SCP, while the nymphal and adult stages are freeze intolerant since the LLT90 and SCP are approximately equal (Bouchard, Carrillo, Kells, & Ferrington, 2006). Sugar alcohols (polyols), primarily glycerol and sorbitol, are cryoprotectants that accumulate in the hemolymph of cold acclimated arthropods (Block & Sømme, 1982; Cannon & Block, 1988). As the concentrations of these cryoprotectants increase, the arthropod becomes more cold tolerant. However, if the temperature could be rapidly increased or decreased in a short time, mites may not be able to acclimate to the change in temperature (Abbar et al., 2016b). Cold temperatures can be used to control mites and reduce chronic infestations if the temperature and required exposure time are reached. This method is

rarely achievable in ham processing facilities since freezing is not commonly part of the process flow, which makes freezers an added cost that is unnecessary in the plant.

2.4.3.3   Relative humidity

T. putrescentiae growth differs with as small as a 1% change in RH within the range of 22% to 78%. Within this range, *T. putrescentiae* preferred higher humidity over lower humidity. *T. putrescentiae* responded to changes in RH more quickly when changes in RH were greater (Sánchez-Ramos et al., 2007). However, at extreme high (80-85%) and low (21-26%) relative humidity, *T. putrescentiae* mites did not choose one humidity over another in any choice tests (Žďárková, 1971).

Different life stages of *T. putrescentiae* respond differently to RH. The smallest mobile stage, the larva, tends to lose more water than the nymph and adult. Eggs are smaller than larvae, but they are less susceptible to relative humidity than larva because of the presence of the exochorion layer that provides a barrier to desiccation (Witaliñski, 1993). The optimal relative humidity for mite survival was approximately 90% (Kheradmand et al., 2007b), with growth and development ceasing below 65% RH. However, mites can utilize several protective and adaptive mechanisms against desiccation at low relative humidity (Cutcher, 1973): 1) mites cluster together to reduce the surface area and the water vapor stress, 2) mites search for a shelter which acts as a barrier against water migration to the environment, and 3) desiccated mites find places where they are surrounded and protected by food components. These strategies allow mites to develop at relative humidity values below their CEH (70-80% at 25 °C for *T. putrescentiae*), which is dependent on temperature. "Vapor pressure deficit" (VPD) was defined by Eaton & Kells (2009) to evaluate the combined effect of relative humidity and

temperature on mite survival. The range of VPD for *T. putrescentiae* development was below 8.2 mbar, which is equivalent to 20 °C and 65% RH, or 25 °C and 74% RH (Cunnington, 1967).

In the laboratory, low relative humidity limited mite populations and mold growth on the surface but accelerated the drying process, which causes the formation of cracks on the ham. Low relative humidity thus forced mites to seek refuge in the cracks and crevices (Sánchez-Ramos & Castañera, 2005). At 60% RH, mites sometimes infested the whole ham and interacted with Micrococcaceae to cause putrid odors (Garcia, 2004). Controlling mites on ham or cheese would be difficult because of the relatively large amount of available water in these products that is available to mites regardless of a lesser water activity.

2.4.3.4   Other physical methods

Other physical methods used for mite control include controlled atmosphere, inert dust, irradiation, sanitation, UV light traps, packaging, exclusion, etc. Inert dusts, such as diatomaceous earth, were effective at controlling mite growth and preventing mite migration in laboratory studies (Collins, 2012; Collins & Cook, 2006; Palyvos & Emmanouel, 2006). The advantages of using inert dust is that it is relatively non-toxic, long lasting, inexpensive, and does not affect product quality (Desmarchelier & Dines, 1987). The disadvantages include reduced effectiveness at 75% RH and higher, and visible residues on the products affect grain product grading (Fields & Korunic, 2000).

2.4.4   Food grade dipping and coating

Edible coatings are suspensions of food grade ingredients that include lipid, protein, carbohydrate, and/or active compounds. The coating can be delivered onto foods by dipping, spreading, or spraying. The coating directly contacts the food and is often regarded as part of the final product (Han & Aristippos, 2005). Edible coatings that are incorporated with natural antimicrobial or antioxidants have been utilized in vegetables, fruits, and meat products to limit moisture loss, improve food safety, and extend the shelf life (Embuscado & Huber, 2009).

Application of food grade coating on dry cured ham is not a common process. The idea originated from using coating agents as a carrier of food grade ingredients that are effective at controlling mite infestations. Abbar et al. (2012) dipped 1-cm ham cubes into vegetable and animal oils, salts of sorbic, propionic and citric acids, short-chain alcohols, organic acids, and butylated phenol preservatives, and evaluated *T. putrescentiae* growth after two weeks of incubation on ham cubes that were dipped in these solutions. *T. putrescentiae* mortality was 100% when cubes were dipped in 50 or 100% propylene glycol, 100% lard, or 10% BHT (butylated hydroxytoluene). Other short chain alcohols including 1,3-propanediol, 1,4-butanediol, 1,3-butanediol, and 1-propanol also significantly reduced the number of mites on treated ham cubes. Based on their findings, food grade coatings including lard or propylene glycol were further developed and evaluated for their efficacy at controlling mites on dry cured hams.

Coating foods with fat, known as 'larding,' was used to prolong the shelf life of meat products in Europe in the 16th century (Cagri, Ustunol, & Ryser, 2004; Pavlath & Orts, 2009). Wax, vegetable oil, and lard have been commercially used as protective

coatings for fresh and frozen meat and poultry products. Vegetable cooking oil has been

used in horticulture to control mites and insect pests (Hoy, 2011). In Spain, coating dry

cures ham with hot lard near the aitch bone and hot vegetable oil is a common practice

for ham producers to reduce mite infestations (Garcia, 2004). However, the

hydrophobicity of the lard coating may reduce water vapor permeability or the air

exchange rate, which limits water loss and affects product quality. Therefore, lard should

be evaluated for water and air permeability when considering the use of lard coating on

whole hams, or lard should be used along with plasticizers or hydrophilic compounds to

compromise the negative effect on water loss. The effect of coatings on the weight loss of

whole hams over 48 days in a simulated aging house was evaluated and it was found that

control hams lost 7.4% of their original weight (Zhao, Abbar, Phillips, & Schilling,

2014). In comparison, hams with 2% carrageenan + 50% PG lost 6.4% of weight, and

hams with a thin layer of lard lost 5.3% of weight. These results confirmed that coating

with lard limited moisture evaporation.

       As mentioned earlier, 50-100 % propylene glycol (PG), a well-known humectant,

was effective at controlling mite infestations (Abbar et al., 2016a; Aldrich, 2014). In their

study, PG was used as a dipping solution of small ham cubes. However, this method is

not a practical application for whole hams. Therefore, Zhao et al. (2016b) developed

polysaccharide/gum and PG coatings, in which the polysaccharide matrix serves as the

carrier of PG. These research found that starch, carrageenan, and sodium alginate (with

added Ca^{2+}) formed a weak gel or did not gel with 50% propylene glycol. Based on the

gelling effect of gum with the presence of PG, xanthan gum (XG) and the combination of

carrageenan (CG) and propylene glycol alginate (PGA) were chosen as carriers for PG in

the coating. Ham cubes were coated with XG (1%) + PG (10, 20, 30, and 50%) and CG (1%) + PGA (1%) + PG (10, 20, 30, and 50%) and then inoculated with 20 mixed sex adult mites. The effectiveness of these coatings at controlling *T. putrescentiae* were evaluated after three weeks of storage at 25 °C and 70% RH. Gum + 10% PG treatments reduced the mite numbers from 476 in the control to 70 in XG + 10% PG and only two for CG + PGA + 10% PG. Treatments containing 20-50% PG had no detectable mites after three weeks of incubation (Zhao et al., 2016b).

2.4.5   Integrated pest management (IPM)

IPM is a pest risk-management program that incorporates physical, chemical, and biological methods to maintain product quality and deal with health, environmental, and economic risks in a sustainable manner. It requires information on target pests, processing, regulations, and effective and practical control methods. Examples of physical and chemical control methods were described above. The biological control methods include using some organisms to attack, infect, or parasitize targeted pests in stored food products. However, use of these organisms in food processing facilities is limited because the potential problems caused by the presence of these organisms. Therefore, it is not going to be discussed in this chapter. In the case of ham mites, the key issues in the IPM development program are the ability to understand mite biology and behavior, to identify and monitor the mite species, and to determine the most appropriate management tactics. There is not an IPM program that is suitable for every situation because of the varied food systems and environmental conditions. To date, there is not a single method that can replace methyl bromide fumigation in the dry cured ham facilities to control *T. putrescentiae*.

Key techniques for IPM program of *T. putrescentiae* include use of food grade coatings, sanitation, traps, diatomaceous earth, temperature and humidity management, methyl bromide stock management, detection, and corrective actions when pest infestations occur. A few steps are recommended as follows:

(1) Prevention of pests on the ham products and in the ham processing environment: sanitation, HACCP (Hazard Analysis and Critical Control Points) and related good manufacturing practices need to be in place to ensure food safety and prevent pest access and build-up.

(2) Monitoring and detection of pests in the dry cured ham plants: specialists should monitor the pest status in the plant on a daily basis and can use food-bait traps to assist with early detection of ham mites and other pests; specialists should also be aware of those locations that pests are present and take possible preventive measures.

(3) Decision-making of control strategies: based on the monitoring information, control measures can be applied when pests are present on the dry cured hams or in the environment; proper actions would be i) separating heavily infested hams from other hams; ii) isolating lightly infested hams with others to avoid cross infestations; iii) Using existing methyl bromide stocks and other practical and economical alternative methods to eliminate the infestations.

(4) Actions after the treatments: specialists should immediately monitor the pest status after the treatments and take further actions if necessary.

CHAPTER III

APPLICATION OF FOOD-GRADE INGREDIENTS TO NETS FOR DRY CURED

HAMS TO CONTROL MITE INFESTATIONS

3.1   Abstract

Infestations of *Tyrophagus putrescentiae* (Schrank) (Sarcoptiformes: Acaridae),

known as the ham mite, may occur on dry cured hams during the aging process. The

fumigant methyl bromide is currently used to control mite infestations, but it will

eventually not be available for use since it contributes to the depletion of the ozone layer.

The use of ham nets treated with xanthan gum, carrageenan, propylene glycol alginate,

and propylene glycol food-grade ingredients on mite orientation to or oviposition on

treated or untreated ham cubes, and mite reproduction and population growth over a 10-

week period was evaluated.

The mite culture of *T. putrescentiae* species that was used in this study was

confirmed by using a PCR-cloning method.

Behavioral tests indicated that more than 95 % of the mites oriented to the ham

cubes that were wrapped in untreated nets when compared to treated nets and no eggs

were laid on the latter. The reproduction assays demonstrated that there were fewer ($P <$

0.05) *T. putrescentiae* produced over a two-week period on ham cubes covered with both

gum and propylene glycol (PG) treated nets, when compared to the untreated or gum-

only treated nets over the 10-week storage period of the experiment. Medium and high

concentrations of PG treatments showed the lowest reproductive rates of mites. No more than four mites could be found on each of these treatments in comparison to 200 to 300 mites that were recorded on the untreated hams.

This study demonstrated the efficacy of using the nets treated with food-grade ingredients during ham aging to control mite infestations on a laboratory scale. Further research will be conducted to determine the effectiveness of the same treated nets on whole hams in commercial aging rooms.

Key words: dry-cured ham, *Tyrophagus putrescentiae*, behavior, reproduction

3.2   Introduction

Dry-cured ham is one of the most popular cured meat products in Spain, Germany, China and the United States (Toldrá, 2008). Dry cured hams are susceptible to mite infestations due to their high fat and protein contents (Hughes, 1976; Lee et al., 2006; Macchioni et al., 2002), water activity, intense flavor, molds that grow on the meat surface (Garcia, 2004), and the environmental temperature and humidity in the curing and aging room.

Tyrophagus putrescentiae (Schrank) (Sarcoptiformes: Acaridae), known as the copra mite, mold mite, cheese mite or ham mite, may infest hams and feed directly on the dry-cured ham surface and the molds that grow on the hams (Cui, 2014). In addition, the presence of mold enhances mite growth and reproduction (Canfield & Wrenn, 2010; Hughes, 1976), and mites distribute molds on the ham surface because they carry viable fungus spores on their bodies and in their digestive tracts (Hoy, 2011). *T. putrescentiae* infestations may decrease the quality of dry-cured hams (Townsend, 2007), and severe infestations lead to a powdery residue on the ham surface. In addition, *T. putrescentiae*

may cause sensitization (Boquete et al., 2000; Garcia, 2004), dermatitis (Quiñones Estévez, 2006; Vidal & Rial, 1998) and occupational asthma (Rodriguez del Rio et al., 2012) in people who work closely with mite-infested products.

Methyl bromide is an odorless, colorless gas that has been used effectively by food storage and processing facilities as a single space fumigant for more than 50 years to control pests, including ham mites. However, the use of methyl bromide is in the process of being phased out, since it was listed as one of several substances that contribute to the depletion of ozone in the atmosphere in the Montreal Protocol (Fields & White, 2002). Although the United States dry-cured ham industry can use existing stocks of methyl bromide, no additional methyl bromide can be produced at this time (EPA, 2017b). In addition, the cost for fumigations has increased by 10 fold from 2000 to 2015, which has contributed to decreased ham production.

Eventually, methyl bromide will likely not be an option for the dry-cured ham industry, which makes it necessary to research effective and economical alternatives to control mites. Alternatives that have been tested include chemical alternatives (Fields & White, 2002), food-safe compounds (Abbar et al., 2016a), the use of hot or cold temperature (Abbar et al., 2016b), and controlled atmosphere (Hasan, Aikins, Schilling, & Phillips, 2016). Sulfuryl fluoride was effective at controlling the adult stage of mites but was not effective at a concentration three times greater than the EPA label rate when applied at room temperature (Phillips et al., 2008). Phosphine fumigation was effective at controlling mites under laboratory conditions. However, phosphine use was not practical in commercial plants due to serious metal corrosion with costly damage from phosphine (Zhao et al., 2015). In Spain, coating the meat surface with lard or vegetable

oil is part of the production process in order to help manage mite infestations (Garcia, 2004). The application of lard to dry-cured ham cubes inhibited mite infestations on the laboratory benchtop, but may affect ham quality due to limited moisture and oxygen permeability (Zhao et al., 2016b).

Application of coatings that were composed of food-grade polysaccharides and propylene glycol to the dry cured ham cubes and slices was effective at controlling mites without affecting sensory quality in laboratory studies (Abbar et al., 2016a; Zhao et al., 2016b). Hams are typically aged by placing them in nets that are composed of natural and/or synthetic fibers and hanging them on racks. Application of ham nets treated with these mite-protective food-grade coatings controlled mites at the benchtop level (Campbell et al., 2016b). Therefore, the primary objective of this study was to determine the most effective formulations of food-grade chemicals for use in ham nets with respect to inhibiting mite contact and controlling mite reproduction over a 10-week storage period.

Mite cultures in the laboratory can be contaminated with other mite species from the environment. Mite identification has been conventionally based on morphological characterization. The techniques involved generally use direct observation of phenotypic differences between organisms. However, using the conventional microscopic technique requires specific expertise and subspecies identification and intraspecies differentiation may be difficult (Zhao, Xu, Hu, Wu, & Wang, 2012). Therefore, molecular techniques have been developed in order to help determine mite species. Phyletic classification based on rDNA are consistent with those of traditional morphological classification (Beroiz et al., 2014). The use of DNA markers is advantageous in that it does not require

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a given developmental stage or a source of living individuals for analysis. Nuclear ribosomal DNA region (rDNA) has been previously used to identify different eukaryote organisms. The nuclear ribosomal DNA (rDNA) cluster consists of three conserved ribosomal subunits, 18S, 5.8S and 28S that are linked by the two internal transcribed spacers (ITS), ITS1 and ITS2 (Fig. 3.1). These three subunits are highly conserved and are useful in discriminating taxonomic groups, whereas the ITS1 and ITS2 regions can be used for species identification (Hillis & Dixon, 1991; Navajas, Gutierrez, Williams, & Gotoh, 2001; Navajas, Lagnel, Fauvel, & De Moraes, 1999) of mites, ticks, and insects. To characterize the ITS regions, PCR primers can be defined based on more conserved genes (28S, 5.8S and 18S) that flank the ITS. Therefore, the secondary objective of this research was to develop a molecular diagnostic procedure to confirm the identity of *T. putrescentiae* in our mite culture.

3.3   Materials and methods

3.3.1   Hams and food additives

Dry-cured hams (6-8 kg) aged for 4-6 months were purchased from a commercial supplier. Ham nets were provided by Ennio International (Aurora, IL). Xanthan gum (XG), carrageenan (CG), and propylene glycol alginate (PGA) were provided by TIC Gums (Belcamp, MD). Food-grade propylene glycol (PG) was purchased from the Essential Depot (Sebring, FL).

3.3.2   Mite cultures

T. putrescentiae were reared in Dr. Phillips's laboratory in the Department of Entomology at Kansas State University as described by Abbar et al. (2016). Prior to use,

the mite containers were shipped overnight to Mississippi State University and maintained on a diet in a latching storage box that contained soap water at the bottom and petroleum jelly smeared on the edges to prevent mites from escaping.

3.3.3   Genomic DNA extraction for mite identification

Approximately 3000 mites were collected into each capped centrifuge tube and stored overnight at -80 °C. The mites were subsequently ground to fine powder with a mortar and a pestle in liquid nitrogen. After transferring the powder into a 1.5-mL microcentrifuge tube, the sample was homogenized with 700 μL standard cetyltrimethylammonium bromide (CTAB) DNA extraction buffer by gently inverting the tube and incubating at 65 °C for 20 min. Next, 5 μL of RNase A (10 mg/ml) was added into the solution followed by incubation at 37 °C for 15 min with periodic, gentle mixing. The sample was subsequently extracted with 700 μL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v). After centrifugation at 13,000 × g for 10 min, the aqueous (upper) phase was transferred to a fresh 1.5-mL microcentrifuge tube and further extracted with 700 μL of chloroform : isoamyl alcohol (24:1, v/v). The mixtures were centrifuged at 13,000 × g for 10 min. The aqueous (upper) phase was transferred into a fresh 1.5-mL microcentrifuge tube. The DNA sample in the aqueous layer was then precipitated by adding 1/9 volume of 3 M sodium acetate (pH 4.8) and two volumes of 96% ethanol. The mixture that contained the DNA sample was stored at -20 °C for 30 min. The DNA sample was then harvested by centrifugation at 13,000 × g for 10 min. The DNA pellet was washed twice with 0.5 mL of 70% (v/v) ethanol and then air-dried for 10 min at room temperature. The dried DNA sample was resuspended in 40 μL of nuclease-free water and stored at -20 °C until use. The purity and concentration were

evaluated by using a NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies, Wilmington, DE). The quality and integrity (degradation) of the DNA sample were evaluated on a 1% (w/v) agarose gel that consisted of 20 mM Tris-HCl (pH 7.5), 7.5 mM sodium acetate, 0.5 mM EDTA, and 0.01% SYBR safe (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was conducted at 70 V for 60 min in a running buffer that contained 20 mM Tris-HCl (pH 7.5), 7.5 mM sodium acetate, and 0.5 mM EDTA. Gel images were taken with a ChemiDoc XRS+ system that was equipped with Image Lab (version 5.0) software (Bio-Rad Laboratories, Hercules, CA).

3.3.4   PCR amplification of conserved regions of mite's rDNA

PCR (Polymerase Chain Reaction) was used to amplify the mite's rDNA by using mite-specific primers that were designed from the conserved region of the 3' end of the rDNA 18S to the 5' end of the rDNA 28S sequence (Navajas et al., 1999) using a T100 Thermal Cycler (Bio-Rad). The primers were mite-specific primers FNav (5'-AGAGGAAGTAAAAGTCGTAACAAG-3'), based on the 3' end of the rDNA 18S sequence (Navajas et al., 1999), and RNav2 (5'- ATATGCTTAAATTCAGCGGG-3'), based on the 5' end of the rDNA 28S sequence (Navajas, Lagnel, Gutierrez, & Boursot, 1998).

The PCR reaction mixture contained 1 μL of mite's genomic DNA (100 ng), 5 μL of 10X PCR reaction buffer, 1 μL of dNTPs (10 mM of each dATP, dTTP, dGTP, and dCTP), 1 μL of forward primer FNav (10 μM), 1 μL of reverse primer RNav (10 μM), 0.5 μL of AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc., Foster City, CA), and 40.5 μL of ddH$_2$O. The PCR reaction was performed by preheating at 95 °C for 5 min, followed by 35 cycles at 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 90 s, with a final

extension at 72 °C for 5 min. The PCR product was electrophoresed on a 1% (w/v) agarose gel at 70 V for 60 min. An amplified 1300-bp DNA fragment was sliced out and purified with a DNA Gel Extraction Kit (Thermo Fisher) by following the manufacturer's protocol. The purified DNA fragment was then ligated into a pGEM-T easy vector (Promega). The ligation reaction was carried out at 4 °C overnight in a mixture containing 0.5 μL of pGEM-T easy vector (50 ng/μL), 5 μL of 2X ligation buffer (60 mM Tris-HCl, pH 7.8, 20 mM MgCl$_2$, 2mM ATP and 10% PEG), 3.5 μL of the purified 1300-bp DNA fragment, and 1 μL of T4 DNA ligase (3 units/μL, Promega).

3.3.5   Transformation of *Escherichia coli* XL1-Blue competent cells

An aliquot (3-5 μL) of ligation mixture was added into a tube containing 200 μL of XL1-Blue competent cells, and incubated on ice for 30 min. After heat shock at 42 °C for 45 s, the transformed cells were mixed with 1 mL of Luria-Bertani (LB) medium and incubated at 37 °C for 45 min with gentle agitation. After adding 10 μL of 0.1 M (IPTG) and 25 μL of 2% (w/v) (X-gal) in N,N-dimethylformamide, the cell mixture was spread onto two LB/ampicillin (50 μg/mL) plates (LB medium with 15 g/L agar). The plates were then inverted and incubated overnight at 37 °C.

Eighteen white colonies and one blue colony were picked and incubated individually in 2 mL of LB/ampicillin (50 μg/mL) medium, and cultured overnight at 37 °C with 200 r/min agitation. The recombinant pGEM-T easy plasmid DNA was isolated using a Plasmid Miniprep Kit (Thermo Fisher).

3.3.6   Sequence analysis

Both strands of each recombinant plasmid were sequenced using M13F and M13R sequencing primers at Eurofins Genomics. The vector sequence fragments were removed, and two sequences were assembled into a full-length sequence. BLAST analysis against the nucleotide collection (nr/nt) was performed at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Sequences were deposited in the GenBank database (GenBank accession nos. MF353994-MF354011). The following sequences available in the GenBank database were also used in the ITS2 alignment: *T. putrescentiae* (AB105000, GQ205623). The boundaries of ITS1 and ITS2 were assigned by conserved sequences at the 18S (5'-AGGATCATTA-3'), 5.8S (5'-CTGYYAGTGG-3' and 5'-TGAGCGTCGT-3') and 28S (5'-CGACCTCAG-3') subunits.

3.3.7   Preparation of food ingredients infused nets

The solution of gum and PG was prepared as follows. Xanthan gum was first mixed with PG (low, medium, and high concentrations) and then the mixture was gradually poured into cold tap water and stirred until the mixture was clear. The combination of carrageenan and PGA was also first mixed with PG (low, medium, and high concentrations). The slurry was then mixed with warm water while stirring and heated to 90-100 °C until the liquid turned from cloudy to clear. The specific methods under which the experimental nets were prepared are reported here as "low", "medium" and "high" concentrations of PG because the information is currently intellectual property with a patent pending.

The nets were cut, weighed, and then dipped in the coating solution. The nets were then pressed between two rollers in the netting machine (Midwest Metal Craft & Equipment Company, Winsor, MO) to minimize the amount of gum and PG that were used. The infused nets were weighed, and the netting solution that each foot of nets absorbed was calculated. The treated nets were then vacuum-packaged into vacuum bags (3 mil. standard barrier, nylon/PE Clarity Vacuum Pouches; Kansas City, MO) with a dual-chamber vacuum packaging machine (Model 2100, Koch Equipment LL., Kansas City, MO) at a full vacuum setting, and stored at room temperature prior to use.

3.3.8   Ham cubes

Whole hams were transversally cut into 2.5 cm or 1.3 cm thick pieces in the meat laboratory. Ham cubes were prepared and used for mite behavior ($1.3 \times 1.3 \times 1.3$ cm³) and mite reproduction assays ($2.5 \times 2.5 \times 2.5$ cm³). The cubes were chosen from *biceps femoris*, *semitendinosus*, *adductor*, and *semimembranosus* muscles to avoid large variations among muscles. The ham cubes were wrapped in untreated nets, gum treated nets, or gum + PG treated nets.

3.3.9   Two choice behavior tests of mites

The two-choice behavior test was conducted according to Abbar et al. (2016) with some modifications. One 1.3 cm ham cube covered with the control (untreated) net and another 1.3 cm ham cube covered with a treated net were offered simultaneously to the mites inside a small arena (as shown in Fig. 3.2). The inside bottom of the plastic Petri dish (150 mm diameter × 15 mm depth) was covered with black construction paper that was cut to the same dimension as the Petri dish. Three specific round areas (C, M, and T)

were assigned along a line passing through the center of the paper, with M (Mite) in the center, and C (control) and T (treatment) 10 mm away from each side wall of the Petri dish (Fig. 3.2). Two ham cubes from the same muscle section were selected and wrapped with control and treated nets, with one control cube placed in area C and one cube placed in area T. Five replications (five pairs of cubes) were tested for each treatment. Among them, two pairs of cubes were taken from the *biceps femoris* muscle, one pair from the *semitendinosus* muscle, and two pairs from the *adductor* and *semimembranosus* muscles. A total of 20 mixed sex adult mites were placed in the M area on the paper, and the Petri dish was placed in the dark in a growth chamber (23 ± 2 °C and 80 ± 5% RH). A thin layer of petroleum jelly was applied on the inner upper 5 mm of the Petri dish to prevent mites from escaping. After 6 h of incubation, mites that were oriented to each of the net-wrapped ham cubes were counted. Orientation was indicated by the number of live mites on the control and treated ham cubes (Abbar et al., 2016). An identical setup was prepared, and 20 mites were placed in the M area. The number of eggs laid on the cubes and nets were counted after 4 days of incubation. Oviposition was determined by counting eggs that were laid on the control and treated ham cubes.

3.3.10   Mite reproduction assays

The 2.5 cm ham cubes were removed from ham muscles and assigned to and packaged with either control or treated nets. Five replications were tested for each treatment. Two cubes were taken from the *biceps femoris* muscle, one from the *semitendinosus* muscle, and two from the *adductor* and *semimembranosus* muscles. Ham cubes wrapped in nets were then placed in ventilated glass Mason jars (216 mL, 65 mm diameter, 55 mm height; Ball Corp., Broomfield, CO). The bottom of the jars was

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covered with black construction paper and the top was covered with filter paper (Whatman No. 1, 90 mm diameter; GE Healthcare, UK) that was sealed with the jar ring.

Two sets of mite infestation studies were conducted. One experiment included XG + PG treated nets, and the other set consisted of CG + PGA + PG treated nets. For each set of experiments, three groups of samples were prepared. Twenty mixed sex adult mites were introduced to ham cubes of each group on the first day (1st group), and at 4 weeks (2nd group) and 8 weeks of storage (3rd group), respectively. This was done to evaluate the long-term effectiveness of treated nets at controlling mite survival and reproduction. This experiment was conducted twice. The first batch was exposed to a relative humidity of 70 ± 5 % for the first four weeks with an increase to 80 ± 5 % from week 5 to week 8 at 23 ± 2 °C. For the second replication, ham cubes and nets were exposed to a relative humidity of 80 ± 5 % at 23 ± 2 °C. Two weeks after inoculation, the ham cube, net, and paper were separated in a Petri dish (90 mm diameter × 15 mm depth) that was placed in a large Petri dish (150 mm diameter × 15 mm depth) that held 90 % ethanol. The mites that were present on ham cubes, nets, black paper, and jars were counted separately under a microscope. Between samples, the Petri dishes that held samples were sprayed with 90 % ethanol and wiped thoroughly. The presence of molds were also recorded based on the mycelium growth and colored spores.

3.3.11   Statistical analyses

Significance of mite preference for a given ham combination (control vs. treated) in the two-choice behavioral test were calculated using a paired, two-sided Student's t-test using Microsoft Excel 2007, assuming unequal variances.

A randomized complete block design with two replications (batch) was used to determine the effect of different treatments on mite reproduction on ham cubes over two weeks in glass jars. The blocking factor was included to account for slightly variable relative humidity throughout the study. When significant differences ($P < 0.05$) occurred among treatments, Tukey's Honestly Significant Difference Test ($P < 0.05$) was used to separate treatment means.

3.4   Results

3.4.1   Mite identification

Gel electrophoresis revealed a single, high molecular weight DNA band with little evidence of shearing or RNA contamination (Fig. 3.3). The primer combination of FNav and RNav2 was successfully used for PCR amplification of rDNA in the DNA that was extracted from mites. The complete ITS1, 5.8S rDNA, and ITS2, and portions of the flanking 3' end of the 18S and the 5' end of the 28S were PCR-amplified. A 1300 bp PCR product was purified (Fig. 3.4) and was then ligated into a pGEM-T easy vector. Nineteen clones (18 insert-containing clones and 1 non insert-containing clone) were randomly selected for sequencing (GenBank accession nos. MF353994-MF354011) after the vector was transformed into *E. coli* XL1-Blue competent cells. The insertions were confirmed by running agarose gel (Fig. 3.5). Sequence analyses confirmed that the amplification of the complete ITS1, 5.8 S and ITS2 rDNA regions were flanked by partial sequences corresponding to the 18S and 28S subunits. The similarity among the sequences obtained and their comparison with sequences available in the GenBank database identified all sequences as *T. putrescentiae* rDNA. The estimated sizes for ITS1, 5.8S and ITS2 are listed in Table 3.1. The 5.8S subunit was highly conserved. It was 151

bp in 17 clones, and 152 bp in 1 clone. Low variation in length at ITS1 (667-671 bp) and

ITS2 (384-388) regions was also found. The complete ITS2 region (Table 3.2) flanked by

the 5.8S and 28S partial sequences from each of 18 clones was compared with a known

ITS2 sequence of *T. putrescentiae* (GeneBank accession no. GQ205623). A total of 28

single nucleotide polymorphisms (SNPs, indicating more than one nucleotide is present

in a nucleotide site in a DNA sequence within a population) were identified across all

samples relative to the reference *T. putrescentiae* GQ205623 sequence. There were 3-10

SNPs in each sample out of 384-388 nucleotides in the ITS2 regions.

3.4.2   Two choice behavior assays

Fewer mites ($P < 0.01$) were found on the treated samples, in comparison to the

control after six hours in the Petri dish arena (Table 3.3), indicating that *T. putrescentiae*

oriented to and remained on the control ham cube that was wrapped in the untreated nets

over those cubes treated with low or medium levels of PG whether in XG or CA+PGA.

In addition, there were no mites found on any of the netted ham cubes at six hours that

were wrapped in nets with either XG or CG+PGA that included the high concentration of

PG. For the XG + PG group, only two and one mite(s) were found on all the ham cubes

treated with low and medium PG, respectively; no mites were found on the high PG

treatment. Similarly, for the CA + PGA + PG group, only three and one mite(s) chose to

remain on the ham cubes treated with low and medium PG, respectively. On average, 8-

12 mites oriented to the ham cubes that were wrapped in the control nets, out of the 20

original mites that were used for inoculation. The total number of mites found on the pair

of control and treated ham cubes was less than the number of mites introduced, which

indicated that some mites did not stay on the ham samples. Since the Petri dish stage was

an open area, there were mites under the black paper and mites that climbed up the Petri dish wall and became stuck in the petroleum jelly.

Adults of *T. putrescentiae* preferred ($P < 0.05$) to lay eggs on ham cubes in the control nets in comparison to ham cubes in treatment nets. Evaluation of the egg placement by mites after four days revealed that all eggs in the arena were laid on control cubes and none were found on the treated cubes, regardless of PG levels. Between 110 and 230 eggs were laid on the control cubes, and no eggs were laid on any treated ham cubes over the four days period regardless of the type of gum treatment used or the PG concentration that were used (Table 3.3). In addition, after four days, only one moving mite was found on one ham cube wrapped in CG + PGA + low PG treated net among all samples observed for oviposition, which suggests that nearly all mites given four days for orientation (compared to six hours in the orientation trial) preferred to settle on untreated cubes where females laid all their eggs.

3.4.3   Reproduction assays

Regardless of the netting duration (1st day, four or eight weeks) prior to mite inoculation, the number of *T. putrescentiae* produced in the assays was less ($P < 0.05$) on ham cubes with nets that were treated with gum and PG after two weeks of culture when compared to any of the treatments lacking PG. These treatments include no net on the cube, untreated nets, and nets treated with gums (Tables 3.4 and 3.5).

Though no difference ($P > 0.05$) existed in mite numbers on ham cubes regardless of PG concentration, the medium PG and high PG treatments only had approximately four live mites on the cubes in comparison to the initial inoculating level of 20 mites, or in comparison to between 280 and 480 mites on the non-PG treated ham cubes. Control

hams without nets had the highest number of mites (195-483 on average) followed by the ones wrapped in gum-treated (95-513 on average) and untreated nets (76-288 on average), respectively. No difference was found ($P > 0.05$) between the ham samples with or without gum-treated nets with respect to mite numbers after two weeks of incubation, with the exception of the third batch of the 8-week old XG-treated sample set, in which XG-treated samples contained fewer mites than ham cubes without nets or with untreated nets. In addition, no differences ($P > 0.05$) were observed between XG and CG + PGA treated nets of the same concentration of PG regarding mite population on net-treated hams.

Molds appeared on most of the ham cubes (without nets, with untreated nets, or with gum treated nets in both batches of experiments). Molds were also found on several ham cubes wrapped in XG + low/medium PG treated nets, and two ham cubes that were wrapped in CG + PGA + low PG treated nets. No molds were found on any of ham cubes wrapped in nets with XG + high PG or CG + PGA + medium/high concentrations of PG in either batch.

3.5   Discussion

ITS2 sequences have been used for the molecular identification of Astigmata mites and phylogenetic studies (Navajas et al., 1998; Yang, Cai, & Cheng, 2011). However, cloning is necessary because intra-individual polymorphism may complicate the readings of direct sequencing (Beroiz et al., 2014). The PCR-cloning method was utilized to identify mite species and confirm that *T. putrescentiae* was the species in the mite culture with no contaminations of other mite species in the subcultures. Sequence alignments of 18 isolated colonies indicated 2-4 deletions (at 261, 262, 363, and 364

positions) and 4-8 nucleotide variations when compared to the reference sequence of GQ205623, which did not pose any problem at the identification of mite species. Results indicate complete homogeneity of ITS2 consensus sequences in polymorphic clones of *T. putrescentiae* in the lab culture, which indicates the invariance of the ITS2 consensus sequence over the species range. Discrepancies in mite ITS2 sequences have been reported in other studies (Beroiz et al., 2014; Yang et al., 2011). Therefore, it would be necessary to investigate the variability in ITS2 sequence of *T. putrescentiae* populations that are collected from different samples or from different geographical areas. Although most SNPs have no effect on mite behavior or development, they may still indicate potential changes in mite behavior and development.

The behavioral choice test is a commonly used experimental design in animal ecology and behavior studies (Raffa, Havill, & Nordheim, 2002). In the present study, PG and gum were infused into net fibers to produce wraps that were applied on ham cubes. When two food choices were provided simultaneously, the free-moving adult *T. putrescentiae* mites exhibited clear preferences. Mites discriminated between untreated and treated ham cubes. Most mites chose to feed (~10 moving mites/cube) and lay approximately 100 eggs on each ham cube that was wrapped with an untreated net within the four-day period. The result of mite orientation to and oviposition on untreated ham cubes was in agreement with the previous study by Abbar et al. (2016). That study tested several food additives that were applied to ham cubes without nets, and *T. putrescentiae* oriented to untreated controls when compared to dry-cured ham pieces that were treated with 25-100% PG. In addition, those researchers reported that fewer eggs ($P < 0.01$) were laid on PG treated ham cubes in comparison to the untreated ham cubes.

In the reproduction assay, mites were contained in the jar where either untreated or treated ham cubes were kept. The total number of mites was the sum of mites from the initial 20 mites placed on the ham cube and all the progeny produced by these mites over the two-week period. When the uncovered ham was provided, *T. putrescentiae* fed on the ham and reproduced. Twenty mites eventually multiplied to 200 or more moving mites and many unhatched eggs. Most mites were found on the meat rather than in the jar or black paper. After the ham cube was covered with untreated nets, fewer moving mites were found after two weeks of incubation, indicating that nets limited their movement to a small extent because of the smaller mesh size and tight attachment to the ham cubes. The nets infused with XG or the combination of CG and PGA did not necessarily reduce mite settling when compared to the uncovered control. However, all net products infused with gum and PG inhibited the population growth and/or reproduction of *T. putrescentiae* when compared to the control samples without nets or with untreated nets. Also, the inhibitory effects did not decline with the application time of gum and PG treated nets on hams, which revealed that the treated nets were effective at controlling mites for a minimum of 10 weeks.

Similar studies that were conducted with gum and PG coatings also indicated the effectiveness of PG at controlling *T. putrescentiae* population growth. According to Zhao et al. (2016), ham cubes coated with XG or CG + PGA plus 20% PG or higher had no live mites after two weeks of incubation. The gum and PG coating was slightly more effective than the gum and PG infused nets, which may be due to the consistent film coating formed directly on the ham cubes providing mites with less access to meat in comparison to the gum and PG infused nets. However, the netting technique could have

two advantages over the coating on a commercial scale. First, treated nets can be produced on a large scale and would be ready to use by ham producers in their current production process. However, applying the PG-infused gel coating to hams would require an additional spraying or dipping operation that would need to be added to the production process. Second, preliminary results with whole hams indicated that the use of netting required approximately half of the amount of PG and gum solution when compared to the coating (Campbell et al., 2016a). Since propylene glycol procured during the last two years was approximately $12 per gallon (http://www.essentialdepot.com/product/CUBE-4PG.html), use of nets would provide an economical advantage over coating the ham surface.

It is evident that PG inhibited the mites from staying on the ham at the benchtop level. PG is affirmed to be GRAS (Generally Recognized as Safe) by the US Food and Drug Administration's (ID Code 57556) and the Code of Federal Regulations (21CFR184.1666). PG has been used as an ingredient in pharmaceuticals, foods (Fiume et al., 2012) and cosmetics (Anonymous, 1994) due to its antifungal and pesticidal qualities. In this study, medium and high concentrations of PG plus gum treated nets controlled mite infestations. In addition, none or only a very small amount of eggs was detectable on these samples, which indicated that the reproductive ability of the mites was also greatly inhibited.

A study conducted in Kansas State University on the whole ham indicated that only the coating of CG + PGA + 40% PG significantly lowered the number of resident mites when compared to the control three months after the gums were applied, whereas lower concentration of PG, as well as the combination of XG + 20%/40% PG did not

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differ from the control (Abbar et al., 2016). These results indicate that the coatings are also effective at controlling mites on whole hams, but that a greater concentration of PG is needed, when used on whole hams in comparison to ham cubes. Therefore, the application of netting on whole hams in order to control mite infestation needs further study to determine the effective PG concentration that is needed to control mites on whole hams.

The inclusion of high concentrations of PG may raise a concern regarding ham sensory quality. Previous studies conducted in our laboratory (Campbell et al., 2016c; Zhao et al., 2016a) indicated that the sensory quality attributes of hams were not affected by coating under laboratory conditions. In addition, minimal sensory differences were detected on whole hams, but ham quality was still excellent. This is logical since the PG was incorporated directly into the netting solution before forming the gel, so it became locked into the gum matrix (Nieto, 2009), which inhibited the ham muscles from absorbing PG.

In addition, the treatments with the highest PG concentration controlled mite infestations and fungal growth or propagation. CG, PGA and PG combination controlled mold better than XG and PG. The ham samples wrapped in CG + PGA + medium and high concentrations of PG treated nets had no visible fungal growth after 4 weeks of storage. However, the XG + low-PG treatment had fungal growth during all experimental periods. This was plausibly due to the killing of most of the potential fungal spores or other contaminating microorganisms in the formula by heating CG + PGA + PG combination during netting. The development of molds is part of ham aging. *T. putrescentiae* has been reported to consume a substantial quantity of molds (Hubert et

al., 2004). In this study, there were several mold species present on the ham cubes, including white, yellow, black, and blue molds. Mites were present on white, blue, and yellow molds but rarely on black molds. In general, molds were divided into the following four groups based on mite preferences or fungal attractiveness and fungal suitability that was for mite development: 1. preferred and suitable for mite growth; 2. preferred but unsuitable; 3. avoided but suitable; and 4. avoided and unsuitable (Hubert et al., 2004). *T. putrescentiae* preferred *Eurotium amstelodami* var. *amstelodami*, *Alternaria alternata*, *Cladosporium cladosporioides*, and *Penicillium aurantiogriseum*, but avoided *Mycocladus corymbifer*, *Aspergillus montevidensis*, and *Aspergillus versicolor*.

3.6   Conclusions

It was confirmed that the mite culture that was reared in our lab was a pure culture of *T. putrescentiae*. Incorporating propylene glycol into ham nets, as a natural food-grade processing aid, inhibited the growth and reproduction of *T. putrescentiae* and mold growth on dry-cured ham cubes in benchtop experiments. Ham nets that were infused with propylene glycol and gums are potential candidates for use during aging to control mites in dry cured ham plants. The infused ingredients are safe for humans and have no adverse effects on the sensory quality attributes of hams (proved by previous studies). The gum and propylene glycol treated nets should be considered for testing and potential use during whole ham aging. Further studies are required to evaluate the efficacy of treated nets in inhibiting mite infestation when they are applied on whole hams in commercial settings.

Table 3.1   Total size (base pairs) of cloned products and estimated full-length size for
              ITS1, 5.8S rDNA subunit, and ITS2

GenBank accession no	PCR product (bp)	ITS1 (bp)	5.8S rDNA subunit (bp)	ITS2 (bp)
GQ205623				388
MF353994	1302	667	151	384
MF353995	1309	670	151	388
MF353996	1306	668	151	386
MF353997	1307	670	151	386
MF353998	1306	668	152	386
MF353999	1305	668	151	386
MF354000	1303	668	151	384
MF354001	1309	670	151	388
MF354002	1305	668	151	386
MF354003	1308	671	151	386
MF354004	1305	668	151	386
MF354005	1305	670	151	384
MF354006	1305	670	151	384
MF354007	1308	671	151	386
MF354008	1310	671	151	388
MF354009	1307	670	151	386
MF354010	1308	671	151	386
MF354011	1310	671	151	388

Table 3.2   Sequence variations of the ITS2 nuclear rDNA region.

GenBank accession no	Variable sites																											
	16	32	53	86	124	128	134	161	185	186	220	240	251	258	261	262	276	277	287	305	313	314	326	345	359	362	363	364
GQ205623	C	C	C	A	A	G	C	T	G	T	A	A	G	T	A	C	C	T	T	G	C	A	G	A	A	T	T	G
MF353994	.	.	.	.	.	.	.	C	.	.	.	.	.	.	–	–	.	.	.	A	.	.	.	.	G	C	–	–
MF353995	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
MF353996	.	.	.	T	G	.	.	.	.	C	G	.	.	.	–	–	.	.	.	.	.	.	.	.	.	.	.	.
MF353997	.	.	.	T	G	.	.	.	.	C	G	.	.	.	–	–	.	.	.	.	.	C	.	.	.	.	.	.
MF353998	.	.	.	.	.	.	.	.	T	C	G	.	.	.	–	–	.	.	.	.	T	.	.	.	.	.	.	.
MF353999	.	.	T	T	G	.	.	.	.	C	G	.	A	C	–	–	.	G	.	.	.	.	.	.	.	.	.	.
MF354000	.	.	.	.	.	.	.	.	.	C	G	T	A	.	–	–	.	.	.	A	.	.	.	.	G	C	.	.
MF354001	T	.	.	.	.	.	T	.	.	C	G	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
MF354002	.	A	.	T	G	.	.	.	.	C	G	.	.	.	–	–	.	.	.	A	.	.	.	.	.	.	.	.
MF354003	.	.	.	.	.	.	.	.	.	C	G	.	.	.	–	–	.	.	C	.	.	.	.	C	.	.	.	.
MF354004	.	.	.	.	.	.	T	.	T	C	G	.	.	.	–	–	.	.	.	.	T	.	.	.	.	.	.	.
MF354005	.	.	.	.	.	.	.	.	.	C	.	.	A	.	–	–	.	.	.	A	T	C	.	.	G	C	–	–
MF354006	.	.	.	.	.	.	.	.	.	.	.	.	A	.	–	–	.	.	.	A	.	.	.	.	G	C	–	–
MF354007	.	.	.	T	.	A	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.
MF354008	.	.	.	.	.	.	.	.	.	C	G	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MF354009	.	.	.	T	G	.	.	.	.	C	G	.	.	.	–	–	.	.	.	.	.	.	.	.	.	.	.	.
MF354010	.	.	.	T	.	A	.	.	.	C	G	.	.	.	–	.	.	.	.	.	.	.	.	C	.	.	.	.
MF354011	.	.	.	G	G	.	.	.	.	C	G	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.

Only variable sites are presented and numbered as in the GenBank submitted sequence. Deletions are indicated by dashes. Dots indicate identity with the sequence of GQ205623

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Table 3.3   Mean numbers (SD) of mites (orientation) and eggs (oviposition) of *T. putrescentiae* on small dry-cured ham cubes wrapped in untreated control and treated nets in a laboratory two-choice behavior bioassay (n=5)

Treatment	Orientation-mites after 6 h			Oviposition-eggs after 4 days		
	Treated	Control	Probability difference	Treated	Control	Probability difference
XG + low PG	0.4 (0.9)	11.0 (2.5)	0.00033	0	129 (36)	0.00133
XG + medium PG	0.2 (0.4)	9.0 (2.1)	0.00055	0	225 (83)	0.00373
XG + high PG	0	9.8 (2.3)	0.00066	0	162 (62)	0.00426
CG + PGA + low PG	0.6 (0.9)	11.8 (3.1)	0.00080	0	150 (64)	0.00625
CG + PGA + medium PG	0.2 (0.4)	10.0 (2.1)	0.00035	0	114 (56)	0.01044
CG + PGA + high PG	0	8.0 (2.6)	0.00250	0	154 (71)	0.00849

XG: xanthan gum, PG: propylene glycol, CG: carrageenan, PGA: propylene glycol alginate.
Pairwise comparison of treated and control orientation and oviposition data followed by a two-sample Student's *t*-test, assuming unequal variances.

Table 3.4   Mean (SD) of population growth of *T. putrescentiae* fed on 0-week-old, 4-week-old, and 8-week-old small dry-cured ham cubes treated with xanthan gum and propylene glycol infused nets after 2 weeks (n=10)

Treatment	0-week-old		4-week-old		8-week-old	
	Mites	Molds	Mites	Molds	Mites	Molds
Control without net	483 (183) a	Yes	240 (172) a	Yes	212 (61) a	Yes
Control with untreated net	281 (70) b	Yes	158 (137) a	Yes	188 (94) a	Yes
XG	383 (237) ab	Yes	167 (142) a	Yes	95 (32) b	Yes
XG + low PG	19.6 (10.9) c	Yes	7.2 (9.7) b	Yes	6.6 (4.8) c	Yes
XG + medium PG	2.9 (2.2) c	No	1.6 (2.0) b	No	1.8 (1.4) c	Yes
XG + high PG	3.3 (3.2) c	No	1.1 (1.7) b	No	1.2 (1.9) c	No
SEM	15.6		10.4		6.0	

XG: xanthan gum, PG: propylene glycol, SEM: standard error of the mean.
Means with same letter within each column are not different ($P > 0.05$) using Tukey's Honestly Significant Difference test.

Table 3.5   Mean (SD) of population growth of *T. putrescentiae* fed on 0-week-old, 4-week-old, and 8-week-old small dry-cured ham cubes treated with carrageenan, propylene glycol alginate, and propylene glycol infused nets after 2 weeks (n=10)

Treatment	0-week-old		4-week-old		8-week-old	
	Mites	Molds	Mites	Molds	Mites	Molds
Control without net	670 (250) a	Yes	196 (134) a	Yes	220 (73) a	Yes
Control with untreated net	287 (243) b	Yes	77 (50) b	Yes	143 (64) b	Yes
CG + PGA	513 (174) a	Yes	129 (101) a	Yes	188 (107) ab	Yes
CG + PGA + low PG	10.3 (15.8) c	Yes	2.1 (2.5) c	No	3.2 (2.5) c	No
CG + PGA + medium PG	2.7 (4.1) c	No	1.1 (1.1) c	No	2.0 (1.5) c	No
CG + PGA + high PG	0.7 (1.1) c	No	0.5 (0.7) c	No	0.8 (1.0) c	No
SEM	19.1		7.6		7.3	

CG: carrageenan, PGA: propylene glycol alginate, PG: propylene glycol, SEM: standard error of the mean.
Means with same letter within each column are not different ($P > 0.05$) using Tukey's Honestly Significant Difference test.
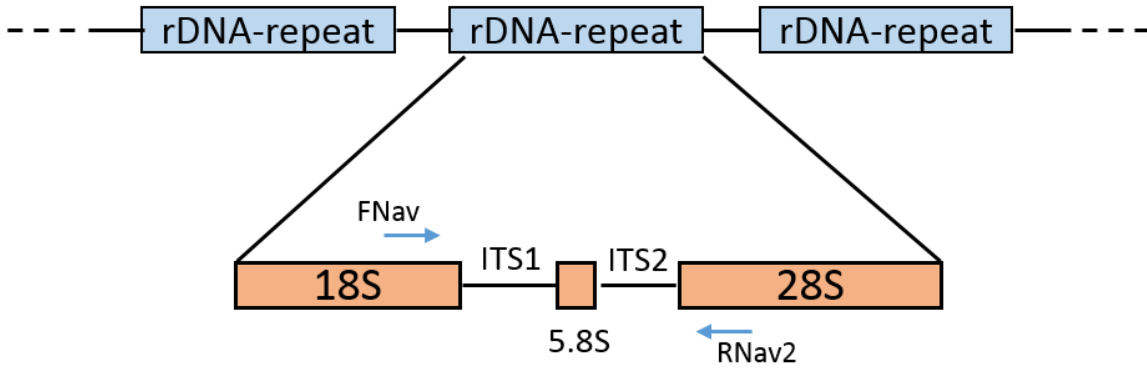
Figure 3.1   Structure of the ITS (internal transcribed spacer) regions of the nuclear ribosomal DNA clusters and schematic location of the primers used in this study
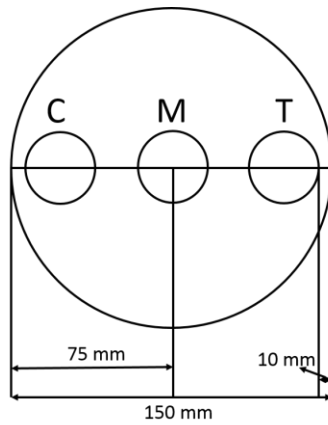
Figure 3.2   Mite two-choice behavior assay.

Mites were released at circle M (Mites), and the ham cubes wrapped in untreated and treated nets were placed in circles C (Control) and T (Treatment), respectively.
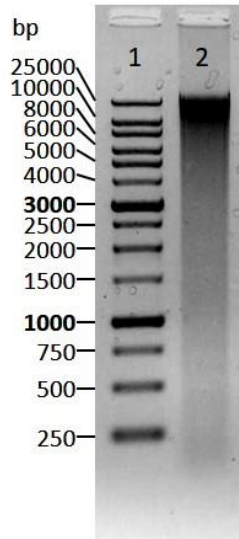
Figure 3.3   Gel electrophoresis of mite genomic DNA extraction

Lane 1: 25 Kb Plus DNA Ladder; Lane 2: purified mites genomic DNA
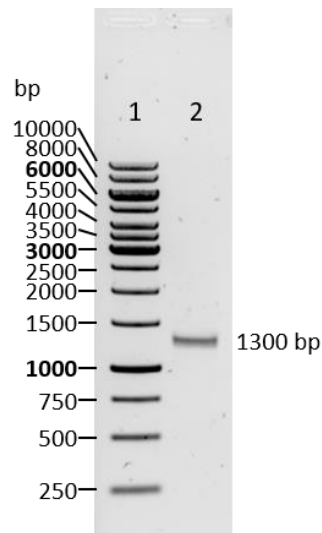
Figure 3.4   Polymerase chain reaction (PCR) amplification of rDNA from mite
                genomic DNA

Lane 1: 10 Kb DNA Ladder; Lane 2: purified PCR product
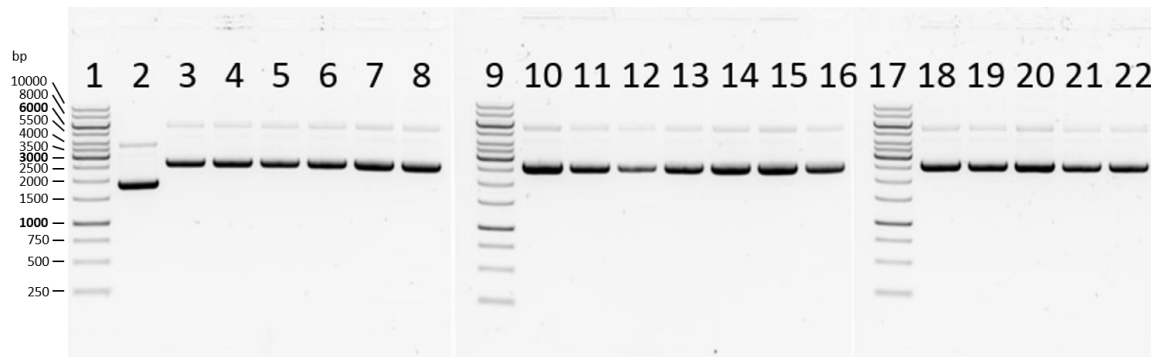
Figure 3.5   Gel electrophoresis of isolated plasmids

Lanes 1, 9, 17: 10 Kb DNA Ladder; Lane 2: negative clone, Lanes 3-8, 10-16, 18-22:
positive clones

CHAPTER IV

APPLICATION OF FOOD-GRADE INGREDIENTS INFUSED INTO NETS TO

CONTROL *TYROPHAGUS PUTRESCENTIAE* (SCHRANK)

(SARCOPTIFORMES: ACARIDAE) INFESTATION

ON DRY CURED HAMS

4.1 Abstract

Background: *Tyrophagus putrescentiae* (Schrank) is controlled in the U.S. dry cured ham industry with methyl bromide (MB) fumigation. However, MB fumigation is being phased out of use since it is an ozone depleting substance. The objective of this research was to evaluate ham nets that were infused with mite-deterring food-grade ingredients for their efficacy at controlling mite infestations on dry cured ham. RESULTS: Fewer *T. putrescentiae* ($P < 0.05$) were on ham cubes with treated nets containing propylene glycol (PG) when compared to the number of mites on ham cubes with untreated nets over 10 weeks of storage. Lard infused nets without PG did not decrease the mite population ($P > 0.05$). The net without coating slowed the growth and reproduction of *T. putrescentiae* since net controls had fewer mites ($P < 0.05$) than controls without nets. Fungi were not present on ham cubes that were treated with PG-containing nets over 10 weeks of storage with a few exceptions. CONCLUSION: Lard and xanthan gum, or carrageenan + propylene glycol alginate treated nets containing the medium concentration of PG effectively inhibited mite

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reproduction and fungal growth on dry cured ham and could potentially be used in an integrated pest management program to control mites on dry cured hams.

Keywords: *Tyrophagus putrescentiae*, mite reproduction, propylene glycol, lard, gum, dry cured ham

4.2   Introduction

Tyrophagus putrescentiae (Shrank, 1781), known as the mold mite, cheese mite, or ham mite, is an ubiquitous and economically important mite species that infests a wide variety of stored foods, particularly those with high protein and fat contents (Gulati & Mathur, 1995; Hughes, 1976; Sánchez-Ramos & Castañera, 2001). This mite is a major pest in nuts, grains, aged cheese, dried eggs, dry cured ham, dog food, mushroom beds, and mycology laboratory cultures (Arnau & Guerrero, 1994; Canfield & Wrenn, 2010; Duek, Kaufman, Palevsky, & Berdicevsky, 2001; Eaton & Kells, 2009; Erban et al., 2016; Garcia, 2004; Gill, McEwan, McGarry, & Nuttall, 2011; Hughes, 1976; Kheradmand, Kamali, Fathipour, & Goltapeh, 2007a; Rentfrow et al., 2008; Sánchez-Ramos & Castanera, 2000). Mite infestations cause damage to stored products (Nayak, 2006). Moving mites and piles of pale brownish dust may appear on heavily infested products. The dust piles are consist of waste material, dead mites, and shed cuticle, which have pungent and/or minty odors (Nayak, 2006; Townsend, 2007).

Some fungi (usually *Penicillium*, *Candida zeylanoides* and *Debaryomices hansenii*) may be found on the outer surface of the ham (Dikeman & Devine, 2014), which positively influences mite viability and contributes to infestations (Okabe & Oconnor, 2001). Moreover, exposure to *T. putrescentiae* may contribute to allergic diseases, such as contact dermatitis and asthma, among farmers and workers who handle

heavily infested stored products (Arlian, Yadav, Kondreddi, Elder, & Morgan, 2004; Armentia et al., 1994; Fernandez-Caldas, Iraola, & Carnes, 2007; Hage-Hamsten et al., 1985; Revsbech & Dueholm, 1990).

The optimum environmental conditions for mite growth and reproduction include 25-32 °C and 70-90% relative humidity (Boczek, 1991), which are similar to the environmental conditions in dry cured ham aging houses. Mite infestations occur in dry cured ham plants in the southeastern United States (Rentfrow et al., 2008), Spain (Arnau & Guerrero, 1994; Sánchez-Ramos & Castañera, 2001), and other countries, resulting in product rejection and economic losses (Armentia et al., 1994).

Methyl bromide is currently the only known effective space fumigant that is effective at controlling ham mites (Rentfrow et al., 2008). However, since methyl bromide is an ozone depleting substance, it was phased out of production and consumption as of January 1, 2005, with the exception of critical use exemptions (EPA, 2017a), which were available to the dry cured ham industry through 2016. Beginning in 2017 the dry cured ham industry was only given approval to purchase and use the remaining stockpiles of methyl bromide until they are depleted (EPA, 2014). Therefore, it is important to explore alternative control measures to prevent mite infestations during dry cured ham aging since methyl bromide will eventually not be possible to use.

Phosphine (Zhao et al., 2015), sulfuryl fluoride (Phillips et al., 2008), controlled atmosphere (Hasan et al., 2016; Sánchez-Molinero et al., 2010), traps (Amoah, 2016), high temperature (Abbar et al., 2016b; Fields, 1992), low temperature (Abbar et al., 2016b), and sanitation (Rentfrow et al., 2006) have been evaluated for their efficacy at controlling mite infestations. Though heat treatments (42-45 ºC for 48 h) or cold

treatments (-10 to -20 ºC for 48 h) in the freezer controlled mites in laboratory studies and could be potentially applied in the building or in the freezer (Abbar et al., 2016b), most treatments are impractical or would be best used in combinations with other practices. Coating or rubbing hams during aging with vegetable oils or hot lard is frequently used in Spain to help prevent mite infestations (Garcia, 2004). Researches determined that lard, propylene glycol, canola oil, calcium sorbate, sodium sorbate, iodate salt, maleic acid, propanol and butanediol decreased *T. putrescentiae* reproduction when compared to untreated controls (Abbar et al., 2016a; Abbar et al., 2012; Zhao et al., 2016b).

Since propylene glycol was the most effective ingredient in the research by Abbar et al. (2012) and Zhao et al. (2016), xanthan gum + propylene glycol and carrageenan + propylene glycol alginate + propylene glycol coatings were developed that were effective at controlling mite reproduction on ham cubes under laboratory conditions (Zhao et al., 2016b). Since gum and propylene glycol coatings were applied to the surface of the hams, an additional processing step is necessary. Therefore, research was conducted on the effectiveness of infusing coatings into special nets that are used to hang dry cured hams on racks during aging, which revealed that gum and propylene glycol infused nets effectively inhibited mite growth and reproduction over a 10-week storage period under laboratory conditions (Zhang et al., 2017). Since lard is used in Europe to control mites and is less costly than propylene glycol, it was hypothesized that incorporating lard into the netting solution, along with propylene glycol to control mites and reduce costs. Therefore, in this study, ham nets were infused with different combinations of gum, lard and propylene glycol. The choice behavior of *T. putrescentiae* between ham cubes

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wrapped with untreated and treated nets were investigated in addition to the effect of using treated nets on mite reproducibility during 10 weeks of storage.

4.3   Materials and methods

4.3.1   Food additives

Xanthan gum (XG) (21 CFR 172.695), carrageenan (CG) (21 CFR 184.1221), and propylene glycol alginate (PGA) (21 CFR 172.858) were provided by TIC Gums, Inc. (Belcamp, MD). Lard (Armour pork fat, 21 CFR 182.70) was purchased from Wal-Mart Stores Inc. (Starkville, MS). Food-grade propylene glycol (PG) (21 CFR 184.1666) was procured from the Essential Depot (Sebring, FL). Ham nets (Ennio International, Aurora, IL) consisted of 50% polyester and 50% cotton (blend nets) with a stitch density of 112 loops/cm^2.

4.3.2   Preparation of food-grade ingredients infused nets

The patent pending netting solutions consisted of gum (XG or CG + PGA), lard (low or medium concentration), and/or PG (low or medium concentration).

To prepare the netting solutions, XG (1%) or CG (1%) + PGA (1%) were mixed with propylene glycol followed by mixing with tap water while stirring. CG + PGA + PG mixtures were then heated to boil while stirring. Lard was melted on a heating plate prior to the addition of the gum and PG solution. The gum, PG, and lard mixtures were blended in a food blender at high speed for 1 min.

Nets were cut, weighed, and then immediately soaked in the newly-prepared netting solution. The soaked nets were pressed through two rollers of a netting machine (Midwest Metalcraft & Equipment Company, Winsor, MO) in order to squeeze out the

extra fluids. The treated nets were weighed, vacuum packaged, and kept at room temperature until use. The weight of absorbed ingredient per foot of net was calculated.

4.3.3   Ham cubes

Dry cured hams (6-8 kg; 4-6 months in age) were purchased from a commercial supplier. The bone-in ham was cut transversally into 1.3 cm and 2.5 cm thick slices. Each slice was vacuum-packaged and stored at 4 °C until use. Ham cubes were prepared for the analysis as described below.

4.3.4   Mite cultures

T. putrescentiae stocks were provided by Dr. Phillips's laboratory in the Department of Entomology at Kansas State University and were maintained on a diet as previously reported (Abbar et al., 2016b). The inoculated rearing jar was placed in a locked storage box that contained soapy water at the bottom, and petroleum jelly smeared on the edges to prevent the mites from escaping. The mite culture was maintained at 23 ± 2 °C and 80 ± 5% RH in a dark cabinet for three to four weeks prior to use. A PCR (polymerase chain reaction) cloning method confirmed that the mite culture raised in the laboratory was a pure culture of *T. putrescentiae*.

4.3.5   Two choice behavior test of mites

A two choice behavior test was conducted according to the methods of Zhang et al. (2017). One 1.3 cm ham cube covered with the control (untreated) net and another 1.3 cm ham cube covered with a treated net were offered simultaneously to the mites inside a small arena (as shown in Fig. 3.2). The inside bottom of the plastic Petri dish (150 mm diameter × 15 mm depth) was covered with black construction paper that was cut to the

same dimension as the Petri dish. Three specific round areas (C, M, and T) were assigned along a line passing through the center of the paper, with M designating the mite release point at the center, C being the ham piece with the untreated net as a control and T as the treated net for a given test, and each ham piece set at 10 mm away from the side wall of the Petri dish. Two ham cubes from the same muscle section were selected and wrapped with control and treated nets, with one control cube placed in area C and one cube placed in area T. Five replications (five pairs of cubes) were tested for each treatment. Two pair of cubes were taken from the *biceps femoris* muscle, one pair from the *semitendinosus* muscle, and two pairs from the *adductor* and *semimembranosus* muscles. A total of 20 mixed sex adult mites (approximately 15 females) were placed in the M area on the paper, and the Petri dish was stored in the dark in a growth chamber (23 ± 2 °C and $80 \pm 5\%$ RH). A thin layer of petroleum jelly was applied on the inner upper 5 mm of the Petri dish to prevent mites from escaping. After 6 h of incubation, mites that were oriented to each of the net-wrapped ham cubes were counted. Orientation was indicated by the number of live mites on the control and treated ham cubes (Abbar et al., 2016a). An identical setup was prepared, and 20 mites were placed in the M area. The number of eggs laid on the cubes and nets were counted after 4 days of incubation. Oviposition was determined by counting eggs that were laid on the control and treated ham cubes.

4.3.6　　Mite reproduction assays

Ham cubes (2.5 cm × 2.5 cm × 2.5 cm) were removed from ham muscles and assigned to and packaged with either control or treated nets. Five replications were tested for each treatment. Two cubes were taken from the *biceps femoris* muscle, one from the

semitendinosus muscle, and two from the *adductor* and *semimembranosus* muscles. Ham cubes that were packaged in control and treatment nets were then placed in ventilated glass Mason jars (216 mL, 65 mm diameter, 55 mm height; Ball Corp., Broomfield, CO). The bottom of the jars was covered with black construction paper and the top was covered with filter paper that was sealed with the jar ring. Three sets of experiments were conducted. The netting formulations in the first set included 100% lard, lard + low-, and medium-PG; the second set included XG + low/medium- lard + low/medium- PG; and the third set included CG + PGA + low/medium- lard + low/medium- PG. Control ham cubes were not covered with nets, while net control ham cubes were wrapped with untreated nets. Each cube was inoculated with 20 large mixed sex adult mites (predominantly females) and incubated in a dark cabinet that was controlled at 23 ± 2 °C with a relative humidity of 80 ± 5 %. In order to evaluate the long-term effectiveness of treated nets at controlling mite infestations, three batches of samples were prepared and each batch was inoculated with mites either on the first day of storage, after four weeks of storage, and after eight weeks of storage, respectively. After two weeks of incubation, the total numbers of moving mites on the ham cubes, nets, black paper, and in the jars were counted under a stereo microscope (Model 568, American Optical Company, Buffalo, NY). The presence of mold and yeast growth on ham cubes were recorded. Molds were characterized by the cottony mycelium growth or colored spores; yeasts were characterized by the growth of slimy, light colored, and defined colonies. The culture of molds or yeasts on ham samples were subcultured on Potato Dextrose Agar (PDA) plates to confirm their morphological traits.

4.3.7   Water activity, moisture content and weight loss

Simultaneous experiments were conducted as described above with non-inoculated ham cubes to evaluate water activity (a_w), moisture content, and weight loss of the ham cubes from each treatment. Each treatment consisted of three replications, two ham cubes from the *biceps femoris* muscle, and one from the *semitendinosus* muscle. After four and eight weeks of storage, water activity of the ham cube was measured at room temperature with a water activity meter (AquaLab Series 3 TE, Decagon Devices, Inc., Pullman, WA). The moisture content of the samples was determined by drying the minced ham sample (2.0 ± 0.1 g) in an oven at 105 ± 2 °C until a constant weight was obtained (AOAC, 2000). The moisture content in percentage was expressed as the difference of the weight before and after drying divided by the initial weight $\times$ 100. The weights of fresh ham cubes and the weights of ham cubes after 10 weeks of storage were taken to calculate the weight loss. Weight loss in percentage was expressed as the difference of the weight before and after the experiment divided by the initial weight (before experiment) $\times$ 100.

4.3.8   Statistical analyses

Significance of mite preference for a given ham combination (control vs. treated) in the two-choice behavioral test were calculated by a paired, two-sided Student's t-test using Microsoft Excel 2007. A p-value > 0.05 was not significant (NS), a p-value < 0.05 was considered to be significant marked with '*', and a p-value < 0.01 was marked with '**', assuming unequal variances.

A randomized complete block design with two replications (batches) and 5 subsamples per replication was used to determine the effect of different treatments on

mite reproduction, water activity, moisture content, and weight loss of ham cubes in glass jars. When significant differences ($P < 0.05$) occurred among treatments, Tukey's Honestly Significant Difference Test ($P < 0.05$) was used to separate treatment means.

A 2^2 factorial arrangement within a randomized complete block design with 2 replications and 5 subsamples per replication was used to evaluate the effects of using combinations of PG (low-, and medium-) and lard (low- and medium-) net treatments on mite reproduction; A 2^3 factorial arrangement within a randomized complete block design with 2 replications and 5 subsamples per replication was used to evaluate the effects of combinations of gums (XG, CG + PGA), PG (low-, and medium-) and lard (low- and medium-) treatments on water activity, moisture content, and weight loss.

4.4   Results

4.4.1   Mite behavioral assays

Mite orientation results indicated that a greater number of *T. putrescentiae* mites were found on the controls than on the treatments of XG + medium lard + medium PG ($P < 0.01$), CG + PGA + low lard + medium PG ($P < 0.01$), or CG + PGA + medium lard + medium PG ($P < 0.05$) (Table 4.1). In addition, there was no difference ($P > 0.05$) between the number of mites on the control and each of the remaining treatments ($P > 0.05$). However, oviposition data indicated a strong egg-laying preference on the control when compared to its treated ham cube pair ($P < 0.01$); on average less than three eggs were found on treated ham cubes whereas 74 - 165 eggs were found on untreated ham cubes (Table 4.1).

4.4.2   Mite reproduction assays

Fewer *T. putrescentiae* mites ($P < 0.05$) were on ham cubes with treated nets containing PG when compared to the number of mites on ham cubes with untreated nets over 10 weeks of storage (Tables 4.2, 4.3, and 4.4). In comparison to the net controls (123-163 mites on average), lard and low- or medium- PG infused net treatments had 19-44 mites (Table 4.2). However, lard infused nets without PG did not decrease the mite population ($P > 0.05$) (Table 4.2). XG + lard + PG infused nets had fewer mites (2-39 mites) ($P < 0.05$) when compared to the net control (77-146 mites) (Table 4.3). Similarly, CG + PGA + lard + PG infused nets also had fewer mites (0-22 mites) ($P < 0.05$) than the net control (88-123 mites) over 10 weeks of storage.

Factorial analysis indicated that medium concentration PG consistently inhibited mite reproduction ($P < 0.05$) in comparison to treatments without PG that were inoculated with mites at storage periods of 0, 4, and 8 weeks after packaging in net treatments (Tables 4.3 and 4.4). In addition, the greatest lard concentration decreased mite reproduction in the CG + PGA + lard + PG treatments when mites were inoculated after 4 weeks of storages ($P < 0.05$).

Nets slowed the growth and reproduction of *T. putrescentiae* since net controls had fewer mites (77-163 mites) ($P < 0.05$) than controls without nets (133-437 mites). Molds or yeasts were not present on ham cubes that were treated with PG-containing nets over 10 weeks of storage, with the exception of lard + PG treatments that were inoculated at 8 weeks of storage, XG + low lard + low PG and CG + PGA + low lard + low PG treatments that were inoculated at 4 weeks of storage. This indicates that using the

greater concentrations of PG and lard prevents mold and yeast growth, which is an important factor in controlling mite infestations.

4.4.3 Water activity, moisture content, and weight loss

There was no difference ($P > 0.05$) between control and net control samples in terms of a_w, moisture content and weight loss (Table 4.5). The water activity was lesser ($P < 0.05$) in the net control at four weeks of storage than the treatments containing gum, lard, and the medium concentration of PG. After eight weeks of storage, the water activity of all samples decreased by 0.02-0.08 on average; and the net control had a lower a_w than all treatments except for the lard + low PG, XG + medium lard + low PG, or CG + PGA + low lard + low PG treatments. Moisture content decreased as ham cubes were stored in the humidity controlled cabinet (Table 4.5). Net control presented the least moisture content, which was less than CG + PGA + medium lard + low/medium PG and XG + low lard + medium PG after four weeks of storage, and less than XG + medium lard + medium PG and CG + PGA + low lard + medium PG treatments after eight weeks of storage. After 10 weeks of storage all treatments had less weight loss ($P < 0.05$) than the net control with CG + PGA + medium lard + medium lard being the lowest (44%). Addition of PG to the net coating treatments led to increased moisture content and decreased weight loss ($P < 0.05$). In addition to the net coating treatments, medium PG treatments retained more moisture than the low PG treatments. However, coating nets with treatments did not prevent moisture loss from the hams. This indicates that hams can be aged and preserved in nets that were infused with the food grade coatings that were used in this study.

4.5   Discussion

The purpose of this study was to develop nets that were infused with food-grade coatings and evaluate their effectiveness at inhibiting mite contact and reproduction. The net used in this study was a blend of 50 % polyester and 50 % cotton with high stitch density (112 loops/cm^2). The nets without a coating decreased the mite population since the nets reduced the meat exposure to mites and limited their actual locomotion, which is in agreement with the previous findings (Campbell et al., 2016a; Zhang et al., 2017). There are a few commercially available mite-proof covers that are made from different materials, including a tightly woven cover with a pore size of 2-10 μm (Mahakittikun, Boitano, Komoltri, Ninsanit, & Wangapai, 2009; Mahakittikun et al., 2006), cotton fabric coated with eugenol loaded chitosan (Jarupaiboon et al., 2007), and Chitosan/Ag$^+$-impregnated textile (Rahel et al., 2013). These anti-mite covers prevent the settlement and development of house dust mites, *Dermatophagoides farinae* Hughes on household fabrics and other surfaces, but have not been tested for ham mites. However, the usability of the tightly woven cover in the ham industry needs to be evaluated because of its low air permeability (4.0 cm^3/s/cm^2); while neither of two fabrics for protection from house dust mites can be applied in the ham industry due to the cost and the usage of non-food-grade materials. The coated nets used in the ham industry should contain only food-grade (Generally Recognized As Safe, GRAS) ingredients or approved for food contact (FDA 21 CFR) (Baldwin, Hagenmaier, & Bai, 2011), which are gum, propylene glycol and lard in this study, and have no side effect on ham aging process and ham quality.

Data from behavior assays indicated that *T. putrescentiae* avoided ham cubes with some of the treated nets, and they showed strong preference for oviposition on control

ham cubes over any of the treated ham cubes. The reproduction test suggested that nets containing PG reduced the mite population and the inhibitory effect was maintained over the 10-week storage period, which is in agreement with previous findings on research conducted on gums and PG infused nets (Zhang et al., 2017). The main differences between these two studies were the incorporation of lard into the netting formulation and the infusion of nets with different combinations of gum, lard, and PG, which was due to the fact that coating ham cubes with lard was effective at inhibiting *T. putrescentiae* reproduction (Abbar et al., 2016a; Zhao et al., 2016b).

The 100% lard infused nets were avoided by *T. putrescentiae* for oviposition but did not decrease the mite population when compared to the net control. Since two ham cubes with untreated and lard treated nets were provided, mites likely chose to lay eggs on control ham cubes because they were preferred by the mites. However, ham cubes with lard infused in the nets could sustain mite growth if mites had no other options for growth. For example, in the reproduction studies, ham cubes that were covered with lard infused net was the only food source to mites. When lard coating was used instead of lard infused nets, the mite population was controlled in the reproduction assay (Zhao et al., 2016b), which might be due to the fact that the lard coating provided a lipid barrier that blocked the access of mites to feed on the meat. In contrast, the mesh size of the nets that were used was not small enough to prevent mites from penetrating through the nets to the ham meat.

Propylene glycol is the key ingredient that inhibits mite infestations on dry cured ham (Abbar et al., 2016a; Zhang et al., 2017; Zhao et al., 2016b). PG is a clear, viscous, and colorless liquid with minimal odor and a slightly bittersweet taste. PG is classified as

a GRAS ingredient by the FDA when used as a direct additive for years in food, cosmetic, and pharmaceutical products (Anonymous, 1994; Fiume et al., 2012). When compared to net control, the lard + low/medium PG infused nets reduced the mite population by 73.1% / 80.0%, 64.5% / 84.6%, and 85.3% / 81.6% for treatments that were immediately inoculated after netting, four weeks after netting and eight weeks after netting ham samples inoculated, respectively. This reinforces the effectiveness of PG at inhibiting mite reproduction.

For the combinations of gum, PG and lard, all treated nets reduced the number of mites compared to net control over 10 weeks of storage, with reductions of the mite population by 63-99%. The inhibitory effect on mite reproduction was dependent on the inclusion of PG and lard. In general, as PG increased, the mite population decreased. Similarly, as lard increased, the mite population decreased, but not at as great of a level as when PG was increased. When the same combination of lard and PG was used, CG + PGA treatments had fewer mites than XG treatments. Although there were no statistical difference among the four different treatments at each storage time point, CG + PGA + medium lard + medium PG had the fewest mites present and would be preferred for use because of the zero tolerance of mites for the dry cured ham products in the United States (USDA 9 CFR301; USDA 9 CFR 416). When compared to previous research from our laboratory with XG/(CG + PGA) + medium PG infused nets (Zhang et al., 2017), the nets containing the same concentration of PG did not improve the inhibitory effect, indicating that the lard in the formulation could be excluded for the purpose of lowered cost and easier operation.

Another important property of PG is that it helps control the water activity and thereby spoilage in intermediate moisture or semi-moist foods (Aldrich, 2014), which may explain no or less growth of fungi on the ham cubes that were covered with PG-containing nets. Nets containing PG inhibited fungal growth with only a few exceptions, including lard + PG treatments after eight weeks of storage and XG / (CG + PGA) + low lard + low PG treatments after four weeks of storage. In comparison, fungal growth was present on ham cubes without nets and with untreated nets as well as with 100% lard treatments. Mold growth usually favored the reproduction of *T. putrescentiae*, which confirms that the *T. putrescentiae* mite is fungivorous (Okabe & Oconnor, 2001). Molds might serve as the food source of mites (Hubert et al., 2004), or a source of free water and shelter for mites (Canfield & Wrenn, 2010). In contrast to the moldy ham cubes, the samples with visible yeasts had a pungent smell and fewer mites than the same treatment without yeast. The typical mold and yeast colonies were picked up and saved in the freezer for species identification, which will be conducted by another student in our laboratory.

Dry cured ham develops the typical flavor through the breakdown of proteins and fatty acids into volatile compounds (Pham et al., 2008). In addition, dry cured hams lose moisture by water evaporation and air exchange during the aging, and the finished dry cured ham product have to lose at least 18% of its original weight (Marriott & Ockerman, 2004). Therefore, any coatings, including coated nets must be permeable to moisture so that the hams can lose more than 18% moisture and become shelf-stable. Nets containing lard, PG or both retained more moisture than other treatments. Therefore, the ham cubes with these nets had higher water activity and lower weight loss than ham cubes with

control nets after four and eight weeks of storage. This is due to the nature of lard and PG. Lard is strictly hydrophobic and presents a barrier to water vapor. PG is a humectant. It is miscible with water and can easily bind water from the atmosphere. The samples used for moisture analysis were small ham cubes, weighing approximately 20 g. Whole hams that are aged for 4-6 months usually weigh 5-7 kg, three hundred times heavier than the small cubes. Therefore the water activity, moisture, and weight loss difference between control and treated samples would be much less in commercial products. However, even though the control treatments had less moisture and a lower a_w, ham cubes were still able to lose moisture and have an acceptable a_w, which is 0.76 after eight weeks of storage. Therefore, these nets could be used in commercial production to inhibit mite infestations since hams would be preserved.

The nets infused with CG + PGA + medium lard + medium PG were the most effective at inhibiting mite growth and reproduction, indicating that the combination of these ingredients was effective at controlling mite infestation and fungal growth. However, there were still mites alive after this treated net was applied, which did not meet the requirement of zero tolerance, even though there were fewer mites than the inoculation level of 20. It is recommended that mite infestations are controlled using an integrated pest management (IPM) program, with the use of coated nets as part of the program. Oviposition data indicated that no moving mites or eggs were found on any ham cubes with medium PG infused nets. In this regard, if we place mite traps that attract mites in the aging house and hang all hams in the treated nets, theoretically mites will move towards the traps and avoid treated hams so that hams are prevented from mite settlement and infestation. In the IPM program, prevention should always take place at

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the earliest time of processing. For example, if the ham is infested, cleaning dry cured ham with vegetable oil mentioned earlier only eliminates the mites on the outer layer but not mites in cracks; it would be much complicated to excavate mites inside the cracks. Thus, multiple hurdles need to be considered to take part in the IPM program, including monitoring, sanitation, prompt action to possible infestations, fumigation, etc.

4.6   Conclusions

Gum and medium PG treated nets were effective at controlling mites during aging. This technique would be a helpful addition to an IPM program to control ham mites and has potential as an alternative to methyl bromide.

4.7   Future research

(1) The data obtained in the present study were from laboratory experiments. Further experiments should include scaling up the use of effective food grade ingredient treated nets at controlling mite infestations in commercial dry cured ham plants.

(2) After two weeks of incubation, there were only a few mites and mite eggs on treatments with medium or high concentration of PG. The incubation period of mites on ham cubes that were wrapped with those treated nets will be increased to three to six weeks and the numbers of mites and eggs will be evaluated, which will provide us with information that if PG would completely inhibit mite growth.

(3) The mite eggs will be inoculated on ham cubes that were wrapped with medium or high concentration of PG treatments to determine if eggs will develop to adult mites, which will provide evidence that if PG inhibits mite development.

(4) The mechanism of the inhibitory effect of PG on controlling mite infestations will be discussed from the sensitivity of mites to PG, the hygroscopic property of PG, the damaging effect of PG on the wax layer of mite enterogument, and the potential pesticidal effect.

Table 4.1   Mean numbers (SD) of mites (orientation) and eggs (oviposition) of *T. putrescentiae* on small dry-cured ham cubes wrapped in untreated control and treated nets in a laboratory two-choice behavior bioassay (n=5)

Treatment	Orientation - mite counts after 6 h			Oviposition - egg counts after 4 d		
	Treated	Control	Difference	Treated	Control	Difference
Lard 100%	4.6 (2.1)	5.4 (3.3)	NS	1.6 (2.3)	84 (26)	**
Lard + low PG	4.2 (2.6)	5.0 (1.6)	NS	0	122 (41)	**
Lard + medium PG	3.4 (2.1)	6.6 (3.0)	NS	0	94 (45)	**
XG + low lard + low PG	4.4 (2.5)	5.2 (3.5)	NS	3.0 (6.7)	69 (27)	**
XG + low lard + medium PG	3.0 (3.3)	6.2 (2.4)	NS	0	80 (18)	**
XG + medium lard + low PG	3.6 (1.9)	3.4 (1.3)	NS	0.6 (1.3)	96 (36)	**
XG + medium lard + medium PG	2.8 (2.5)	8.2 (2.2)	**	0	74 (30)	**
CG + PGA + low lard + low PG	3.0 (2.4)	6.0 (2.1)	NS	1.2 (1.8)	111 (31)	**
CG + PGA + low lard + medium PG	2.6 (1.5)	7.8 (2.6)	**	0	165 (73)	**
CG + PGA + medium lard + low PG	3.4 (2.7)	5.2 (2.9)	NS	0	103 (29)	**
CG + PGA + medium lard + medium PG	1.4 (1.3)	6.8 (3.8)	*	0	97 (41)	**

PG: propylene glycol, XG: xanthan gum, CG: carrageenan, PGA: propylene glycol alginate.

Pairwise comparison of treated and control orientation and oviposition data followed by a two-sample Student's *t*-test, assuming unequal variances: NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$.

Table 4.2 Mean (SD) population growth of *T. putrescentiae* inoculated on ham cubes that were stored for either 0, 4, or 8 weeks after applying the nets with lard and/or propylene glycol infused nets after two weeks (n=10)

Treatment	Fresh		4-week-old		8-week-old	
	Mites	Fungi	Mites	Fungi	Mites	Fungi
Control	404.8 (155.1)*	M	260.2 (191.9)*	M	193.7 (111.4)	M, Y
Net control	163.1 (65.0) a	M	123.1 (39.2) a	M	158.0 (126.1) a	M
Lard 100%	121.9 (143.0) ab	M	81.2 (66.7) ab	M	118.1 (78.7) ab	M
Lard + low PG	43.9 (34.8) b	ND	43.7 (14.8) bc	ND	23.2 (38.5) bc	M
Lard + medium PG	32.7 (16.3) c	ND	18.9 (18.6) c	ND	29.1 (36.4) c	M

PG: propylene glycol, M: molds, Y: yeasts, ND: not detected.

Means with same letter within each column are not different ($P > 0.05$) using Tukey's Honestly Significant Difference test.

* indicates that control and net control are different ($P < 0.05$).

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Table 4.3   Mean (SD) population growth of *T. putrescentiae* inoculated on ham cubes that were stored for either 0, 4, or 8 weeks after applying the nets infused with xanthan gum, lard and propylene glycol after two weeks (n=10)

Treatment	Fresh		4-week-old		8-week-old	
	Mites	Fungi	Mites	Fungi	Mites	Fungi
Control	436.6 (132.4) *	M	132.7 (118.1) *	M, Y	157.9 (134.7)	M, Y
Net control	146.2 (83.7) a	ND	76.6 (57.2) a	M, Y	126.5 (147.6) a	Y
XG + low lard + low PG	38.6 (32.7) b	ND	28.6 (14.0) b	M	17.5 (17.9) b	ND
XG + low lard + medium PG	20.9 (18.8) b	ND	4.6 (4.6) b	ND	3.1 (2.4) b	ND
XG + medium lard + low PG	25.3 (24.6) b	ND	17.9 (15.0) b	ND	11.2 (13.2) b	ND
XG + medium lard + medium PG	12.5 (10.2) b	ND	1.9 (1.7) b	ND	2.0 (2.1) b	ND

XG: xanthan gum, PG: propylene glycol, M: molds, Y: yeasts, ND: not detected.

Means with same letter within each column are not different ($P > 0.05$) using Tukey's Honestly Significant Difference test.

* indicates that control and net control are different ($P < 0.05$).

Table 4.4   Mean (SD) population growth of *T. putrescentiae* inoculated on ham cubes that were stored for either 0, 4, or 8 weeks after applying the nets infused with carrageenan, propylene glycol alginate, lard, and propylene glycol after two weeks (n=10)

Treatment	Fresh		4-week-old		8-week-old	
	Mites	Fungi	Mites	Fungi	Mites	Fungi
Control	415.0 (155.2) *	M	277.1 (203.5) *	M, Y	88.8 (51.6)	M, Y
Net control	123.1 (74.0) a	M	88.1 (55.5) a	M, Y	108.5 (81.0) a	M, Y
CG + PGA + low lard + low PG	21.7 (16.4) b	ND	13.9 (14.0) b	M	7.8 (4.7) b	ND
CG + PGA + low lard + medium PG	1.7 (1.6) b	ND	0.5 (1.0) b	ND	0.9 (1.4) b	ND
CG + PGA + medium lard + low PG	9.6 (7.9) b	ND	1.4 (2.8) b	ND	4.9 (9.9) b	ND
CG + PGA + medium lard + medium PG	3.6 (3.4) b	ND	0.4 (0.7) b	ND	0.4 (0.7) b	ND

CG: carrageenan, PGA: propylene glycol alginate, PG: propylene glycol, M: molds, Y: yeasts, ND: not detected.

Means with same letter within each column are not different ($P > 0.05$) using Tukey's Honestly Significant Difference test.

*indicates that control and net control are different ($P < 0.05$).

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Table 4.5   Water activity (a$_w$) and moisture content of dry cured ham cubes wrapped with different treated nets after 4 and 8 weeks of storage and weight loss of dry cured ham cubes after 10 weeks of storage at 23 ± 2 °C and relatively humidity of 80 ± 5%

Treatments	Water activity		Moisture content %		Weight loss %
	4-week	8-week	4-week	8-week	10-week
Net control	0.84 (0.02) c	0.76 (0.03) cd	43.0 (6.5) b	36.8 (2.8) b	58.8 (1.0) a
Lard 100%	0.86 (0.02) bc	0.81 (0.02) b	48.3 (5.1) ab	41.8 (1.5) ab	51. 8 (2.9) bc
Lard + low PG	0.85 (0.02) bc	0.79 (0.02) bc	47.0 (7.7) ab	40.1 (3.9) ab	53.5 (3.7) b
Lard + medium PG	0.85 (0.02) bc	0.82 (0.05) ab	47.2 (4.3) ab	43.1 (6.2) ab	48.6 (1.7) bcde
XG + low lard + low PG	0.87 (0.01) abc	0.82 (0.03) ab	47.8 (4.1) ab	43.8 (5.6) ab	52.2 (4.1) bc
XG + low lard + medium PG	0.88 (0.02) ab	0.84 (0.03) ab	50.1 (2.3) a	45.2 (5.3) ab	50.9 (2.3) bc
XG + medium lard + low PG	0.86 (0.02) bc	0.80 (0.02) bc	47.7 (5.0) ab	39.3 (3.5) ab	51.9 (1.4) bc
XG + medium lard + medium PG	0.88 (0.01) ab	0.86 (0.02) a	49.1 (3.1) ab	47.0 (4.7) a	45.4 (3.3) de
CG + PGA + low lard + low PG	0.84 (0.01) c	0.80 (0.02) bc	47.6 (2.1) ab	43.5 (8.2) ab	50.0 (1.3) bcd
CG + PGA + low lard + medium PG	0.87 (0.02) ab	0.82 (0.01) ab	48.4 (2.4) ab	47.8 (8.1) a	47.1 (1.1) cde
CG + PGA + medium lard + low PG	0.87 (0.01) abc	0.82 (0.01) ab	50.5 (3.3) a	41.7 (1.9) ab	49.1 (4.5) bcde
CG + PGA + medium lard + medium PG	0.90 (0.01) a	0.86 (0.02) a	51.3 (1.9) a	46.2 (1.9) ab	44.0 (0.9) e
SEM	0.002	0.003	0.400	0.584	0.295

PG: propylene glycol, XG: xanthan gum, CG: carrageenan, PGA: propylene glycol alginate.
Means with same letter within each column are not different (*P* > 0.05) using Tukey's Honestly Significant Difference test.

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