PURIFICATION AND CHARACTERIZATION OF *ACHETA DOMESTICUS* AND *GRYLLODES SIGILLATUS* CRICKET CHITIN AND CHITOSAN FOR BIOACTIVE AND BIODEGRADABLE FOOD PACKAGING APPLICATIONS

by

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ABSTRACT

The production of insects for protein is projected to reach a market share of 1.33 billion USD, a rapid increase from the estimated 144 million USD share of 2019 market. The isolation of insect protein produces by-products, including chitin. Currently chitin is extracted from aquaculture by-products, such as shrimp and crab shells, and used to produce chitosan for various applications in the supplement and food industry. With the insect market expected to continue its growth, the feasibility of sourcing commercial chitin and chitosan from reared crickets', and the application properties of its counterpart, chitosan, was investigated in this dissertation. In the first part of this dissertation, chitin from two commonly reared crickets in the Unites States, *Acheta domesticus* and *Gryllodes sigillatus*, was successfully extracted, purified, and identified as a commercially viable option for chitin and chitosan. Extensive crustacean chitin studies served as the foundation of purification steps, however durations were adjusted to account for intrinsic differences between insects and crustacean exoskeletons. Furthermore, cricket chitosan was prepared and optimized with varying degrees of deacetylation. As expected, cricket chitosan had lower molecular but did not have a detectable effect on the bioactive properties tested. All cricket chitosan produced had similar lipid binding capacity *in vitro*. Additionally, the microbial inhibition of cricket chitosan and commercial chitosan (~70% DDA) were not significantly different when evaluated against *L. innocua* and *E. coli*. High DDA cricket chitosan showed greatest bacterial inhibition as expected. In the second part of this dissertation, cricket derived chitosan showed similar and improved food packaging properties, when evaluated against commercial shrimp chitosan. microstructure analysis provided by scanning electron microscopy showed greater compaction and agglomeration of cricket chitosan films. The change in microstructure may be attributed to the increased complexity generally attributed to insect chitosan materials, a result of remaining melanin and protein in close association with insect exoskeleton chitosan. As a result, cricket films had similar or increased tensile strengths but decreased elongation percentages when compared to shrimp films. Water vapor permeability of cricket films was decreased due to tortuosity. Residual melanin likely played an important role in increasing cricket film surface hydrophobicity and providing enhanced light barrier properties. Overall, this dissertation successfully shows the potential of crickets as insect derived chitin and chitosan, and its effectiveness as a lipid binding and antibacterial agent, as well as its potential use in biobased food packaging.

CHAPTER 1. INTRODUCTION

1.1 Crickets for human consumption

The Food and Agriculture Organization for the United Nations (FAO) estimates that by 2050 the world's population will reach 9 billion, and as a result food security will amplify as it is expected that a 70% increase in food production will be necessary. Since it is estimated that animal protein production cannot be sustained (Odegard & van der Voet, 2014), one consideration to aid in food security, while minimizing the effects on the environment, is the production of alternative protein sources such as insects, *in vitro* meat, and algae (F. G. Hall, Jones, O'Haire, & Liceaga, 2017). Specifically, farming of crickets as a protein source is an exceptional solution and alternative as it requires less input of resources for their production like water, and land, and have less detrimental effects on the environment compared to livestock proteins (Van Huis, 2013). Although there are many benefits of cricket rearing which have been extensively detailed, there is an overwhelming psychological aversion to insect consumption in western society outside those who consume them for their novelty. Specifically, studies report consumers lack willingness to consume whole insects. However, consumers are more willing to consume and try insect products when they are incorporated into a product they are already familiar with (Gmuer, Nuessli Guth, Hartmann, & Siegrist, 2016). When crickets were ground into whole flour and then incorporated into tortilla chips, consumers were much more accepting to this cricket product compared to the tortilla chips mixed with whole crickets (Gmuer et al., 2016; Luna, 2019). As crickets have exceptional, total protein composition (~70% dwb) (Zielińska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015), research has extended its focus to the production of cricket protein hydrolysates and isolates, similar to the whey and pea protein isolate products consumers currently use to supplement protein in their diet (F. Hall, Johnson, & Liceaga, 2018; F. G. Hall et al., 2017; Luna, 2019; Nongonierma, Lamoureux, & FitzGerald, 2018; Zielińska, Baraniak, & Karaś, 2017; Zielińska, Karaś, & Jakubczyk, 2017).

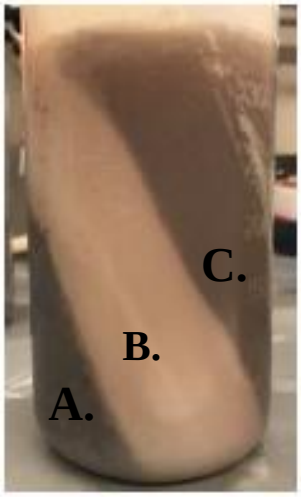

Figure 1. Centrifuged *A. domesticus* crickets after Alcalase hydrolysis of its proteins and centrifugation producing a). chitin rich pellet, b). fat layer, and c). protein hydrolysate supernatant

One benefit cricket protein-rich products have over ground whole cricket powders is the removal of the cricket's chitin found in its exoskeleton. Chitin is reported to have negative gastrointestinal effects likely due to the fact that not all humans produce chitinase, the route which humans breakdown chitin (Paoletti, Norberto, Damini, & Musumeci, 2007). Total dietary fiber of cricket powders have been estimated to be about 8.5%, of this fiber 87% is due to insoluble chitin present (Stull et al., 2018). Gastrointestinal effects of chitin consumption such as nausea, diarrhea, and constipation have been reported and linked to chitin containing products like cricket-based biscuits, or whole crickets (Homann, 2015; Jayanegara, Sholikin, Sabila, Suharti, & Astuti, 2017). As a result, chitin removal during cricket protein isolation is also of interest. Therefore, as cricket protein hydrolysates are produced, the byproduct and waste stream of the chitin-rich byproduct is collected with currently no research on its use (highlighted in Figure 1a). As cricket protein hydrolysate popularity and production increases, the burden of this chitin-rich byproduct will also increase and require innovation for the creation of value-added products.

1.2 Chitin

1.2.1 Structure

Poly (ß-(1→4)-N-acetyl-D-glucosamine, also known as chitin, is a linear polysaccharide native to various living organisms. Chitin provides mechanical strength to the shells and exoskeletons of arthropods like shrimp, crabs, insects, as well as in the cell walls of fungi (Merzendorfer & Zimoch, 2003; Sivashankari & Prabaharan, 2017). The most prevalent form of chitin found in crustacean shells and insects is alpha chitin, which arrange in two antiparallel molecules that allows for highly organized and efficient packing of chains held together strongly through hydrogen bonding (Moratti & Cabral, 2017). Chitin is an abundant polysaccharide but its inability to dissolve in water due to this tight packing structure limits its applicability. As a result, for commercial applications, chitin is frequently converted to its counterpart chitosan due to its advanced properties.

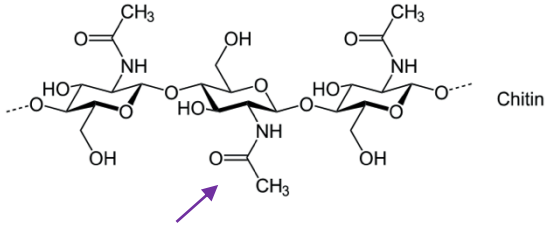

Figure 2. Chitin structure from Zagar, Asghari et al., 2015

1.2.2 Extraction and purification from insects

Current commercial manufacturing of chitosan, first extracts chitin from waste streams of aquaculture production. This includes the wasted shells in the production of shrimp (Aranaz et al., 2009; Bumgardner et al., 2017). Much research is available on chitin obtained from shrimp and has expanded to evaluate other crustacean sources of chitin such as that from crab, cuttle fish, prawn, lobster, squid pen and others (Al Sagheer, Al-Sughayer, Muslim, & Elsabee, 2009; Hajji et al., 2014; Santos, Seabra, Veleirinho, Delgadillo, & Lopes da Silva, 2006; Shepherd, Reader, & Falshaw, 1997; Yen, Yang, & Mau, 2008). However, more recently, research has expanded to evaluate chitin sourced from insects, which can be obtained either from insect farms or collected from the wild. To date research is available on chitin obtained from bumblebees, grasshoppers, crickets, hornets, wasps, centipedes, velvet worms and other species of cockroaches and beetles

(Chae, Shin, & Shin, 2018; Greven et al., 2019; Ibitoye et al., 2018; Murat Kaya et al., 2014; Murat Kaya, Lelešius, et al., 2015; Murat Kaya et al., 2017; Murat Kaya, Sofi, Sargin, & Mujtaba, 2016; Majtán et al., 2007; Zelencova, Erdoğan, Baran, & Kaya, 2015).

The demineralization process is an important first step to remove the minerals present in the exoskeleton matrix. Traditionally, crustacean shells have been demineralized with hydrochloric acid (HCl) solutions, to react with the calcium carbonate intrinsically present at high concentrations (30-50%) (Kurita, 2006; Percot, Viton, & Domard, 2003; Zargar, Asghari, & Dashti, 2015). In the case of insects, due to their environmental differences, they do not contain a large amount of minerals in their exoskeletons when compared to crustaceans (Berezina & Hubert, 2019). Table 1 outlines and compares the demineralization methods that have been employed in different research studies of insect chitin, with shrimp chitin extraction methods as a reference.

Ash composition analysis is used to monitor mineral concentration present in chitin and evaluate the efficacy of the demineralization step (Table 1), and generally ash contents in the range of 2-5% are commercially acceptable (Ibitoye et al., 2018; No & Meyers, 1995). Literature shows insect and crustacean demineralization steps as being similar, with nearly all studies utilizing HCl as the acid treatment with the exception of two cricket studies (Chae et al., 2018; Ibitoye et al., 2018). Demineralization of cicada sloughs, silkworms, beetles, and black soldier flies were performed at 100°C for 20-30 minutes with 1 M HCl and resulted in ash values of 3-5%. Whereas silkworms, mealworms, and grasshoppers were treated with the same acid concentration at lower temperatures (25-30°C) for more than 2 hours, and ash contents were reported to be less than 1%. Overall, insect chitin exposed to longer durations of acid treatment led to increased demineralization efficacy as shown by decreased ash contents. Insect chitin extractions, with increased acid treatment duration and lowered temperature, were likely aiming to protect the insect chitin from hydrolysis.

Due to the decreased mineral content in insects' exoskeletons, the commercial manufacture of insect chitin may be advantageous, in comparison to the demineralization of crustacean chitin (Berezina & Hubert, 2019). Such advantages include decreased processing time and decreased chemicals (acid) required, which may produce a chitin product free of hydrolysis (Percot et al., 2003). Furthermore, patents on technology, which do not require an acid treatment step for cricket chitin have been filed (Berezina & Hubert, 2019; Berezina et al., 2018a, 2018b, 2019). However, the majority of insect chitin studies reviewed commonly employ the demineralization procedures

optimized for crustaceans (Berezina & Hubert, 2019). Two studies on grasshoppers (*Decticus verrucivorus*) and cockroach wings (*Blaberus. giganteus*), employed HCl demineralization parameters that were highly concentrated (2 M), hot (75°C), and prolonged (>2 hours) (Murat Kaya, Lelešius, et al., 2015; Murat Kaya et al., 2017). Therefore, additional optimization studies would be beneficial to determine the ideal demineralization processes parameters for insect chitin. Additionally, precautions should be taken before undergoing intense demineralization processes.

Deproteinization, following the demineralization step, removes proteins found within the chitin matrix. A thorough optimization study of shrimp chitin extraction was performed and found deproteinization to be completed after 6 hours of 1 M sodium hydroxide (NaOH) treatment at 70°C (Percot et al., 2003). For insects, NaOH solutions are applied at varying concentrations, times, and temperatures similarly following established crustacean treatments (Table 1). Two cricket chitin studies removed the proteins from the chitinous matrix following the same NaOH concentration and duration, with increased temperatures of ~95°C (Chae et al., 2018; Ibitoye et al., 2018). Other insect chitin deproteinization steps however, employed extended durations and increased temperatures. Cicada sloughs, beetles, and black soldier flies were treated at 80°C for 24 hours or more with 1 M NaOH (Liu et al., 2012; Purkayastha & Sarkar, 2020; Sajomsang & Gonil, 2010), while cockroaches and grasshoppers had higher concentrated alkali treatments (4 M NaOH) at 150°C for 20 hours (Murat Kaya & Baran, 2015; Murat Kaya, Lelešius, et al., 2015).

Table 1. Extraction, yield, and composition of chitin obtained from insects

Source	**Extraction processing steps and parameters**			**Chitin yield (dwb)**	**Proximate composition**		**Ref.**
	Demineralization	**Deproteination**	**Decolor**		**Ash (%)**	**Protein (%N)**	
Shrimp (shells)	0.25 M HCl, 15 min	1 M NaOH 70°C, 6h	N/A	20%	0.01% (calcium)	<1 %	(Percot et al., 2003)
Cricket (whole)	Oxalic acid, 3h	1M NaOH at 95° for 6h	1% sodium hypochlorite, 3h	4.3%	1%	<6.9	(Ibitoye et al., 2018)
Cricket (whole)	Oxalic acid, 3h	1M NaOH at 95°, 6h	50% APS, 30 min	5.1%	N/A	N/A	(Chae et al., 2018)
Cockroach (wings)	4 M HCl at 75°C, 2h	4M NaOH at 150°C, 20h	N/A	18%	N/A	N/A	(Murat Kaya & Baran, 2015)

Table 1 continued

Beetle (pupae and exuviae)	1 M HCl at 100°C, 30 min	1M NaOH at 80°C, 24h	1% potassium permanganate solution for 1h	15%	2.2%	<6.89	(Liu et al., 2012)
Cicada (sloughs)	1 M HCl at 100°C, 20 min	1 M NaOH at 80°C, 36h	6% sodium hypochlorite	37%	3.2%	<6.89	(Sajomsang & Gonil, 2010)
Silkworm (pupa exuviae)	1 N HCl at 100°C, 20 min	1 N NaOH at 80°C, 36hr & refluxing with Na_2CO_3, 20h	N/A	~10-15%	N/A	N/A	(M. Zhang, Haga, Sekiguchi, & Hirano, 2000)
Beetle (larva)							
Pine weevil	2 M HCl at 25°C, duration unspecified	2 M NaOH at unspecified temperature, 2h	0.5% sodium hypochlorite, 1h	27.9%	N/A	N/A	(M. Kaya et al., 2019)
Cicada (slough)	1 M of HCl at 30°C, 2h	1 M NaOH at 90°C, 2h	1% potassium permanganate solution, 1h & 2% oxalic acid, 2h	~ 28.2%	0.03%	N/A	(Luo et al., 2019)
Silkworm (chrysalis)				~ 3.1%	0.05%		
Mealworm				~ 2.5%	0.89%		
Grasshopper				~ 5.7%	0.95%		
Beetle	1 M HCl at 25°C, duration unspecified	1 M NaOH, at 100°C, 8h	N/A	5.0%	~ 2.0 %	N/A	(Marei, El-Samie, Salah, Saad, & Elwahy, 2016)
Honey bee				2.5%	~ 9.2%		
Desert locust				12.2%	~ 1.6%		
Grasshopper (female)	4 M HCl at 75°C, 2h	4 M NaOH at 150°C, 20h	N/A	10.03%	N/A	<6.89	(Murat Kaya, Lelešius, et al., 2015)
Grasshopper (male)				11.84%		<6.89	
Wasp (larvae)	1 M of HCl at 50°C, 6h	1 M NaOH, at 60°C, 16h	Water, methanol, chloroform (4:2:1), 40 min	2.2%	N/A	N/A	(Murat Kaya, Sofi, et al., 2016)
Wasp (pupa)				6.2%			
Wasp (adult)				10.3%			
Black soldier fly (larvae)	1 M of HCl at 100°C, 30 min	1 M NaOH, at 80°C, 24h	1% potassium permanganate solution, 1h	9%	3.3%	<6.89	(Purkayastha & Sarkar, 2020)
Black soldier fly (imagoes)				23%	5.6%	<6.89	

~ represent values reported by the studies for chitosan, obtained from the insect chitin reported in the table; dwb refers to composition based on a dry weight basis; and N/A indicates no value was available to be reported.

Nitrogen content analyses are employed to monitor the effectiveness of this purification step, as a measure of remaining protein present. Typically, nitrogen content (%N) are obtained through elemental analysis or using the Kjeldahl method; protein content is calculated using the 6.25 conversion factor (Sajomsang & Gonil, 2010). Due to the chitin's intrinsic nitrogen present on the acetyl functional groups, adjustment to the calculations must be made to adequately determine protein amount. A completely acetylated chitin will have a %N value of 6.89% and thus, this value would represent chitin with successful deproteinization (Sajomsang & Gonil, 2010). Whereas, a value higher than 6.89% suggests residual proteins are still present and can be calculated using the appropriate equation described elsewhere (Sajomsang & Gonil, 2010). Frequently, %N values are reported below the 6.89% threshold, as shown in Table 1, for crickets, beetles, cicada sloughs, and black solider flies. Such results imply low residual protein with the possibility residual inorganic materials may still be present (Ibitoye et al., 2018; Sajomsang & Gonil, 2010). This seems likely as these cases of insect chitin, shown in Table 1, still have residual minerals present with ash content roughly between 2-5%.

Although many of the insect chitin studies reported ash contents to demonstrate effective demineralization, most of these studies did not report protein content (or %N) values to monitor the effectiveness of deproteinization. As a result, it is difficult to understand the optimal deproteinization duration required for insect chitin. The insect chitin studies that evaluated the final protein content, also employed long treatment times. Beetle, cicada slough, grasshopper, and black soldier fly chitin studies had long deproteinization treatments of 24, 36, 20, and 24 hours, respectively. Whereas pine weevil, cicada slough, silkworm, mealworm, grasshopper, honeybee, and desert locust chitin studies used shortened treatment duration (8 hours or less), but the protein content was not reported. African field crickets were treated for 6 hours and is one of the only insect chitin studies with a shortened treatment duration that reported protein content (%N) below 6.89 (Chae et al., 2018). Therefore, insect chitin research could potentially choose less excessive demineralization treatments; however, more research is required to determine with certainty the optimal treatment duration for insect chitin extraction.

Current insect chitin studies show almost the entirety of research has been performed based on parameters previously optimized for crustacean sources. Although this is an intuitive approach, the change of species necessitates consideration for the determined parameters since the chitin exoskeletons are intrinsically formed differently from crustaceans (Berezina & Hubert, 2019).

Crustacean chitin studies have shown physicochemical properties, such as molecular weight, to be altered under improperly optimized demineralization and deproteinization steps. Therefore, research focused on the optimization of chitin purification steps would increase fundamental knowledge and understanding for commercial manufacturing of chitin from insects. The results of such research may elucidate the benefits, such as time efficiency and decreased cost of materials used for the chitin extraction.

Overall, of the insect chitin studies available, some insects had similar chitin content compared to chitin from crustacean shells (~20-40%) (Table 1). Particularly, cicada sloughs, cockroach wings, beetle exuviae, and pine weevils had the highest chitin content yields reported. These sources in particular, with the exception of the pine weevils, started with the insect exoskeletons rich in chitin, either by collecting those that had naturally molted (sloughs and exuviae), or chose to manually remove them from the insect (e.g. cockroach wings). The equation below shows the general calculation used to determine overall chitin yield after isolation and extraction steps:

$$chitin\ content\ \% = \frac{final\ purified\ chitin\ weight\ (dry)}{initial\ \&\ unpurified\ chitin\ containing\ material\ (dry)} \quad (1)$$

In this context, these specific insect studies reported higher yields, similar to crustacean sources, due to the chitin-rich starting material. Whereas other insect studies such as those on crickets, grasshoppers, and honeybees report much lower chitin content. These decreased yields may be a direct result of using whole insects as the starting material, which includes the mass of insect tissues that are not rich in chitin. As a result, the chitin content for these insect species represents the amount of chitin in the whole insect, but consideration must be made when comparing to crustacean chitin yields, as those yields do not represent the chitin content of the whole shrimp. For example, the shrimp chitin yield shown in Table 1 was calculated from the weight of the starting material (shrimp shells) and not the whole shrimp (i.e. body included).

1.3 Chitosan

1.3.1 Conversion to chitosan

The conversion of purified chitin occurs by deacetylation, with the removal and replacement of chitin's acetyl group with an amino group (Sivashankari & Prabaharan, 2017) (Figure 3). The conversion is commercially performed in the presence of a hot concentrated NaOH solution for an extended period of time (Bumgardner et al., 2017). Insect chitosan, like crustacean chitosan, has been produced through the traditional reaction process, with conditions such as duration, temperature, and NaOH concentration varied (Table 2). These processing conditions are known to have an effect on the final chitosan physicochemical properties, such as molecular weight and the degree of deacetylation (DDA), regardless of the starting material (Aranaz et al., 2009; Baskar & Kumar, 2009).

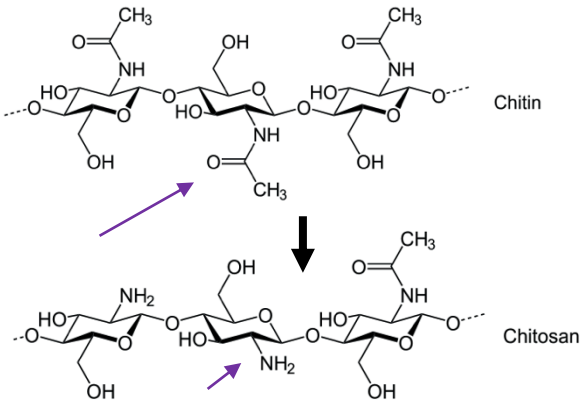

Figure 3. Conversion from Chitin to Chitosan adapted from Zagar, Asghari et al., 2015

Table 2. Insect chitosan conversion process and final properties

Insect species	Deacetylation experimental conditions	Chitosan properties			Reference
		Degree of Deacetylation (%)	Molecular weight (kDa)	Crystallinity Index (%)	
Cricket (*B. bimaculatus*)	67% NaOH at 95°C, 9h	85.0%	0.56–1.28	57.8	(Chae et al., 2018)
Cricket (*B. bimaculatus*)	50% NaOH at 105°C, 3h	95.5%	308	N/A	(Kim et al., 2017)
Cricket (*B. portentosus*)	50% NaOH (1:20 w/v) at 121°C, 4h	80.5%	N/A	86.6	(Ibitoye et al., 2018)
Pine weevil (*H. abietis*)	60% NaOH at 100°C, 4h	81.3%	7.30	N/A	(M. Kaya et al., 2019)
Colorado beetle (*L. decemlineata*)	50% NaOH at 100°C, 3h.	71%	2.72	72	(Murat Kaya et al., 2014)
Silkworm chrysalides (*B. mori)*	40% NaOH (undefined ratio to chitosan) at 100°C for 6h	96.8%	3.29-5.9x10^3	N/A	(Paulino, Simionato, Garcia, & Nozaki, 2006)
Desert locust (*S. gregaria*)	50% NaOH (1:15 w/v) at 100°C for 8h	98%	N/A	69	(Marei et al., 2016)
Honeybee (*A. melifera*)		96%		59	
Beetle (*C. rugosa*)		95%		49	
Black soldier fly (*H. illucens*)	50% NaOH (1:33 w/v) at 100°C for 2h	90%	15	N/A	(Khayrova, Lopatin, & Varlamov, 2019)
House fly larvae (*M. domestica*)	40% NaOH (undefined ratio to chitosan) at 70°C for 8h	90.3%	426	N/A	(Ai, Wang, Yang, Zhu, & Lei, 2008)
Blow fly larvae (*C. megacephala*)	67% NaOH (undefined ratio to chitosan) at 90°C for 9h	88.2%	501	N/A	C. Song, Yu, Zhang, Yang, and Zhang (2013)
Cicada slough (*undefined*)	60% NaOH (1:15 w/v) at 100°C for 8h	84.1%	3.78	64.8	(Luo et al., 2019)
Grasshopper (*undefined*)		89.7%	3.99	50.1	
Mealworm (*undefined*)		85.9%	3.98	51.9	
Silkworm chrysalis (*undefined*)		85.5%	4.09	32.9	

Table 2 provides a list of selected studies on chitosan derived from different insect species, including the deacetylation process conditions used and final chitosan properties. Additional insect chitosan studies have been performed and may be found elsewhere (Murat Kaya, Akyuz, et al., 2016; Kim et al., 2016; Ma, Xin, & Tan, 2015; Shin, Kim, & Shin, 2019; Y. S. Song et al., 2018; Soon, Tee, Tan, Rosnita, & Khalina, 2018; A.-J. Zhang et al., 2011).

1.3.2 Chitosan characterization

1.3.3 Degree of Deacetylation

The degree of deacetylation (DDA) of chitosan is an important physicochemical characteristic, as it quantifies the amount of chitin's acetyl groups that have been replaced with an amino group (Figure 3). Chitosan can have different degrees of deacetylation and generally can be varied between 50 and 100% depending on the reaction conditions and progress (Bumgardner et al., 2017). Literature describes increased treatment times, temperature, and NaOH concentrations produces chitosan polymers with higher DDA% values (Aranaz et al., 2009; Bumgardner et al., 2017). Since chitosan's DDA has implications on its functionality and applications (Aranaz et al., 2009), its characterization is important and consistently reported. Insect chitin deacetylation procedures are similar to that of crustaceans (Table 2). Overall, the reviewed insect chitosan research used a minimum of 40% NaOH solutions and were performed at temperatures between 70°-121°C. The hot and concentrated solutions were typically present at volumes 15-20 times the insect chitosan weight. Chitin from the Colorado potato beetle (*Leptinotarsa decemlineata*) was deacetylated for 3 hours and resulted in chitosan with the lowest DDA (71%), of all reported insect chitosans (Murat Kaya et al., 2014). Other studies deacetylated chitin, from desert locust, beetles, and grasshoppers among others, for 8 hours and obtained chitosan with 85-95% DDA. Whereas the field cricket and pine weevil produced approximately 80% DDA chitosan when deacetylation was performed for 4 hours.

Chae et al. (2018) published one of the few insect chitosan optimization studies reported in literature. Chitin from the African field cricket was converted to chitosan by varying the NaOH solution concentration (50-67%) and the reaction time (1-15 hours). At 50% NaOH and an increase of reaction time from 6 to 9 hours resulted in no subsequent increase of DDA (66%).

However, at 9 hours, increasing the alkaline concentration to 55% and 67% increased chitosan's DDA to 75% and 85%, respectively.

Two studies, each evaluating multiple insects, chose similar processing conditions for the conversion of chitin to chitosan. However, the chitosan obtained from desert locusts (*Schistcerca gregaria*), honeybees (*Apis melifera*), and beetles had 10% DDA more than that obtained from cicada sloughs, grasshoppers, mealworms, and silkworms (Luo et al., 2019; Marei et al., 2016). The differences of DDA may be a result of the species studied; however, it could also be a result of the method used to determine the DDA. The methods of deacetylation determination have previously been studied (Czechowska-Biskup, Jarosińska, Rokita, Ulański, & Rosiak, 2012; Yuan, Chesnutt, Haggard, & Bumgardner, 2011), and have shown that although FTIR spectroscopy is commonly used due to its facile measurements, although it is not always the preferred method for fundamental characterization of chitosan. Other methods, including NMR and titration, may be just as accessible and provide more accurate results (Czechowska-Biskup et al., 2012). The DDA reported for chitosan from cicada slough, grasshopper, mealworm, and silkworm was characterized using FTIR, which could have an effect on the lower deacetylation values reported; in comparison the study on chitosan from desert locust, honeybee, and beetle that used titration reported about 10% higher DDA values.

1.3.4 Molecular weight

Similar to chitosan's deacetylation determination, the molecular weight of chitosan is also an important physicochemical characteristic. It is reported to have an effect on both the solubility and bioactivity of chitosan, for example, influencing its antimicrobial mode of action and antioxidant capacity (Bumgardner et al., 2017; Hosseinnejad & Jafari, 2016; Vinsova & Vavrikova, 2011; Xing et al., 2005). The molecular weight of crustacean chitin can vary between 50 to 2000 kDa (Raafat & Sahl, 2009; Varun et al., 2017). In contrast, insect derived chitosan has been reported to be consistently smaller than that derived from crustaceans, ranging from 2.72 to $5.9x10^3$ kDa (Table 2). Accordingly, it is an important to determine the molecular weight of chitosan as new sources are evaluated. Chitosan from the black soldier fly, blowfly larvae (*Chrysomya megacephala*), house fly larvae (*Musca domestica*), and silkworm chrysalis were found to have some of the largest molecular weights reported at 15, 501, 425, and $5.9x10^3$ kDa, respectively. Kim et al. (2017) reported a molecular weight of 308 kDa for the African field

cricket chitosan; whereas, Chae et al. (2018) determined a molecular weight distribution of 0.56 to 1.28 kDa for the same cricket species. Pine weevil chitosan had a molecular weight of 7.3 kDa, whereas cicada sloughs, grasshoppers, mealworms, and silkworm chrysalis had chitosan with a smaller molecular weight at approximately 4 kDa (Luo et al., 2019).

Many of the insect chitosan studies available agree that insect chitosan generally has a smaller molecular weight than that of crustacean sources. However, the authors of these studies do not hypothesize why this change in subphylum considerably affects the polymer's molecular weight. Others have reported that the chitin matrix may be different due to biological differences and function of chitin when present in the cuticle of insects, in contrast to chitin in crustaceans' shells (Berezina & Hubert, 2019). In summary, crustaceans generally have strong exoskeletons due to their aquatic environment and being exposed to higher pressure in water; as a result, their exoskeletons organize into a "network of nodules" capable of resisting these higher pressure conditions (Berezina & Hubert, 2019). Since insects have different living environments that are not necessarily under water, in addition to requiring aerodynamism for flying and jumping, their chitin cuticles are arranged differently. Instead, insect chitin is present in the form of fibers within the exoskeleton, which are reported to have lengths at the microscale (Berezina & Hubert, 2019). These intrinsic differences in crustacean and insect chitin physical forms may be the cause for the consistently observed differences of chitosan's molecular weight.

1.4 Cricket chitin and chitosan

An extensive literature search found only two research groups who have published studies in scientific journals, which have studied the conditions of extracting chitin from whole crickets followed by its conversion to chitosan for varying applications. Weon-Sun Shin's research group (Hanyang University, Republic of Korea) has two published studies with Kyo-Sung Chae and Vita Jarolimkova on the field cricket (*Gryllus bimaculatus*) (Chae et al., 2018; Jarolimkova, 2015). Jarolimkova performed optimization studies related to the chitin purification processes and reported a novel decolorization technique that produced nearly colorless chitin; however, they did not disclose the chemical solution or conditions of the decolorization step other than the duration of 5 hours and 40 minutes. Later, Chae published a study where decolorization of chitin with 50% ammonium persulfate solution (APS) was performed for 6 hours and it is assumed this is the novel colorization Jarolimkova was referring to. Although Jarolimkova studied the properties of

chitosan produced with 67% NaOH for 3 hours repeated in triplicate for a total of 9 hours, the author cited low chitosan yields (~0.2%) and as a result chose another deacetylation method which was not disclosed, but ultimately resulted in a DDA% of 68% and referred to Chae et al.'s study's conditions. Based on Chae et al.'s reported deacetylation conditions (Table 2 and 3), Jarolimkova likely used 50% NaOH solutions between 6 to 9 hours to produce chitosan for the study's prepared chitosan films from crickets (Jarolimkova, 2015).

After successful isolation and conversion of chitin to chitosan, Jarolimkova produced cricket chitosan films for active food packaging applications with control shrimp films for comparison, with and without added polyphenols from magnolia berry (*S. chinesis*) which have antimicrobial properties. Cricket chitosan films, plasticized with glycerol, were comparable in properties to the commercially available chitosan films and suggested that crickets may be an adequate alternative to shrimp chitosan. Additionally, the control chitosan cricket films without *S. chinesis* showed antimicrobial properties against *B. cereus*, whereas the commercial control shrimp chitosan films did not. The author describes this is a result of the improved mobility of cricket chitosan, from its smaller molecular weight (between 0.56 to 1.28 kDa) compared to the commercially available crab and shrimp chitosan (50 to 2000 kDa) (Jarolimkova, 2015). The study did not however evaluate the surface properties of films, such as water contact angles or surface free energy, or mechanical and permeability properties of the cricket chitosan films to understand potential changes in functional properties as result of different sources.

In the study published by Chae et al. (2018), the deacetylation processing conditions such as NaOH solution concentrations (50-67%), and reaction time (1-15 hours) were studied for their effect on chitosan's DDA% and are shown in Table 3. It was reported that as the conversion duration, at 50% NaOH, was increased the DDA% value also increased. Additionally, the increase in alkaline concentration also caused an increase in chitosan's DDA% value. These results are in good alignment with other studies which show that as time, temperature, and concentration of NaOH are increased, chitosan's degree of deacetylation also increases (Bumgardner et al., 2017).

Table 3. Effect of time and NaOH concentration on Chitosan's degree of deacetylation

Reaction time (h)	NaOH concentration (%)		
	50	55	67
1	56.47[a]	–	–
2	57.72	–	–
3	57.92	–	–
6	66.94	–	–
9	66.54	75.18	84.98
15	–	77.63	–

[a]Degree of deacetylation (%) value

* (Chae et al., 2018)

After the optimization of the deacetylation of chitin to produce chitosan, Chae et al. evaluated the fabrication of nanoparticles (NPs) from cricket chitosan and compared its particle size, zeta potential, and polydisperse index (PDI) to commercial chitosan nanoparticles produced identically. Cricket chitosan NPs were found to have similar zeta potential to shrimp NPs, however cricket chitosan led to NPs which were smaller in size with more stable size distributions an effect thought to be a result of its lower molecular weight (Chae et al., 2018). Although Chae et al.'s study on cricket chitosan use for nanoparticles is the first of its kind, the authors failed to publish which preparation conditions for cricket chitosan production (detailed in Table 3) were used for the nanoparticle fabrication. Therefore, it is unknown the DDA% of the cricket chitosan utilized and compared to the shrimp chitosan NPs, therefore resulting differences between the NPs could be a difference in DDA% and treatment conditions rather than the species sourced from (Mw).

The third study in the literature published by Ibitoye et al. (2018), studied a different cricket species called the Taiwan giant cricket (*Brachytrupes portentosus*) reared in Malaysia. The study characterized and compared the extracted chitin/chitosan to that of commercially available shrimp chitin/chitosan (Ibitoye et al., 2018). The cricket chitin was purified following the steps described in Table 1, and its deacetylation was performed with 50% NaOH solution at 121°C for 5 hours. The resulting chitosan had a DDA% of 80% and determined to be pure due to low ash contents (<1%) and absent of protein as confirmed by FTIR. Total yield of chitosan was

roughly 2.4-5.8% on a dry weight basis of whole crickets, and the authors attribute this lower yield to the decreased presence of chitin possibly due to the cricket species' smaller wings (Ibitoye et al., 2018). The chitosan was determined to be in alpha form through FTIR and XRD, with a crystallinity index value of 86%.

Although these two studies researched the purification and conversion of cricket chitin into chitosan, there has yet to be any studies published in scientific journals on the species *Gyrllus sigillatus or Acheta domesticus*, which are the two most commonly reared crickets in the United States. In addition, the available cricket chitosan studies have yet to explore the known bioactive properties of chitosan and its polymer film properties characterization for food packaging.

1.5 Chitosan applications

1.5.1 Anti-obesity

One effective and proven option to treat obesity is through minimizing a person's ability to absorb lipids, as many studies have shown the correlation between obesity and caloric intake which is the highest for lipids (Hu, Tao, Wang, Xiao, & Wang, 2016). Lipids are typically consumed in diets as triglycerides that are later broken by pancreatic juices, of which pancreatic lipase is responsible for hydrolysis into both glycerol and fatty acids to then be absorbed by enterocytes. Therefore, one treatment option for obesity is to prevent this metabolism reaction.

Commercial crustacean chitosan, and its water-soluble derivatives, have been researched for their anti-obesity or hyperlipidemic properties with *in vitro* and *in vivo* studies (Chiu, Chan, Yang, Liu, & Chiang, 2015; Do et al., 2018; Egan, Sweeney, Hayes, & O'Doherty, 2015; Han, Kimura, & Okuda, 1999; Huang et al., 2015; Pan et al., 2018; Si, Strappe, Blanchard, & Zhou, 2017; Sumiyoshi & Kimura, 2006; Walsh, Sweeney, Bahar, & O'Doherty, 2013; Xia, Liu, Zhang, & Chen, 2011). Chitosan serves as a reaction inhibitor due to its ability of physically entrapping and thus reducing substrate access (lipid) to lipase. Specifically, under acidic conditions of the stomach, chitosan's positively charged glucosamine binds to negatively charged triglycerides and fatty acids, which become physically entrapped when chitosan precipitates under duodenum alkaline conditions (Czechowska-Biskup, Rokita, Ulanski, & Rosiak, 2005; Dimzon, Ebert, & Knepper, 2013; Panith, Wichaphon, Lertsiri, & Niamsiri, 2016; Xia et al., 2011; Zhou, Xia, Zhang, & Yu, 2006). To quantity the degree of entrapment of chitosan, or rather its lipid binding

capacity, lipid-chitosan gels or unbound lipids can be gravimetrically measured after *in vitro* digestion (Czechowska-Biskup et al., 2005; Panith et al., 2016; Zhou et al., 2006). Furthermore, these studies report binding capacities between 20-1200g lipid per g chitosan and disagree on the effect of chitosan's molecular weight and DDA have on binding capacities. These results, paired with the inability to locate any previous evaluation of insect chitosan binding capacity, suggests new sources of insect chitosan will require evaluation to determine feasibility for use as a lipid binding agent.

1.5.2 Antimicrobial

Research spanning over the past two decades has revealed a consensus that chitosan, such as that of crustaceans, exhibits antimicrobial activity against many bacteria and fungi through a variety of mechanisms (Hosseinnejad & Jafari, 2016; Moratti & Cabral, 2017; Sahariah & Másson, 2017). Antimicrobial mechanisms of chitosan include but are not limited to the charge difference between chitosan and bacterial cell wall components, penetration and binding of chitosan to microbial DNA/RNA for nucleic acid synthesis prevention, metal ion binding, as well as the formation of an impermeable layer around the microbial cell preventing required cell transportation (Kong, Chen, Xing, & Park, 2010; Moratti & Cabral, 2017). Chitosan especially has rendered a high amount of interest due to its natural derivation and its low human toxicity. To date, studies have shown DDA and molecular weight as important factors in contributing to chitosan's antimicrobial mechanisms (Kong et al., 2010; Sahariah & Másson, 2017).

Chitosan from two different grasshopper species, *Calliptamus barbarous and Oedaleus decorus*, were found to have antimicrobial activity against a variety of microorganisms (Murat Kaya, Baran, et al., 2015). Disk diffusion tests showed chitosan to be the most effective against two Gram-negative organisms *Salmonella enteritidis* and *Vibiro alginolyticus* with similar inhibition zones. The grasshopper derived chitosan was also reported to be more effective against some of the test microorganisms when compared to traditional antibiotics. For example, the zones of inhibition of the grasshopper chitosan (19.4 and 17.5 mm) against *S. enteritidis* was greater than the standard antibiotics Gentamicin (12.1 mm), Amikacin (15.2 mm), Erythromycin (12.3 mm) and Kanamycin (14.9 mm). Interestingly, the study also found that the minimal bactericidal concentrations (MBC), as determined by the micro-dilution method, were affected by the grasshopper species. For example, the MBC of chitosan from *C. barbarus* against *Listeria*

monocytogenes was 0.32 mg/mL, whereas *O. decorus* had a MBC of 0.63 mg/mL (Murat Kaya, Baran, et al., 2015). A similar study on cockroach chitosan found that the species also played a role in activity; the American cockroach (*P. americana*) exhibited greater antimicrobial activity than the German cockroach (*B. germanica*) (Basseri et al., 2019). Another study reported chitosan obtained from both larvae and adult Colorado potato beetle had good antimicrobial properties when eight different organisms were examined through disk diffusion tests. For example, the inhibition zone diameter of beetle chitosan against *S. enteritidis* was 26.03 mm (larvae) and 23.90 mm (adult), which was greater than the commercial antibiotics Gentamicin (13.79 mm) and Erythromycin (20.39 mm) (Murat Kaya et al., 2014). Whereas, the antifungal activity of the larvae and adult beetle chitosan against *Candida albicans* and *Candida glabrata* were moderate (~14.18-15.57 mm) compared to the traditional antifungal agent Fluconazole, which had greater activity against *C. albicans* (21.85 mm) and *C. glabrata* (25.06 mm). Additionally, the larvae and adult beetle chitosan antimicrobial activity against *L. monocytogones* were similar in the disk diffusion tests (~14 mm), whereas the larvae chitosan had much lower MBC values (0.32 mg/mL) than that of the adults (1.25 mg/mL). In another beetle chitosan study, Komariah, Tatara, and Bustami (2017) successfully formed chitosan nanoparticles from the Rhinoceros beetle (*Xylotrupes gideon*). The chitosan nanoparticles were then utilized in a mouthwash formulation with nano-calcium, and were found to decrease the number of bacteria colonies in children's oral cavities.

African field cricket chitosan films, designed for food packaging, were studied and compared to commercial shrimp chitosan films using a disk diffusion test. It was found that the cricket chitosan films inhibited growth of *Bacillus cereus* (280 mm), whereas the commercial shrimp chitosan films did not (Jarolimkova, 2015). The authors report this to be a result of cricket chitosan's low molecular weight, which allowed its diffusion out of the film, whereas the shrimp chitosan that had a higher molecular weight was unable to diffuse. However, the cricket chitosan did not inhibit the growth of either *Escherichia coli* or *L. monocytogenes*, whereas insect chitosan from mealworms and beetles did have an antimicrobial effect towards these two bacteria. Another study evaluated chitosan from the pine weevil in solution against 28 microbial strains and also reported bactericidal effects against *E. coli* or *L. monocytogenes* (M. Kaya et al., 2019).

1.5.3 Food Packaging materials

In addition to the bioactive and antimicrobial properties of chitosan, chitosan has also been determined to have excellent film forming properties. Currently synthetic packaging has excellent mechanical and barrier properties, providing both functionality and convenience to consumers in various industries. However the load of these plastics on the environment is approximately 335 million tons per year (Guillard et al., 2018) and their manufacture relies on fossil fuels.

Extensive research shows chitosan as an alternative polymer that is both biobased and biodegradable, with potential to replace synthetic polymers (Aider, 2010; H. Wang et al., 2018). Chitosan is commonly dissolved in slightly acidic solvents (pH<6.2), normally dilute solutions of acetic acid, due to the protonation and thus dissolution of chitosan into the solvent (Q. Wu, Therriault, & Heuzey, 2018). Additionally many plasticizers such as glycerol, ethylene glycol, poly(ethylene glycol), propylene glycol, sorbitol, sucrose, oleic acid, and hydrated salts have been explored to produce plasticized chitosan films with varying mechanical properties (Arvanitoyannis, Kolokuris, Nakayama, Yamamoto, & Aiba, 1997; Bourtoom, 2008; Butler, Vergano, Testin, Bunn, & Wiles, 1996; Hirase, Higashiyama, Mori, Takahara, & Yamane, 2010; Lavorgna, Piscitelli, Mangiacapra, & Buonocore, 2010; Sabbah et al., 2019; Srinivasa, Ramesh, & Tharanathan, 2007; Suyatma, Tighzert, Copinet, & Coma, 2005; Vlacha et al., 2016). Of these plasticizers, glycerol is considered to be a good plasticizer for chitosan due to the strong hydrogen bonding which occurs between the two materials (Domjan, Bajdik, & Pintye-Hodi, 2009). Following plasticization, the solutions are typically filtered, degassed, and cast onto surfaces to dry and form a film for a variety of applications such as wound scaffolds and food packaging as shown in Figure 4 (Ahmed & Ikram, 2016; Zargar et al., 2015). Commercial chitosan has been used to create films for packaging that serves as a physical barrier to food, as well as blended with other active ingredients such as antimicrobials to create active and biodegradable food packaging with shelf life extension abilities (Ashrafi, Jokar, & Nafchi, 2018; de Moraes Crizel et al., 2018; Murat Kaya et al., 2018; Rai, Dutta, & Mehrotra, 2017; Râpă et al., 2016; Riaz et al., 2018; Shahbazi, 2017; Siripatrawan & Vitchayakitti, 2016; H. Wang et al., 2018; Q. Wang et al., 2015; C. Wu et al., 2016).

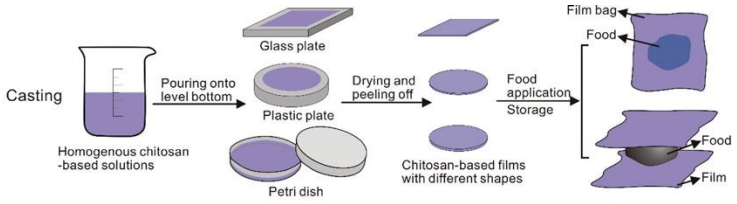

Figure 4. Schematic of chitosan based food packaging and its applications (H. Wang, Qian, & Ding, 2018)

Although there are many film studies available on chitosan derived from shrimp, crab, prawn, and other crustacean sources, currently there is limited literature available on the functionality of chitosan films produced from insects. One study by Jarolimkova (2015) successfully fabricated chitosan films from the African field crickets; however, only the antimicrobial and color properties were investigated with no research performed to determine mechanical and barrier properties for food packaging applications. Another study on pine weevil chitosan produced solution cast films plasticized with glycerol (M. Kaya et al., 2019). The mechanical properties were analyzed, and the films were reported to exhibit both antioxidant and antimicrobial properties. However, the authors did not comment on similarities or differences of these films to crustacean chitosan films. Heterogenous and homogeneous chitosan from black soldier fly larvae were obtained and formed into films through solution casting, with glycerol as a plasticizer (Hahn, Roth, Ji, Schmitt, & Zibek, 2020). The study is the first available on chitosan films derived from black solider fly larvae, however only the thickness of the films was reported with qualitative observations of film color and transparency. No functional properties of the films were quantitatively identified.

Therefore, additional insect chitosan film studies are needed to reasonably assess the prospective of insect chitosan within the packaging industry. Studies of insect chitosan with varying degrees of deacetylation and lower molecular weight, would be beneficial to identify insect chitosan's potential and feasibility for use as biobased packaging materials.

1.6 Objective

The overall objective of this dissertation is to produce cricket chitin and chitosan, from protein production waste streams of two cricket species, with competitive functionality when compared to commercial crustacean chitosan. To achieve this objective, two research studies were

conducted and are presented in this dissertation. In the first study, the chemical structure of purified cricket chitin was determined, and chemical modification was optimized for variable physicochemical properties. Furthermore, the cricket chitosans were evaluated for selected bioactivities, including their lipid binding and antimicrobial activities. In the second study presented in this dissertation, the cricket chitosans were formed into bio-based polymer films with performance determined as it related to food packaging characteristics.

1.7 Hypothesis

The main hypothesis of this dissertation is that cricket chitin-rich byproducts, a result of cricket protein extraction, can be 1) extracted and purified for chitin and 2) converted to chitosan with variable degrees of chemical modification. Furthermore, it is hypothesized cricket chitosan will have similar functional properties to commercial crustacean chitosan, including 1) bioactive properties and 2) performance as a bio-based packaging material, and serve as an alternative for future commercial chitosan sourcing as cricket rearing and consumption increases.

1.8 References

Ahmed, S., & Ikram, S. (2016). Chitosan Based Scaffolds and Their Applications in Wound Healing. *Achievements in the Life Sciences, 10*(1), 27-37. doi:https://doi.org/10.1016/j.als.2016.04.001

Ai, H., Wang, F., Yang, Q., Zhu, F., & Lei, C. (2008). Preparation and biological activities of chitosan from the larvae of housefly, Musca domestica. *Carbohydrate Polymers, 72*(3), 419-423. doi:https://doi.org/10.1016/j.carbpol.2007.09.010

Aider, M. (2010). Chitosan application for active bio-based films production and potential in the food industry: Review. *LWT - Food Science and Technology, 43*(6), 837-842. doi:https://doi.org/10.1016/j.lwt.2010.01.021

Al Sagheer, F., Al-Sughayer, M., Muslim, S., & Elsabee, M. (2009). Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydrate Polymers, 77*(2), 410-419.

Aranaz, I., Mengíbar, M., Harris, R., Paños, I., Miralles, B., Acosta, N., . . . Heras, Á. (2009). Functional characterization of chitin and chitosan. *Current chemical biology, 3*(2), 203-230.

Arvanitoyannis, I., Kolokuris, I., Nakayama, A., Yamamoto, N., & Aiba, S.-i. (1997). Physico-chemical studies of chitosan-poly (vinyl alcohol) blends plasticized with sorbitol and sucrose. *Carbohydrate Polymers, 34*(1-2), 9-19.

Ashrafi, A., Jokar, M., & Nafchi, A. M. (2018). Preparation and characterization of biocomposite film based on chitosan and kombucha tea as active food packaging. *International Journal of Biological Macromolecules, 108*, 444-454. doi:https://doi.org/10.1016/j.ijbiomac.2017.12.028

Baskar, D., & Kumar, T. S. (2009). Effect of deacetylation time on the preparation, properties and swelling behavior of chitosan films. *Carbohydrate Polymers, 78*(4), 767-772.

Basseri, H., Bakhtiyari, R., Hashemi, S. J., Baniardelani, M., Shahraki, H., & Hosainpour, L. (2019). Antibacterial/Antifungal Activity of Extracted Chitosan From American Cockroach (Dictyoptera: Blattidae) and German Cockroach (Blattodea: Blattellidae). *Journal of Medical Entomology, 56*(5), 1208-1214. doi:10.1093/jme/tjz082

Berezina, N., & Hubert, A. (2019). Marketing and Regulations of Chitin and Chitosan from Insects. *Chitin and Chitosan: Properties and Applications*, 477-489.

Berezina, N., Hubert, A., Berro, F., Levon, J.-G., Le Roux, K., Socolsky, C., . . . Laurent, S. (2018a). Chitin, hydrolysate and method for the production of one or more desired products by means of enzymatic hydrolysis, including pre-treatment with an oxidising agent. In: Google Patents.

Berezina, N., Hubert, A., Berro, F., Levon, J.-G., Le Roux, K., Socolsky, C., . . . Laurent, S. (2018b). Chitin, hydrolysate and method for the production of one or more desired products from insects by means of enzymatic hydrolysis. In: Google Patents.

Berezina, N., Hubert, A., Berro, F., Levon, J.-G., Le Roux, K., Socolsky, C., . . . Laurent, S. (2019). Chitin, hydrolysate and production of at least one desired product from insects by means of enzymatic hydrolysis, comprising a combination of steps performed prior to the enzymatic hydrolysis. In: Google Patents.

Bourtoom, T. (2008). Plasticizer effect on the properties of biodegradable blend film from rice starch-chitosan. *Songklanakarin Journal of Science & Technology, 30*.

Bumgardner, J. D., Murali, V. P., Su, H., Jenkins, O. D., Velasquez-Pulgarin, D., Jennings, J. A., . . . Jayakumar, R. (2017). 4 - Characterization of chitosan matters. In J. A. Jennings & J. D. Bumgardner (Eds.), *Chitosan Based Biomaterials Volume 1* (pp. 81-114): Woodhead Publishing.

Butler, B., Vergano, P., Testin, R., Bunn, J., & Wiles, J. (1996). Mechanical and barrier properties of edible chitosan films as affected by composition and storage. *Journal of Food Science, 61*(5), 953-956.

Chae, K. S., Shin, C. S., & Shin, W. S. (2018). Characteristics of cricket (Gryllus bimaculatus) chitosan and chitosan-based nanoparticles. *Food Science and Biotechnology, 27*(3), 631-639. doi:10.1007/s10068-018-0314-4

Chiu, C.-Y., Chan, I.-L., Yang, T.-H., Liu, S.-H., & Chiang, M.-T. (2015). Supplementation of Chitosan Alleviates High-Fat Diet-Enhanced Lipogenesis in Rats via Adenosine Monophosphate (AMP)-Activated Protein Kinase Activation and Inhibition of Lipogenesis-Associated Genes. *Journal of Agricultural and Food Chemistry, 63*(11), 2979-2988. doi:10.1021/acs.jafc.5b00198

Czechowska-Biskup, R., Jarosińska, D., Rokita, B., Ulański, P., & Rosiak, J. M. (2012). Determination of degree of deacetylation of chitosan-comparision of methods. *Progress on Chemistry and Application of Chitin and its Derivatives, 17*, 5-20.

Czechowska-Biskup, R., Rokita, B., Ulanski, P., & Rosiak, J. M. (2005). Radiation-induced and sonochemical degradation of chitosan as a way to increase its fat-binding capacity. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 236*(1-4), 383-390.

de Moraes Crizel, T., de Oliveira Rios, A., Alves, V. D., Bandarra, N., Moldão-Martins, M., & Flôres, S. H. (2018). Active food packaging prepared with chitosan and olive pomace. *Food Hydrocolloids, 74*, 139-150. doi:https://doi.org/10.1016/j.foodhyd.2017.08.007

Dimzon, I. K. D., Ebert, J., & Knepper, T. P. (2013). The interaction of chitosan and olive oil: Effects of degree of deacetylation and degree of polymerization. *Carbohydrate Polymers, 92*(1), 564-570. doi:https://doi.org/10.1016/j.carbpol.2012.09.035

Do, A. R., Cho, S.-J., Cho, Y.-Y., Kwon, E.-Y., Choi, J.-Y., Lee, J. H., . . . Choi, M.-S. (2018). Antiobesity Effects of Short-Chain Chitosan in Diet-Induced Obese Mice. *Journal of Medicinal Food, 21*(9), 927-934. doi:10.1089/jmf.2017.4115

Domjan, A., Bajdik, J., & Pintye-Hodi, K. (2009). Understanding of the plasticizing effects of glycerol and PEG 400 on chitosan films using solid-state NMR spectroscopy. *Macromolecules, 42*(13), 4667-4673. doi:https://doi.org/10.1021/ma8021234

Egan, Á. M., Sweeney, T., Hayes, M., & O'Doherty, J. V. (2015). Prawn Shell Chitosan Has Anti-Obesogenic Properties, Influencing Both Nutrient Digestibility and Microbial Populations in a Pig Model. *PLOS ONE, 10*(12), e0144127. doi:10.1371/journal.pone.0144127

Gmuer, A., Nuessli Guth, J., Hartmann, C., & Siegrist, M. (2016). Effects of the degree of processing of insect ingredients in snacks on expected emotional experiences and willingness to eat. *Food Quality and Preference, 54*, 117-127. doi:https://doi.org/10.1016/j.foodqual.2016.07.003

Greven, H., Kaya, M., Sargin, I., Baran, T., Kristensen, R. M., & Sorensen, M. V. (2019). Characterisation of chitin in the cuticle of a velvet worm (Onychophora). *Turkish Journal of Zoology, 43*(5), 416-424. doi:10.3906/zoo-1903-37

Guillard, V., Gaucel, S., Fornaciari, C., Angellier-Coussy, H., Buche, P., & Gontard, N. (2018). The Next Generation of Sustainable Food Packaging to Preserve Our Environment in a Circular Economy Context. *Frontiers in Nutrition, 5*(121). doi:https://doi.org/10.3389/fnut.2018.00121

Hahn, T., Roth, A., Ji, R., Schmitt, E., & Zibek, S. (2020). Chitosan production with larval exoskeletons derived from the insect protein production. *Journal of Biotechnology, 310*, 62-67. doi:https://doi.org/10.1016/j.jbiotec.2019.12.015

Hajji, S., Younes, I., Ghorbel-Bellaaj, O., Hajji, R., Rinaudo, M., Nasri, M., & Jellouli, K. (2014). Structural differences between chitin and chitosan extracted from three different marine sources. *International Journal of Biological Macromolecules, 65*, 298-306.

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food Chemistry, 262*, 39-47. doi:https://doi.org/10.1016/j.foodchem.2018.04.058

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food Chemistry, 224*, 414-422. doi:https://doi.org/10.1016/j.foodchem.2016.11.138

Han, L. K., Kimura, Y., & Okuda, H. (1999). Reduction in fat storage during chitin-chitosan treatment in mice fed a high-fat diet. *Int J Obes Relat Metab Disord, 23*(2), 174-179. doi:10.1038/sj.ijo.0800806

Hirase, R., Higashiyama, Y., Mori, M., Takahara, Y., & Yamane, C. (2010). Hydrated salts as both solvent and plasticizer for chitosan. *Carbohydrate Polymers, 80*(3), 993-996.

Homann, A. M. (2015). Acceptability of cricket-based biscuits and assessment of gut microbiota composition in schoolchildren. A study in Bondo, Kenya.

Hosseinnejad, M., & Jafari, S. M. (2016). Evaluation of different factors affecting antimicrobial properties of chitosan. *International Journal of Biological Macromolecules, 85*, 467-475.

Hu, X., Tao, N., Wang, X., Xiao, J., & Wang, M. (2016). Marine-derived bioactive compounds with anti-obesity effect: A review. *Journal of Functional Foods, 21*, 372-387. doi:https://doi.org/10.1016/j.jff.2015.12.006

Huang, L., Chen, J., Cao, P., Pan, H., Ding, C., Xiao, T., . . . Su, Z. (2015). Anti-obese effect of glucosamine and chitosan oligosaccharide in high-fat diet-induced obese rats. *Marine drugs, 13*(5), 2732-2756. doi:10.3390/md13052732

Ibitoye, E. B., Lokman, I. H., Hezmee, M. N. M., Goh, Y. M., Zuki, A. B. Z., & Jimoh, A. A. (2018). Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. *Biomed Mater, 13*(2), 025009. doi:10.1088/1748-605X/aa9dde

Jarolimkova, V. (2015). *Preparation and characterization of antimicrobial packaging films from cricket chitosan enriched with schisandra chinensis extract*.

Jayanegara, A., Sholikin, M. M., Sabila, D. A., Suharti, S., & Astuti, D. A. (2017). Lowering chitin content of cricket (Gryllus assimilis) through exoskeleton removal and chemical extraction and its utilization as a ruminant feed in vitro. *Pak. J. Biol. Sci, 20*, 523-529.

Kaya, M., Akyuz, B., Bulut, E., Sargin, I., Eroglu, F., & Tan, G. (2016). Chitosan nanofiber production from Drosophila by electrospinning. *International Journal of Biological Macromolecules, 92*, 49-55.

Kaya, M., & Baran, T. (2015). Description of a new surface morphology for chitin extracted from wings of cockroach (Periplaneta americana). *International Journal of Biological Macromolecules, 75*, 7-12. doi:https://doi.org/10.1016/j.ijbiomac.2015.01.015

Kaya, M., Baran, T., Asan-Ozusaglam, M., Cakmak, Y. S., Tozak, K. O., Mol, A., . . . Sezen, G. (2015). Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnology and bioprocess engineering, 20*(1), 168-179.

Kaya, M., Baran, T., Erdoğan, S., Menteş, A., Aşan Özüsağlam, M., & Çakmak, Y. S. (2014). Physicochemical comparison of chitin and chitosan obtained from larvae and adult Colorado potato beetle (Leptinotarsa decemlineata). *Materials Science and Engineering: C, 45*, 72-81. doi:https://doi.org/10.1016/j.msec.2014.09.004

Kaya, M., Khadem, S., Cakmak, Y. S., Mujtaba, M., Ilk, S., Akyuz, L., . . . Deligöz, E. (2018). Antioxidative and antimicrobial edible chitosan films blended with stem, leaf and seed extracts of Pistacia terebinthus for active food packaging. *RSC Advances, 8*(8), 3941-3950. doi:https://doi.org/10.1039/C7RA12070B

Kaya, M., Lelešius, E., Nagrockaitė, R., Sargin, I., Arslan, G., Mol, A., . . . Bitim, B. (2015). Differentiations of Chitin Content and Surface Morphologies of Chitins Extracted from Male and Female Grasshopper Species. *PLOS ONE, 10*(1), e0115531. doi:10.1371/journal.pone.0115531

Kaya, M., Sargin, I., Mulercikas, P., Labidi, J., Salaberria, A. M., Cakmak, Y. S., . . . Baublys, V. (2019). Conversion of Waste Parasitic Insect (Hylobius abietis L.) into Antioxidative, Antimicrobial and Biodegradable Films. *Journal of Renewable Materials, 7*(3), 215-226. doi:10.32604/jrm.2019.00002

Kaya, M., Sargin, I., Sabeckis, I., Noreikaite, D., Erdonmez, D., Salaberria, A. M., . . . Tubelytė, V. (2017). Biological, mechanical, optical and physicochemical properties of natural chitin films obtained from the dorsal pronotum and the wing of cockroach. *Carbohydrate Polymers, 163*, 162-169. doi:https://doi.org/10.1016/j.carbpol.2017.01.022

Kaya, M., Sofi, K., Sargin, I., & Mujtaba, M. (2016). Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of Vespa crabro (wasp). *Carbohydrate Polymers, 145*, 64-70. doi:https://doi.org/10.1016/j.carbpol.2016.03.010

Khayrova, A., Lopatin, S., & Varlamov, V. (2019). Black Soldier Fly Hermetia illucens as a Novel Source of Chitin and Chitosan. *International Journal of Sciences, 8*(04), 81-86.

Kim, M. W., Han, Y. S., Jo, Y. H., Choi, M. H., Kang, S. H., Kim, S. A., & Jung, W. J. (2016). Extraction of chitin and chitosan from housefly, Musca domestica, pupa shells. *Entomological research, 46*(5), 324-328.

Kim, M. W., Song, Y. S., Han, Y. S., Jo, Y. H., Choi, M. H., Park, Y. K., . . . Jung, W. J. (2017). Production of chitin and chitosan from the exoskeleton of adult two-spotted field crickets (Gryllus bimaculatus). *Entomological research, 47*(5), 279-285.

Komariah, A., Tatara, R. A., & Bustami, D. A. (2017). Efficacy of Rhinoceros Beetle (Xylotrupes Gideon) Nano Chitosan and Calcium Mouthwash in Reducing Quantity Oral Cavity Bacteria among Elementary School Age Children. *International Journal of Advanced Biological and Biomedical Research (IJABBR), 5*, 41-47.

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology, 144*(1), 51-63. doi:https://doi.org/10.1016/j.ijfoodmicro.2010.09.012

Kurita, K. (2006). Chitin and Chitosan: Functional Biopolymers from Marine Crustaceans. *Marine Biotechnology, 8*(3), 203. doi:10.1007/s10126-005-0097-5

Lavorgna, M., Piscitelli, F., Mangiacapra, P., & Buonocore, G. G. (2010). Study of the combined effect of both clay and glycerol plasticizer on the properties of chitosan films. *Carbohydrate Polymers, 82*(2), 291-298.

Liu, S. F., Sun, J., Yu, L. N., Zhang, C. S., Bi, J., Zhu, F., . . . Yang, Q. L. (2012). Extraction and Characterization of Chitin from the Beetle Holotrichia parallela Motschulsky. *Molecules, 17*(4), 4604-4611. doi:10.3390/molecules17044604

Luna, G. C. (2019). *CRICKET (ACHETA DOMESTICUS) PROTEIN HYDROLYSATES: FUNCTIONAL PROPERTIES AND APPLICATION IN A FOOD MATRIX*. (Unpublished. Master's thesis). Purdue University,

Luo, Q., Wang, Y., Han, Q., Ji, L., Zhang, H., Fei, Z., & Wang, Y. (2019). Comparison of the physicochemical, rheological, and morphologic properties of chitosan from four insects. *Carbohydrate Polymers, 209*, 266-275. doi:https://doi.org/10.1016/j.carbpol.2019.01.030

Ma, J., Xin, C., & Tan, C. (2015). Preparation, physicochemical and pharmaceutical characterization of chitosan from Catharsius molossus residue. *International Journal of Biological Macromolecules, 80*, 547-556. doi:https://doi.org/10.1016/j.ijbiomac.2015.07.027

Majtán, J., Bíliková, K., Markovič, O., Gróf, J., Kogan, G., & Šimúth, J. (2007). Isolation and characterization of chitin from bumblebee (Bombus terrestris). *International Journal of Biological Macromolecules, 40*(3), 237-241. doi:https://doi.org/10.1016/j.ijbiomac.2006.07.010

Marei, N. H., El-Samie, E. A., Salah, T., Saad, G. R., & Elwahy, A. H. (2016). Isolation and characterization of chitosan from different local insects in Egypt. *International Journal of Biological Macromolecules, 82*, 871-877.

Merzendorfer, H., & Zimoch, L. (2003). Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *J Exp Biol, 206*(Pt 24), 4393-4412. doi:10.1242/jeb.00709

Moratti, S. C., & Cabral, J. D. (2017). 2 - Antibacterial properties of chitosan. In J. A. Jennings & J. D. Bumgardner (Eds.), *Chitosan Based Biomaterials Volume 1* (pp. 31-44): Woodhead Publishing.

No, H. K., & Meyers, S. P. (1995). Preparation and characterization of chitin and chitosan—a review. *Journal of aquatic food product technology, 4*(2), 27-52.

Nongonierma, A. B., Lamoureux, C., & FitzGerald, R. J. (2018). Generation of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides during the enzymatic hydrolysis of tropical banded cricket (Gryllodes sigillatus) proteins. *Food & function, 9*(1), 407-416.

Odegard, I. Y. R., & van der Voet, E. (2014). The future of food — Scenarios and the effect on natural resource use in agriculture in 2050. *Ecological Economics, 97*, 51-59. doi:https://doi.org/10.1016/j.ecolecon.2013.10.005

Pan, H., Fu, C., Huang, L., Jiang, Y., Deng, X., Guo, J., & Su, Z. (2018). Anti-Obesity Effect of Chitosan Oligosaccharide Capsules (COSCs) in Obese Rats by Ameliorating Leptin Resistance and Adipogenesis. *Marine drugs, 16*(6). doi:10.3390/md16060198

Panith, N., Wichaphon, J., Lertsiri, S., & Niamsiri, N. (2016). Effect of physical and physicochemical characteristics of chitosan on fat-binding capacities under in vitro gastrointestinal conditions. *LWT - Food Science and Technology, 71*, 25-32. doi:https://doi.org/10.1016/j.lwt.2016.03.013

Paoletti, M. G., Norberto, L., Damini, R., & Musumeci, S. (2007). Human gastric juice contains chitinase that can degrade chitin. *Ann Nutr Metab, 51*(3), 244-251. doi:10.1159/000104144

Paulino, A. T., Simionato, J. I., Garcia, J. C., & Nozaki, J. (2006). Characterization of chitosan and chitin produced from silkworm crysalides. *Carbohydrate Polymers, 64*(1), 98-103.

Percot, A., Viton, C., & Domard, A. (2003). Optimization of Chitin Extraction from Shrimp Shells. *Biomacromolecules, 4*(1), 12-18. doi:10.1021/bm025602k

Purkayastha, D., & Sarkar, S. (2020). Physicochemical Structure Analysis of Chitin Extracted from Pupa Exuviae and Dead Imago of Wild Black Soldier Fly (Hermetia illucens). *Journal of Polymers and the Environment, 28*(2), 445-457.

Raafat, D., & Sahl, H. G. (2009). Chitosan and its antimicrobial potential–a critical literature survey. *Microbial biotechnology, 2*(2), 186-201.

Rai, S., Dutta, P., & Mehrotra, G. (2017). Lignin incorporated antimicrobial chitosan film for food packaging application. *Journal of Polymer Materials, 34*(1), 171.

Râpă, M., Miteluţ, A. C., Tănase, E. E., Grosu, E., Popescu, P., Popa, M. E., . . . Vasile, C. (2016). Influence of chitosan on mechanical, thermal, barrier and antimicrobial properties of PLA-biocomposites for food packaging. *Composites Part B: Engineering, 102*, 112-121. doi:https://doi.org/10.1016/j.compositesb.2016.07.016

Riaz, A., Lei, S., Akhtar, H. M. S., Wan, P., Chen, D., Jabbar, S., . . . Zeng, X. (2018). Preparation and characterization of chitosan-based antimicrobial active food packaging film incorporated with apple peel polyphenols. *International Journal of Biological Macromolecules, 114*, 547-555. doi:https://doi.org/10.1016/j.ijbiomac.2018.03.126

Sabbah, M., Di Pierro, P., Cammarota, M., Dell'Olmo, E., Arciello, A., & Porta, R. (2019). Development and properties of new chitosan-based films plasticized with spermidine and/or glycerol. *Food Hydrocolloids, 87*, 245-252.

Sahariah, P., & Másson, M. (2017). Antimicrobial Chitosan and Chitosan Derivatives: A Review of the Structure–Activity Relationship. *Biomacromolecules, 18*(11), 3846-3868. doi:10.1021/acs.biomac.7b01058

Sajomsang, W., & Gonil, P. (2010). Preparation and characterization of α-chitin from cicada sloughs. *Materials Science and Engineering: C, 30*(3), 357-363. doi:https://doi.org/10.1016/j.msec.2009.11.014

Santos, C., Seabra, P., Veleirinho, B., Delgadillo, I., & Lopes da Silva, J. A. (2006). Acetylation and molecular mass effects on barrier and mechanical properties of shortfin squid chitosan membranes. *European Polymer Journal, 42*(12), 3277-3285. doi:https://doi.org/10.1016/j.eurpolymj.2006.09.001

Shahbazi, Y. (2017). The properties of chitosan and gelatin films incorporated with ethanolic red grape seed extract and Ziziphora clinopodioides essential oil as biodegradable materials for active food packaging. *International Journal of Biological Macromolecules, 99*, 746-753. doi:https://doi.org/10.1016/j.ijbiomac.2017.03.065

Shepherd, R., Reader, S., & Falshaw, A. (1997). Chitosan functional properties. *Glycoconjugate journal, 14*(4), 535-542.

Shin, C.-S., Kim, D.-Y., & Shin, W.-S. (2019). Characterization of chitosan extracted from Mealworm Beetle (Tenebrio molitor, Zophobas morio) and Rhinoceros Beetle (Allomyrina dichotoma) and their antibacterial activities. *International Journal of Biological Macromolecules, 125*, 72-77. doi:https://doi.org/10.1016/j.ijbiomac.2018.11.242

Si, X., Strappe, P., Blanchard, C., & Zhou, Z. (2017). Enhanced anti-obesity effects of complex of resistant starch and chitosan in high fat diet fed rats. *Carbohydrate Polymers, 157*, 834-841. doi:https://doi.org/10.1016/j.carbpol.2016.10.042

Siripatrawan, U., & Vitchayakitti, W. (2016). Improving functional properties of chitosan films as active food packaging by incorporating with propolis. *Food Hydrocolloids, 61*, 695-702. doi:https://doi.org/10.1016/j.foodhyd.2016.06.001

Sivashankari, P. R., & Prabaharan, M. (2017). 5 - Deacetylation modification techniques of chitin and chitosan. In J. A. Jennings & J. D. Bumgardner (Eds.), *Chitosan Based Biomaterials Volume 1* (pp. 117-133): Woodhead Publishing.

Song, C., Yu, H., Zhang, M., Yang, Y., & Zhang, G. (2013). Physicochemical properties and antioxidant activity of chitosan from the blowfly Chrysomya megacephala larvae. *International Journal of Biological Macromolecules, 60*, 347-354. doi:https://doi.org/10.1016/j.ijbiomac.2013.05.039

Song, Y. S., Kim, M. W., Moon, C., Seo, D. J., Han, Y. S., Jo, Y. H., . . . Kim, Y. W. (2018). Extraction of chitin and chitosan from larval exuvium and whole body of edible mealworm, Tenebrio molitor. *Entomological research, 48*(3), 227-233.

Soon, C. Y., Tee, Y. B., Tan, C. H., Rosnita, A. T., & Khalina, A. (2018). Extraction and physicochemical characterization of chitin and chitosan from Zophobas morio larvae in varying sodium hydroxide concentration. *International Journal of Biological Macromolecules, 108*, 135-142.

Srinivasa, P. C., Ramesh, M. N., & Tharanathan, R. N. (2007). Effect of plasticizers and fatty acids on mechanical and permeability characteristics of chitosan films. *Food Hydrocolloids, 21*(7), 1113-1122. doi:https://doi.org/10.1016/j.foodhyd.2006.08.005

Stull, V. J., Finer, E., Bergmans, R. S., Febvre, H. P., Longhurst, C., Manter, D. K., . . . Weir, T. L. (2018). Impact of Edible Cricket Consumption on Gut Microbiota in Healthy Adults, a Double-blind, Randomized Crossover Trial. *Sci Rep, 8*(1), 10762. doi:10.1038/s41598-018-29032-2

Sumiyoshi, M., & Kimura, Y. (2006). Low molecular weight chitosan inhibits obesity induced by feeding a high-fat diet long-term in mice. *Journal of Pharmacy and Pharmacology, 58*(2), 201-207. doi:10.1211/jpp.58.2.0007

Suyatma, N. E., Tighzert, L., Copinet, A., & Coma, V. (2005). Effects of Hydrophilic Plasticizers on Mechanical, Thermal, and Surface Properties of Chitosan Films. *Journal of Agricultural and Food Chemistry, 53*(10), 3950-3957. doi:https://doi.org/10.1021/jf048790+

Varun, T. K., Senani, S., Jayapal, N., Chikkerur, J., Roy, S., Tekulapally, V. B., . . . Kumar, N. (2017). Extraction of chitosan and its oligomers from shrimp shell waste, their characterization and antimicrobial effect. *Veterinary world, 10*(2), 170-175. doi:10.14202/vetworld.2017.170-175

Vinsova, J., & Vavrikova, E. (2011). Chitosan derivatives with antimicrobial, antitumour and antioxidant activities-a review. *Current pharmaceutical design, 17*(32), 3596-3607.

Vlacha, M., Giannakas, A., Katapodis, P., Stamatis, H., Ladavos, A., & Barkoula, N.-M. (2016). On the efficiency of oleic acid as plasticizer of chitosan/clay nanocomposites and its role on thermo-mechanical, barrier and antimicrobial properties–Comparison with glycerol. *Food Hydrocolloids, 57*, 10-19.

Walsh, A. M., Sweeney, T., Bahar, B., & O'Doherty, J. V. (2013). Multi-functional roles of chitosan as a potential protective agent against obesity. *PLOS ONE, 8*(1), e53828.

Wang, H., Qian, J., & Ding, F. (2018). Emerging Chitosan-Based Films for Food Packaging Applications. *Journal of Agricultural and Food Chemistry, 66*(2), 395-413. doi:https://doi.org/10.1021/acs.jafc.7b04528

Wang, Q., Tian, F., Feng, Z., Fan, X., Pan, Z., & Zhou, J. (2015). Antioxidant activity and physicochemical properties of chitosan films incorporated with Lycium barbarum fruit extract for active food packaging. *International Journal of Food Science & Technology, 50*(2), 458-464. doi:https://doi.org/10.1111/ijfs.12623

Wu, C., Tian, J., Li, S., Wu, T., Hu, Y., Chen, S., . . . Ye, X. (2016). Structural properties of films and rheology of film-forming solutions of chitosan gallate for food packaging. *Carbohydrate Polymers, 146*, 10-19. doi:https://doi.org/10.1016/j.carbpol.2016.03.027

Wu, Q., Therriault, D., & Heuzey, M.-C. (2018). Processing and properties of chitosan Inks for 3D printing of hydrogel microstructures. *ACS Biomaterials Science & Engineering, 4*(7), 2643-2652.

Xia, W., Liu, P., Zhang, J., & Chen, J. (2011). Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloids, 25*(2), 170-179. doi:https://doi.org/10.1016/j.foodhyd.2010.03.003

Xing, R., Liu, S., Guo, Z., Yu, H., Wang, P., Li, C., . . . Li, P. (2005). Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities in vitro. *Bioorganic & Medicinal Chemistry, 13*(5), 1573-1577. doi:https://doi.org/10.1016/j.bmc.2004.12.022

Yen, M.-T., Yang, J.-H., & Mau, J.-L. (2008). Antioxidant properties of chitosan from crab shells. *Carbohydrate Polymers, 74*(4), 840-844. doi:https://doi.org/10.1016/j.carbpol.2008.05.003

Yuan, Y., Chesnutt, B. M., Haggard, W. O., & Bumgardner, J. D. (2011). Deacetylation of chitosan: material characterization and in vitro evaluation via albumin adsorption and pre-osteoblastic cell cultures. *Materials, 4*(8), 1399-1416.

Zargar, V., Asghari, M., & Dashti, A. (2015). A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications. *Chembioeng Reviews, 2*(3), 204-226. doi:10.1002/cben.201400025

Zelencova, L., Erdoğan, S., Baran, T., & Kaya, M. (2015). Chitin extraction and chitosan production from Chilopoda (Scolopendra cingulata) with identification of physicochemical properties. *Polymer Science Series A, 57*(4), 437-444. doi:10.1134/S0965545X15040161

Zhang, A.-J., Qin, Q.-L., Zhang, H., WANg, H.-T., Li, X., Miao, L., & Wu, Y.-J. (2011). Preparation and characterisation of food-grade chitosan from housefly larvae.

Zhang, M., Haga, A., Sekiguchi, H., & Hirano, S. (2000). Structure of insect chitin isolated from beetle larva cuticle and silkworm (Bombyx mori) pupa exuvia. *International Journal of Biological Macromolecules, 27*(1), 99-105. doi:https://doi.org/10.1016/S0141-8130(99)00123-3

Zhou, K., Xia, W., Zhang, C., & Yu, L. (2006). In vitro binding of bile acids and triglycerides by selected chitosan preparations and their physico-chemical properties. *LWT - Food Science and Technology, 39*(10), 1087-1092. doi:https://doi.org/10.1016/j.lwt.2005.07.009

Zielińska, E., Baraniak, B., & Karaś, M. (2017). Antioxidant and anti-inflammatory activities of hydrolysates and peptide fractions obtained by enzymatic hydrolysis of selected heat-treated edible insects. *Nutrients, 9*(9), 970.

Zielińska, E., Baraniak, B., Karaś, M., Rybczyńska, K., & Jakubczyk, A. (2015). Selected species of edible insects as a source of nutrient composition. *Food Research International, 77,* 460-466. doi:https://doi.org/10.1016/j.foodres.2015.09.008

Zielińska, E., Karaś, M., & Jakubczyk, A. (2017). Antioxidant activity of predigested protein obtained from a range of farmed edible insects. *International Journal of Food Science & Technology, 52*(2), 306-312.

CHAPTER 2. PHYSICOCHEMICAL PROPERTIES OF CHITOSAN FROM TWO COMMONLY REARED EDIBLE CRICKET SPECIES, AND ITS APPLICATION AS A HYPOLIPIDEMIC AND ANTIMICROBIAL AGENT

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2.1 Abstract

Insect-derived chitin and chitosan have gained interest as alternative sources to that derived from crustaceans; however, little information is available on chitin from the house cricket *(Acheta domesticus*) and tropical banded cricket (*Gryllodes sigillatus*), two cricket species commonly reared in the United States for human consumption. In this study, chitin was successfully isolated and purified from these two cricket species; using FTIR, chitins were found to be in alpha-crystalline form. Cricket chitosan was produced from both species with varying degrees of deacetylation (DDA) by varying alkaline conversion duration. *G. sigillatus* chitosan was larger (524 kDa) than *A. domesticus* chitosan (344 kDa). Both cricket chitosans showed similar ($p > 0.05$) lipid-binding capacity to that of shrimp chitosan. Both chitosans were as effective at inhibiting microbial growth of surrogate foodborne pathogens as the commercial shrimp chitosan. At a concentration of 0.50 mg/mL cricket chitosan, approximately 100% of *Listeria innocua* growth was inhibited, due to a contribution of both chitosan and the solvent-acetic acid. At the same concentration, growth of *Escherichia coli* was inhibited 90% by both cricket chitosan samples with ~80% DDA, where a decrease in the DDA led to decreased antimicrobial activity. However, varying the DDA had no effect on chitosan's lipid-binding capacity. As more edible insects become a normalized protein source in our diet, the use of by-products, such as chitin and chitosan, derived from insect protein processing, show promising applications for the pharmaceutical and food industries.

2.2 Introduction

Chitin is a polysaccharide consisting of *N*-acetyl-D-glucosamine units that form a polymer through covalent β-1,4-linkages; it is commonly converted to its counterpart chitosan through replacement of its acetyl group with an amino group (Sivashankari & Prabaharan, 2017). Chitin is naturally occurring in the exoskeletons of arthropods from the largest animal phylum Arthropoda, which accounts for 80% of the species in the animal kingdom. Crustacean (crab and shrimp) shells, a by-product of the food industry, are mainly used as commercial sources of chitin and chitosan (Hamed, Özogul, & Regenstein, 2016; Kumar, 2000; No & Meyers, 1995; Zargar, Asghari, & Dashti, 2015).

Another subphylum of Arthropoda is the Hexapoda, which contains the class Insecta with over 1 million species. To date, research is available on chitin obtained from bumblebees, grasshoppers, crickets, hornets, wasps, centipedes, velvet worms and other species of cockroaches and beetles, among others (Chae, Shin, & Shin, 2018; Greven et al., 2019; Ibitoye et al., 2018; Murat Kaya et al., 2014; Murat Kaya, Lelešius, et al., 2015; Murat Kaya et al., 2017; Murat Kaya, Sofi, Sargin, & Mujtaba, 2016; Majtán et al., 2007; Zelencova, Erdoğan, Baran, & Kaya, 2015). In this context, edible insects have gained recent attention as emerging protein sources to help alleviate the demand of food in a growing world population (Liceaga, 2019). In Europe, Canada and the United States, interest on edible insects has surged due to consumers' willingness to eat more sustainable and environmentally friendly proteins, driving insect-focused food product development (Liceaga, 2019). For example, protein bars, baked goods, and pasta products made with cricket flour (ground whole crickets) have launched in the western market. In addition to the consumption and product development of cricket-based products, technology has been developed for enzymatic hydrolysis of cricket protein (Berezina et al., 2018a, 2018b, 2019; F. Hall, Johnson, & Liceaga, 2018; F. Hall & Liceaga, 2019; F. G. Hall, Jones, O'Haire, & Liceaga, 2017; Liceaga, 2019). These cricket protein hydrolysates, provide concentrated protein powders rich in essential amino acids that can be incorporated into low or poor quality protein foods to enhance the overall protein content/quality. Most importantly, a large by-product of this manufacturing technology is the chitin-rich exoskeletons of the crickets (Liceaga, 2019). The giant cricket (*Brachytrupes portentosus*) and the African field cricket (*Gryllus bimaculatus*) have been used successfully as sources of chitin and chitosan (Chae et al., 2018; Ibitoye et al., 2018; Kim et al., 2017). However, there is currently no information on chitin isolated from edible cricket species commonly farmed

in the United States, the house cricket *(Acheta domesticus*) and tropical banded cricket (*Gryllodes sigillatus*); as a result, little is known on the physicochemical properties of chitin derived from these two species.

Evidence suggests that crustacean (shellfish) chitosan may be an alternative treatment for obesity due to its impressive lipid binding capacity (Jin, Yu, Wang, Li, & Li, 2017), among other modes of action (Xia, Liu, Zhang, & Chen, 2011). In addition, numerous studies are available detailing shellfish chitosan's antimicrobial activity, with potential applications in the food industry. Research suggests chitosan's physicochemical properties, such as degree of deacetylation (DDA) and molecular weight, have significant effects on its bioactivity (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Nevertheless, a research gap remains on the characteristics and bioactivity of edible insect chitin and chitosan. The aim of this study is to investigate chitin derived from two edible cricket species commercially reared in the United States, and its conversion into chitosan with focus on its physicochemical properties. In addition to its characterization, this research will also explore the bioactive properties of cricket chitosan related to its entrapment of lipids under *in vitro* digestion and its ability to inhibit the growth of two bacteria important to the food industry.

2.3 Materials and Methods

2.3.1 Materials

All materials and chemical reagents were purchased from Fisher Scientific (Waltham, MA, USA) and Sigma Aldrich (St. Louis, MO, USA), unless specified. The two cricket species, *Acheta domesticus* (house cricket) and *Gryllodes sigillatus* (tropical banded cricket), were obtained from two edible cricket rearing facilities, Ovipost, Inc. (Labelle, FL, USA) and Three Cricketeers, LLC (St. Louis Park, MN, USA), respectively. Each cricket species was shipped frozen and stored in a −20 °C freezer until needed. Commercial chitosan sourced from shrimp shells and Alcalase® (protease from *Bacillus licheniformis*) was purchased from Sigma Aldrich. *Escherichia coli* ATCC 25922 and *Listeria innocua* ATCC 33090 were obtained from the American Type Culture Collection (Manassas, VA, USA).

2.3.2 Cricket Chitin Extraction

Chitin rich pellets were obtained through enzymatic treatment of each cricket species *Acheta domesticus* and *Gryllodes sigillatus* using the procedure previously described by Hall et al. (2018) and Luna, Martin-Gonzalez, Mauer, and Liceaga (2021), which was shown to most effectively separate protein from the chitin matrix. Briefly, frozen crickets (100 g) and 250 mL of water were homogenized for 2 min in a commercial blender (Waring Commercial, CT), followed by pasteurization in a 90°C water bath to inactive endogenous enzymes. The slurry's pH was adjusted to 8 with 6 M NaOH solution and equilibrated 55°C to produce optimum conditions for the enzyme. Protein hydrolysis was performed with 3% (w/w) Alcalase®for 80 min and ended by pasteurizing slurries at 90°C for 15 min. The slurries were then centrifuged at 17,636 × g for 15 min at 4°C (Avanti J-26S Centrifuge, Beckman-Coulter INC., CA) to separate the chitin-rich pellet from the protein supernatant. Chitin pellets were stored at 5°C prior to purification

2.3.3 Chitin Demineralization and Deproteinization

Cricket chitin pellets were demineralized in 0.25 mol L^{-1} HCl (1:2 w/v) in a 85 °C shaking water bath for 15 minutes following procedures previously established (Elshaarawy, Mustafa, Herbst, Farag, & Janiak, 2016; Percot, Viton, & Domard, 2003). Chitin was filtered (100 mesh) and washed with distilled water to neutrality. Demineralized cricket chitin was deproteinized with an alkali treatment of 1 mol L^{-1} NaOH (1:2 w/v) at 70 °C in a water bath for 22 hours as previously described (Chae et al., 2018; Ibitoye et al., 2018; Percot et al., 2003). The purified chitin samples were filtered (100 mesh), washed again to neutrality, lyophilized for 4 days, ground in a blender (Waring Commercial, CT), and stored in a -20 °C freezer until further use.

The efficiency of the purification process was determined through moisture (AOAC 950.46b), ash (AOAC 920.153), and total N(%) (AOAC methods 984.13 (A-D)) analysis. The % protein content remaining in the chitin was calculated using Equation 1 (Sajomsang & Gonil, 2010):

$$Protein\ (\%)\ = (N\ (\%) - 6.9\%)x\ 6.25 \qquad (1)$$

where N(%) is the chitin's total nitrogen content determined via composition analysis, 6.9% represents the nitrogen content of pure and fully acetylated chitin, and 6.25 is the average nitrogen content in proteins. All proximate composition analyses are reported as percentages on a dry weight basis.

Chitin yield was determined on a dry weight basis (dwb) for each cricket species using Equation 2:

$$Chitin\ content\ (\%) = \frac{weight\ of\ chitin\ (g)}{weight\ of\ whole\ crickets\ (g)} x\ 100 \qquad (2)$$

2.3.4 Chitosan Conversion and Characterization by Fourier Transform Infrared Spectroscopy (FTIR) and Degree of Deacetylation

Purified, lyophilized cricket chitin was converted to chitosan with 67% w/v NaOH (1:20 w/v) for 2 ,4, 6, and 10 hours to vary the degree of deacetylation (Chae et al., 2018). Due to employing the traditional conversion method for chitosan with concentrated alkali solution, chitosan was first filtered (100 mesh) with water until effluent ran clear. Chitosan was then washed with a minimum of 5 L of water, until effluent reached neutrality (pH 6.5), to reduce the presence of residual NaOH. Finally, chitosan was collected, lyophilized, and stored at -20 °C until needed. Chitosan conversion yield (dwb) for each duration treatment was determined using Equation 3:

$$Chitosan\ yield\ (\%) = \frac{mass\ chitosan\ (g)}{mass\ chitin\ (g)} x\ 100 \qquad (3)$$

The eight cricket chitosan samples were evaluated by FT-IR (ThermoScientific) from 3500 to 800 cm^{-1} with a resolution of 8 cm^{-1} (n=8) to determine the chemical structure and degree of deacetylation (DDA) (Chae et al., 2018; Ibitoye et al., 2018; Sivashankari & Prabaharan, 2017). Intensity of FTIR peaks with appropriate baselines at 1650 cm^{-1} (A_{1652}) and 3350 cm^{-1} (A_{3450}) were used to determine DDA (Equation 4) (Chae et al., 2018; Czechowska-Biskup, Jarosińska, Rokita, Ulański, & Rosiak, 2012; Domard & Rinaudo, 1983).

$$DDA\ (\%) = 100 - \left(\frac{A_{1650}}{A_{3350}}\ x\ \frac{100}{1.33}\right) \qquad (4)$$

Additionally, commercial shrimp chitosan spectra were used as a reference. Cricket chitosan samples from each species, which were determined to have similar DDA values (~72%, 76%, and 80%), were used in the lipid binding capacity and antibacterial activity experiments.

2.3.5 Molecular weight determination

The average molecular weight of cricket chitosan was determined viscometrically following the method of Czechowska-Biskup, Wach, Rosiak, and Ulański (2018) and Roberts and Domszy (1982). In brief, solutions (1.5-4.5 mg/mL) were prepared from ~80% DDA cricket chitosan (10 hr samples) in a solvent system consisting of equal parts of 0.1 mol L^{-1} CH_3COOH and 0.2 mol L^{-1} NaCl, and stirred overnight. The flow time of each chitosan solution and solvent system (5 mL) was measured in triplicate using a capillary viscometer (Cannon-Fenske, Size 75) in a 25 °C water bath. Relative viscosity, specific viscosity, and reduced viscosity were calculated using measured flow times (Chen & Tsaih, 1998). The linear relationship between reduced viscosity and concentration for each cricket chitosan was extrapolated to determine its intrinsic viscosity ($[\eta]$). Finally, using the averaged intrinsic viscosity (n=3), the viscosity average molecular weight (M_v) for each chitosan was calculated using the Mark-Houwink Equation:

$$[\eta] = KM_v^{\alpha} \qquad (5)$$

with previously determined solvent system constants: K =1.81x10^{-3} α =0.93 (Murat Kaya et al., 2014; Roberts & Domszy, 1982).

2.3.6 Anti-Obesity Effects (Lipid Binding Capacity) of Cricket Chitosan

The *in vitro* lipid binding capacity of shrimp and cricket (72, 76, and 80% DDA) chitosan samples was determined in triplicate following established procedures (Panith, Wichaphon, Lertsiri, & Niamsiri, 2016; Zhou, Xia, Zhang, & Yu, 2006). Each chitosan sample (20 mg) was dissolved in 1.25 mL 0.6 mol L^{-1} HCl and incubated for 30 minutes in a 37 °C water bath with constant shaking. Then, 25 g of olive oil was added to each tube, vortexed, and incubated under the same conditions for 2 hours. After incubation, 8 mL of phosphate buffer (pH 7.4) was added and the solution's pH was adjusted to 6.8 with 1 mol L^{-1} NaOH, and incubated again for 30

minutes to mimic duodenal conditions. Finally, tubes were centrifuged at 697 × g for 10 min and the supernatant representing unbound lipids was gravimetrically measured. The lipid binding capacity of chitosan was calculated using Equation 6:

$$lipid\ binding\ capacity\ (\frac{g\ oil}{g\ chitosan}) = \frac{(25\ g\ oil - g\ unbound\ oil)}{0.02\ g\ chitosan} \qquad (6)$$

2.3.7 Determination of Antimicrobial Activity

The antimicrobial activity of cricket and shrimp chitosan samples against two bacteria strains was determined using methods described by Aguilar-Toalá, Deering, and Liceaga (2020) with slight modifications (Murat Kaya, Baran, et al., 2015). Specifically, antibacterial activity against *Escherichia coli* ATCC 25922 and *Listeria innocua* ATCC 33090, as surrogates for foodborne pathogens *Escherichia coli* O157:H7 and *Listeria monocytogenes*, were studied (Hu & Gurtler, 2017).

2.3.8 Bacterial stock cultures and sample preparation

The lyophilized bacteria were inoculated in Brain Heart Infusion (BHI) and Mueller Hinton Broth (MHB) for *L. innocua* and *E. coli*, respectively, following ATCC guidelines. Inoculums were used to prepare freezer stocks with a final glycerol concentration of 20% (w/v), cryovials were stored at -80 °C until needed. Prior to use, 50 μL of each stock was transferred into 5 mL MHB and BHI, for *E. coli* and *L. innocua*, grown to stationary phase. As determined by plate counts, *E. coli* inoculums were 1.1 x 10^8 cfu/mL and *L. innocua* inoculums were 7.2 x 10^8 cfu/mL. Fresh cultures were prepared from freezer stocks following the same incubation procedure and used immediately for each assay.

Shrimp and cricket chitosan (72, 76 and 80% DDA) solutions (8 mg/mL) were prepared in 1% (v/v) acetic acid, and stirred overnight to ensure homogenous dissolution. Solutions were then sterilized by autoclaving for 20 min at 121 °C. Additionally, 1% acetic acid was sterilized under the same conditions.

2.3.9 Antibacterial determination

The antimicrobial activity of cricket chitosan samples was determined using 96-well sterile microplates. Chitosan samples, positive and negative control wells were prepared aseptically with a final volume of 220 µL (n=6). For chitosan wells, 13.75 µL of each chitosan sample was added with 204.25 µL BHI, resulting in a final chitosan sample concentration of 0.5 mg/mL in each well. Positive control and negative control wells were prepared with 220 µL and 218 µL BHI, respectively. Chitosan sample and negative control wells were inoculated with 2 µL of bacterial culture (~10^6 cfu/mL) (n=6). Finally, an additional solvent control was prepared to deconvolute its effects from chitosan on bacterial growth. Similar to the chitosan wells, 13.75 µL of 1% CH_3COOH was combined with 204.25 µL BHI, and inoculated (2 µL) (n=6).

Following preparation, microplates were incubated for 24 hours at 37°C with optical density measurements (OD= 620 nm) conducted at 0, 6, 12, and 24 hours. Growth curves of samples were created by plotting optical density over the duration of the experiment. Additionally, the percent of bacterial growth inhibition at 24 hours was calculated (n=6) using Equation 7:

$$\%\ inhibition = 1 - \frac{\Delta OD_{sample,24\ hr}}{OD_{control,24\ hr}} \times 100 \qquad (7)$$

2.3.10 Statistical Analysis

All experiments and analyses were conducted in triplicate, unless otherwise indicated. Statistical analysis of chitosan samples' degree of deacetylation, chitosan yield, lipid binding capacities, and percent of bacterial inhibition were performed using a one way ANOVA with 95% confidence level (Minitab 18® State College, PA, USA).

2.4 Results and Discussion

2.4.1 Cricket Chitin and Chitosan Processing

Cricket chitin and chitosan were successfully extracted, purified, and converted with similarities to the commercial products. Overall, the extraction and purification processing steps

produced cricket chitin with low protein and ash compositions, in comparison to their original compositions (Table 4).

The demineralization process is an important first step to remove the minerals present in the exoskeleton matrix. Traditionally, crustacean shells are demineralized with hydrochloric acid solutions, to react with the calcium carbonate intrinsically present at high concentrations (30-50%) (Kurita, 2006; Percot et al., 2003; Zargar et al., 2015). In the case of insects, due to their environmental differences, they do not contain a large amount of minerals in their exoskeletons when compared to crustaceans (Berezina & Hubert, 2019). Yet, the majority of insect chitin studies commonly employ the demineralization procedures optimized for crustaceans (Berezina & Hubert, 2019). For example, two studies on grasshoppers (*Decticus verrucivorus*) and cockroach wings (*Blaberus. giganteus*), employed HCl demineralization parameters that were highly concentrated (2 M), hot (75 °C), and prolonged (>2 hours) (Murat Kaya, Lelešius, et al., 2015; Murat Kaya et al., 2017). Demineralization of cicada sloughs, silkworms, beetles, and black soldier flies were performed at 100 °C for 20-30 minutes with 1 mol L^{-1} HCl and resulted in ash values of 3-5% (Purkayastha & Sarkar, 2020; Sajomsang & Gonil, 2010; Zhang, Haga, Sekiguchi, & Hirano, 2000). Whereas silkworms, mealworms, and grasshoppers were treated with the same acid concentration at lower temperatures (25-30 °C) for more than 2 hours, and ash contents were reported to be less than 1% (Luo et al., 2019). In this study, *A. domesticus and G. sigillatus* chitin was demineralized with 0.25 mol L^{-1} HCl for 15 minutes at 85 °C with final chitin ash contents less than 0.09% (Table 4). The results of this study show that cricket chitin, and likely other insect derived chitin, do not need extensive demineralization treatment as those used for crustacean (shellfish) sources.

Table 4. Chitin composition and yield, after proteolysis and purification, with reference to whole crickets

Composition analysis (%)	*A. domesticus*	*G. sigillatus*
Whole crickets, ground		
Protein	67.4 ± 1.5[1]	56.8 ± 0.01[2]
Ash	4.0 ± 0.90[1]	18.1 ± 0.60[2]
Chitin[3]	5.7 ± 0.10	3.4 ± 0.10
Chitin, extracted & purified		
Nitrogen	<6.9	7.1
Protein (calculated)[4]	ND	1.4
Ash	0.09	<0.01

[1]*(Luna et al., 2021)*
[2]*(F. G. Hall et al., 2017)*
[3]*Yield of chitin, extracted and purified, following Equation 2*
[4]*Corrected protein concentration following Equation 1, where protein content in chitin with N%<6.9% (due to residual inorganic materials), is determined to be "none detected" (ND) (Mohan et al., 2020).*

A. domesticus chitin had comparable yields (5.68%) to that extracted in other studies for other insects including other cricket species (5.1%), grasshoppers (5.7%), beetles (5.0%), and wasp pupa (6.2%) (Chae et al., 2018; Murat Kaya et al., 2016; Luo et al., 2019; Marei, El-Samie, Salah, Saad, & Elwahy, 2016). In contrast, the *G. sigillatus* had lower chitin yields (3.38%); however, it was still comparable to chitin extracted from silkworm chrysalis (3.1%), mealworms (2.5%), honeybees (2.5%), and wasp larvae (2.2%) (Murat Kaya et al., 2016; Luo et al., 2019; Marei et al., 2016). This chitin yield difference between the two cricket species is likely a result of the larger body size and exoskeleton of the *A. domesticus* in comparison to *G. sigillatus*. Other insect chitin studies reported higher chitin yields (18-37%); however, these studies used molted exoskeletons or wings, which naturally have higher chitin contents, when compared to the intact insect (Murat Kaya & Baran, 2015; Luo et al., 2019; Sajomsang & Gonil, 2010).

2.4.2 Cricket Chitin and Chitosan Characterization

FTIR analysis of all cricket chitin and chitosan samples showed strong chemical and structural similarities to those from shrimp. Chitin from both cricket species (Figure 5) contained characteristic peaks known to chitin from both crustaceans and other insects (Murat Kaya et al., 2014; Kumirska et al., 2010). The two cricket chitins did not differ from each other. Chitin has three crystalline forms, either alpha, beta, or gamma, although chitin is most commonly present

in its alpha form (Kumirska et al., 2010). The FTIR spectra peak between 1700-1500 cm^{-1}, known as the Amide I band, shows that cricket chitin from both species is in the alpha crystalline form, due to the two peaks observed at ~1660 and ~1630 cm^{-1}. These two peaks represent alpha chitin's intra- and inter- molecular hydrogen bonds forming its antiparallel chain arrangement (Kumirska et al., 2010; Liu et al., 2012). The hydrogen bonds between -C=O (Amide I) and -NH- (Amide II) are responsible for the peak at ~1660 cm^{-1}, while the second peak occurring at ~1630 cm^{-1} is due to hydrogen bonding between the $-CH_2O$ side chain and -C=O (Jang, Kong, Jeong, Lee, & Nah, 2004).

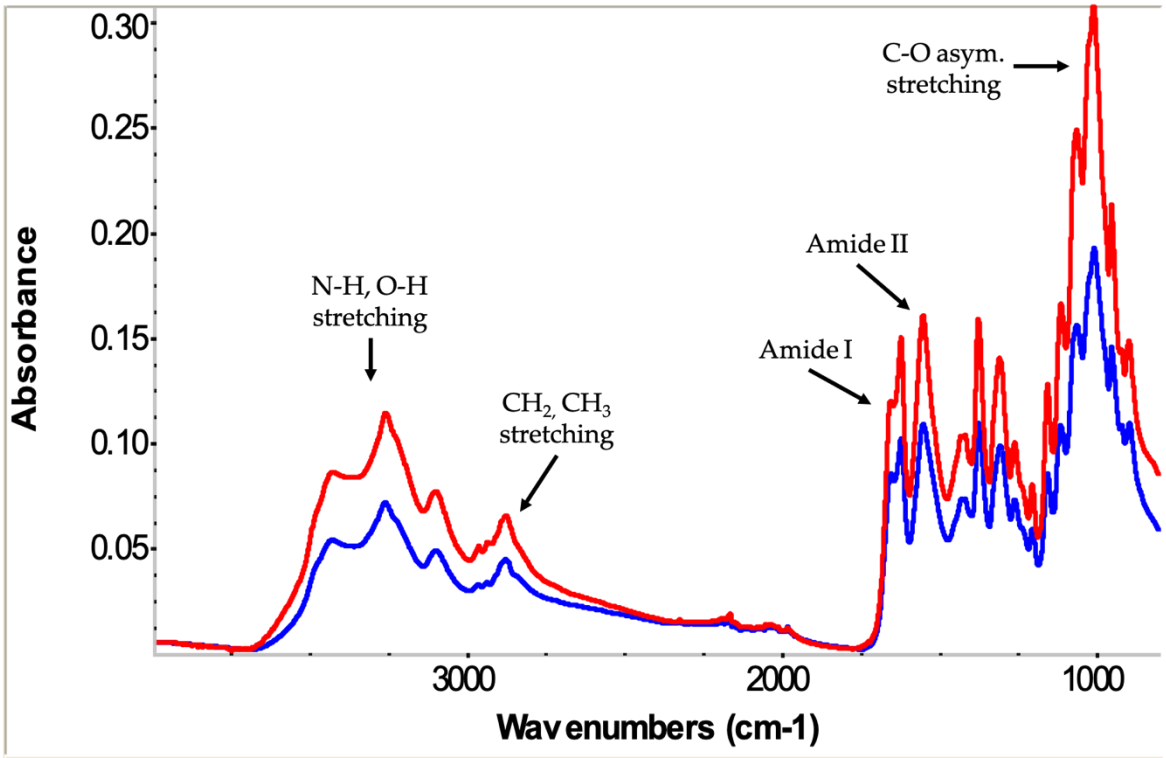

Figure 5. FT-IR spectra of *A. domesticus* (blue) and *G. sigillatus* (red) purified chitin.

Spectral peaks related to chitosan's chemistry have been extensively reported. Following deacetylation of chitin, chitosan samples produced from both cricket species were in good alignment with commercially purchased shrimp chitosan (Figure 6). Two spectral peaks, the OH peak (~3450 cm^{-1}), and the -CO-NH (1650 cm^{-1}) of the Amide I band, were used to calculate the DDA of the cricket and shrimp chitosans (Chae et al., 2018; Czechowska-Biskup et al., 2012; Domard & Rinaudo, 1983) (Table 5). Deacetylating both the *A. domesticus* and *G. sigillatus* chitin

for 2 hours resulted in chitosan with a DDA of ~73%; while the commercial shrimp chitosan was ~70%. Colorado potato beetle (*Leptinotarsa decemlineata*) and larvae chitin processed under similar conditions for 3 hours produced chitosan with 71% and 64% DDA, respectively (Murat Kaya et al., 2014). *A. domesticus* chitin reached 76% DDA after 4 hours, whereas *G. sigillatus* chitin required 6 hours to reach a similar DDA. After 10 hours of deacetylation, cricket chitosan reached a DDA of ~ 80%, similar to that reported by Chae et al. (2018), which produced 84% DDA chitosan from the African field cricket (*Gryllus bimaculatus*) chitin after 9 hours of deacetylation under similar conditions. In Figure 6, as the deacetylation time of cricket chitin increased, acetyl groups were continuously replaced with amine groups leading to a higher DDA (Czechowska-Biskup et al., 2012). Shrimp chitosan had a lower DDA (70%) and therefore a greater peak intensity at ~1650 cm^{-1} compared to the two cricket chitosans deacetylated for 10 hours (~80% DDA).

The chemical deacetylation of cricket chitin to chitosan had similar yields (Table 5) comparable to other insects and crustaceans (Mohan et al., 2020). Approximately 70-80% of the *A. domesticus* chitin mass was recovered after deacetylation, whereas of *G. sigillatus* cricket chitin yielded about 60-65% chitosan. Additionally, deacetylation duration had little effect on the overall conversion yields. *G. sigillatus* chitosan conversion yields were not affected by duration time, and *A. domesticus* chitosan yield was the highest after 6 hours and the lowest after 10 hours. However, the 6 and 10 hour deacetylation had chitosan yields that were not significantly different after 2 and 4 hours of deacetylation.

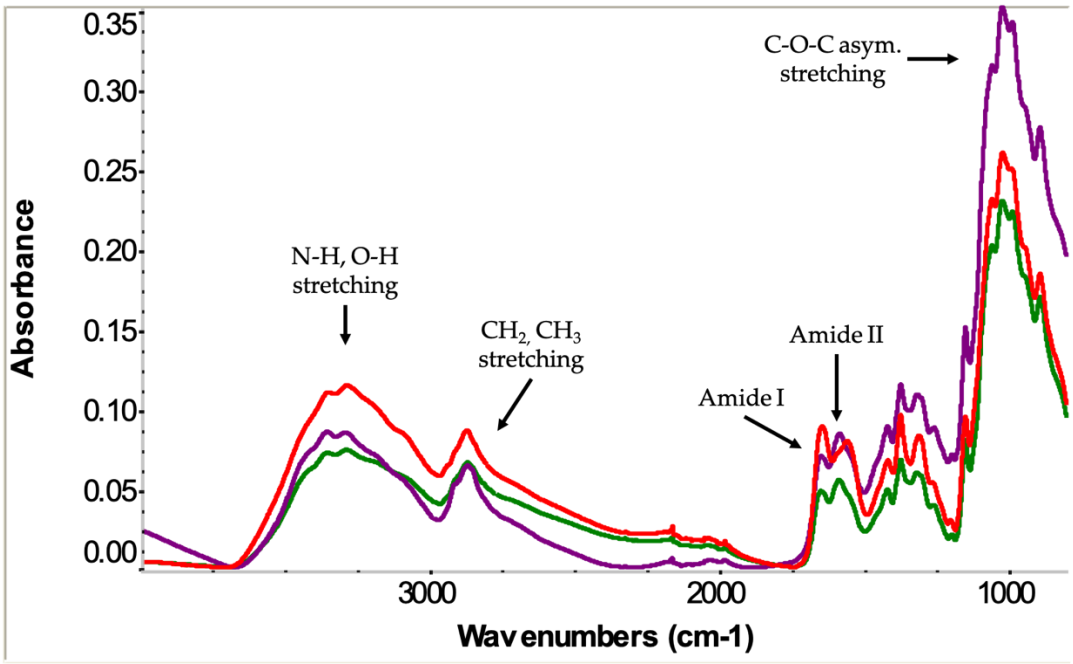

Figure 6. FT-IR Characterization of *A. domesticus* (green), and *G. sigillatus* (purple) chitosan samples after 10 hours of deacetylation, compared to commercial shrimp chitosan (red).

Table 5. Yield and degree of deacetylation (DDA) of cricket chitin converted to chitosan.

Conversion time (hours)	***A. domesticus*[3]**	***G. sigillatus*[3]**
***DDA (%)*[1]**		
2	72.5 ± 1.0[a]	73.5 ± 1.4[a]
4	76.3 ± 1.3[b]	74.9 ± 1.3[a]
6	79.1 ± 1.9[c]	77.2 ± 1.8[b]
10	79.4 ± 1.3[c]	81.3 ± 1.1[c]
***Chitosan yield (%)*[2]**		
2	76.0 ± 6.7[ab]	65.0 ±1.6[a]
4	77.3 ± 1.9 [ab]	63.7 ± 1.2[a]
6	80.5 ± 2.1[b]	60.3 ± 3.3[a]
10	69.0 ± 2.2[a]	62.3 ± 0.9[a]
***Chitosan molecular weight*[4]** -	344 kDa	524 kDa

[1]*DDA (%) for each cricket species, average of triplicate determinations ± standard deviation, was calculated using eq. 4.*

[2]*Chitosan yield (%), average of triplicate determinations ± standard deviation, was calculated using eq. 3.*

[3]*Values that do not share the same letter (a, b, c) within a column, for DDA% or chitosan yield, are statistically different (P<0.05).*

[4]*Intrinsic viscosity, measured in triplicate, was used to calculate molecular weight average using eq. 5.*

2.4.3 Molecular Weight Determination

The average molecular weight was determined to be 344 kDa for *A. domesticus* chitosan, while *G. sigillatus* chitosan had a larger molecular weight of 524 kDa (Table 5). Although the *A. domesticus* cricket is physically larger than *G. sigillatus*, its molecular weight was determined to be smaller. This suggests that *A. domesticus* may have more chitin fibers in its exoskeleton of a smaller weight, while *G. sigillatus cricket's* exoskeleton may have larger, but fewer, chitin fibers in its exoskeleton. The size of chitosan from these two cricket species is in good agreement with another cricket chitosan study. Kim et al. (2017) studied the molecular weight of *G. bimaculatus* crickets using dynamic light scattering and found its molecular weight to be ~308 kDa. As a result, *A. domesticus* and *G. bimaculatus* crickets have similar molecular weights, but are smaller than *G. sigillatus* crickets. Additionally, cricket chitosan is relatively similar in size to other insect chitosan previously studied, such as that sourced from blowfly (501 kDa) and housefly larvae (426 kDa) (Ai, Wang, Yang, Zhu, & Lei, 2008; Song, Yu, Zhang, Yang, & Zhang, 2013). However, compared to other species of insect chitosan, *A. domesticus* and *G. sigillatus* cricket chitosan are much larger in size. Cicada sloughs, grasshoppers, mealworms, silkworm chrysalis, black soldier flies, and beetles ranged in molecular weights between 2.7-15 kDa (Murat Kaya et al., 2014; Khayrova, Lopatin, & Varlamov, 2019; Luo et al., 2019). As a result, insect chitosan has repeatedly been stated to be of low molecular weight compared to crustacean chitin and chitosan, which can range from 50 to 2000 kDa (D. Raafat & H. G. Sahl, 2009; Varun et al., 2017). The results of this study, as well as others, confirm that insect chitosan may also be characterized as high molecular weight.

2.4.4 Lipid-Binding Capacity

Commercial shrimp chitosan, and its water-soluble derivatives, has been reported as an anti-obesity or hyperlipidemic treatment in a variety of *in vitro* and *in vivo* studies (Xia et al., 2011). Chitosan exhibits both hypocholesteremic and hypolipidemic properties through its ability to regulate lipid metabolism, achieved by electrostatic interaction with, or physical entrapment of, targeted molecules (Xia et al., 2011). The focus of this study was to evaluate the lipid binding capacity of chitosan through interaction and entrapment of dietary lipids *in vitro*, one of the reported mechanisms of chitosan to reduce lipid metabolism in a high lipid diet. The consumption

of lipids in the presence of chitosan, is thought to lead to the glucosamine in chitosan to be positively charged in the stomach due to its low pH, followed by its binding to negatively charged lipid molecules such as triglycerides and fatty acids (Czechowska-Biskup, Rokita, Ulanski, & Rosiak, 2005; Dimzon, Ebert, & Knepper, 2013); this emulsion formation has been demonstrated previously with fluorescent microscopy (Panith et al., 2016). When the lipid-chitosan emulsion is transferred from the acidic stomach conditions to the alkaline conditions of the duodenum, the chitosan precipitates and physically entraps the emulsified lipid droplets through gel formation (Czechowska-Biskup et al., 2005; Panith et al., 2016; Zhou et al., 2006). Ultimately, this entrapment prevents lipids to be accessed and digested by the body and results in their excretion through feces.

Table 6. Cricket and shrimp chitosan lipid binding capacity.

	Lipid Binding Capacity (g oil per g chitosan)[1]		
DDA (%)	*A. domesticus*	*G. sigillatus*	*Shrimp*
~ 72	210.8 ± 21.1	163.5 ± 17.8	168.5 ± 36.8
~ 76	221.8 ± 25.4	159.0 ± 15.9	-
~ 80	168.7 ± 10.2	180.5 ± 21.6	-

[1]Average of triplicate determinations ± standard deviation, calculated with Equation 3. DDA= degree of deacetylation. Values were not statistical different (P>0.05).

Chitosan from both cricket species showed high lipid binding capacity, between ~160 to 220 g of oil per gram of chitosan (Table 6). To the best of our knowledge, this is the first study evaluating the lipid binding capacity of chitosan from edible insects. There were no significant differences on lipid binding capacity between the different samples, therefore no correlation between physicochemical properties such as molecular weight or DDA can be made from this study. However, these results suggest that edible cricket chitosan could be as effective as commercial shrimp chitosan in binding lipids under gastric conditions. Panith et al. (2016) showed that chitosan from shrimp shells, produced with a low molecular weight (~30 kDa), had inferior lipid binding capacity compared to medium and high molecular weight chitosan samples (890 kDa and 8900 kDa). In contrast, low molecular weight chitosan (~25 kDa), from an unspecified marine species, had superior binding capacity compared to larger molecular weight chitosan (408 kDa) (Czechowska-Biskup et al., 2005). In this study, the molecular weight of cricket and shrimp chitosans were determined to be high molecular weight. Therefore, it is

possible the molecular weights of the samples of this study were too similar to result in significant changes between their lipid binding capacities. Further research on this topic is required since many insect chitosan studies report low molecular weights (Murat Kaya et al., 2014; M. Kaya et al., 2019; Luo et al., 2019), and it is unknown how cricket chitosans' lipid binding capacities would compare to other insect-based chitosans due to opposing literature conclusions.

Overall, the lipid binding capacities of edible cricket and shrimp chitosan in this study were found to be different than those reported for crustacean chitosan. Panith et al. (2016) showed the highest binding capacity to be at ~28 g oil/g chitosan, which is much lower than the results of this study. These differences are likely due to the different *in vitro* methods applied. For example, our study gravimetrically measured unbound oil, whereas Panith et al. (2016) solvent-extracted entrapped lipids and then gravimetrically measured bound oil. These differences in methods could lead to some over-estimation in our study. Additionally, Panith et al. (2016) centrifuged at 4000 × *g*, whereas in this study, our method called for 696 × *g* to separate bound lipids from unbound lipids. It is possible the larger centrifugal force used in other studies destabilized the chitosan-lipid gel, and physically removed chitosan entrapped lipids; this could result in an under-estimation of lipid binding capacity reported by Panith et al. (2016). In contrast, Zhou et al. (2006) reported a much larger lipid binding capacity value of ~1200 g of oil bound per gram of chitosan. However, the methods for determining the binding capacity did not differ, making it difficult to understand the differences in values reported.

2.4.5 Antimicrobial Activity

The effect of edible cricket chitosan on inhibiting growth of *Listeria innocua* ATCC 33090 and *Escherichia coli* ATCC 25922 over 24 hours is shown in Figure 7. For each bacteria strain, the effect of shrimp chitosan on growth was also evaluated as a reference for current commercial (shrimp) chitosan. Additionally, the solvent used, 1% acetic acid, is an antimicrobial agent itself, and thus its effect on measured antibacterial properties was evaluated (Table 7) (Fraise, Wilkinson, Bradley, Oppenheim, & Moiemen, 2013; Halstead et al., 2015). At a concentration of 0.50 mg/mL, all chitosan samples were able to effectively inhibit growth of *L. innocua* over 24 hours, with ~100% inhibition at hour 24 (Table 7), as shown by no increase in the optical density of the samples during the duration of the experiment (Figure 7). Acetic acid, at a final concentration of 0.06%, resulted in *L. innocua* growth to be inhibited by 38% (Table 7).

Therefore, *L. innocua* inhibition by chitosan samples was due to both the presence of chitosan, as well as acetic acid.

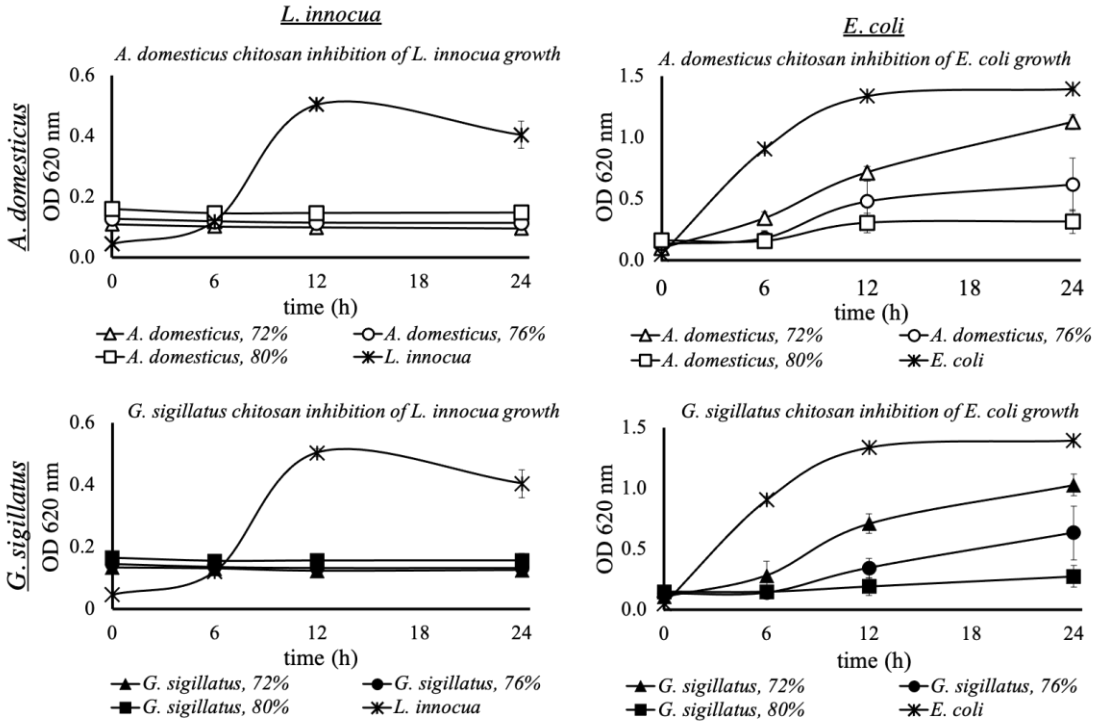

Figure 7. Antimicrobial effect of chitosan from edible *A. domesticus* (empty shapes) and *G. sigillatus* (filled shapes) crickets, with varying degrees of deacetylation including 72% (triangles), 76% (circles), and 80% (squares), on the growth of *Listeria innocua* (left column) and *Escherichia coli* (right column) over 24 hours of incubation (37 °C).

One generally accepted mechanism of chitosan's antibacterial activity is a result of the positive charged $+NH_3$ glucosamines interacting with negatively charged surface components of bacterial cells, which ultimately interrupts or prevents vital cell functions (D. Raafat & H.-G. Sahl, 2009). Therefore, it is thought that an increase of DDA, increases the number of positively charged glucosamines that are able to bind with cell components, leading to an increase in antimicrobial activity. This was seen when the DDA of *G. sigillatus* cricket chitosan increased from 72 to 76 and 80%, which resulted in a sequential increase of growth inhibition of *E. coli*; showing approximately 90% growth inhibition at 80% DDA (Table 7). However, DDA did not have an apparent effect on the inhibition of *L. innocua* growth at the chitosan concentration chosen (Figure 7). Decreasing the chitosan sample concentrations sequentially may allow for

differentiation between chitosan samples with different degrees of deacetylation on the efficacy of inhibiting *L. innocua* growth, while minimizing the contribution due to acetic acid. For both bacteria, growth inhibition achieved by 72% DDA cricket chitosan was similar to that of commercial shrimp chitosan, suggesting that cricket chitosan can be as effective as current market options.

Table 7. Effect of edible cricket chitosan (0.50 mg/mL) with varying degrees of deacetylation on bacteria growth inhibition (%) after 24 hours incubation.

	Bacteria Growth Inhibition (%)[1]			
Inhibitors		Degree of Deacetylation (%)		
		72	76	80
Escherichia coli ATCC 25922				
A. domesticus chitosan	-	26.6 ± 0.7[a]	65.6 ± 2.5[b]	89 ± 1.2[c]
G. sigillatus chitosan	-	33.8 ± 1[a]	64.2 ± 2.7[b]	94.2 ± 0.9[c]
Commercial shrimp chitosan	21.4 ± 2.4	-	-	-
Acetic acid[3]	5.4 ± 0.2	-	-	-
Listeria innocua ATCC 33090				
A. domesticus chitosan	-	100[2]	100	100
G. sigillatus chitosan	-	100	100	100
Commercial shrimp chitosan	100	-	-	-
Acetic acid[3]	37.7 ± 0.7	-	-	-

[1] Inhibition (%) values represent mean of six replicates ± standard error, determined using eq. 7, where different letters (a, b, c) indicate significant difference (P<0.05) between degree of deacetylation for each cricket chitosan (rows).
[2] 100% represents complete inhibition in chitosan samples inoculated with L. innocua, where no change in optical density at 620 nm was observed (Figure 7, left).
[3] Acetic acid (0.06% v/v final concentration) quantifies the contribution of solvent to chitosan's antimicrobial activity.

The results of this study are similar to other insect chitosan antimicrobial assays, however direct comparisons are difficult to make due to differing microorganisms, chitosan concentrations employed, and assaying methods (i.e. MIC, MBC, zone of inhibition, etc.). For example, chitosan derived from larvae and adult Colorado potato beetles were found to have minimal bactericidal concentrations (MBC) of 0.32 and 1.25 mg/mL against *L. monocytogenes*, where MBC values represent the lowest concentration of an antimicrobial agent required to kill bacteria in an inoculated sample. Chitosan derived from two different grasshopper species were found to have

a MBC of 0.32 and 0.63 mg/mL against *L. monocytogenes*. Another study quantified the effect of mealworm (*Tenbrio molitor*) and crustacean derived chitosan on cell counts of foodborne pathogens *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella enterica* serovar Typhimurium, over 48 hours (Ibañez-Peinado, Ubeda-Manzanaro, Martínez, & Rodrigo, 2020). The study found in general that crustacean and insect chitosan antibacterial activity at 1.5 mg/mL led to unchanged or reduced following 24 hours after inoculation for all three bacteria, with recovery of bacterial counts detected between 24 – 48 hours (when inoculated with 10^6 cfu/mL, pH 6.2). At 24 hours, the antibacterial activity of crustacean chitosan was slightly greater for *E. coli* and *S.* Typhimurium, whereas mealworm chitosan was more effective against *L. monocytogenes*. However, the study did not characterize or report the specifications of the chitosan samples, such as molecular weight or % deacetylation, so it is difficult to understand the basis of these differences. Another study evaluated the antimicrobial efficacy of two different grasshopper species against a variety of organisms, including that of *L. monocytogenes*. The MBC of one grasshopper chitosan (*Calliptamus barbarous*) was 0.32 mg/mL, whereas the MBC of chitosan from the other grasshopper species (*Oedaleus decorus*) was 0.63 mg/mL (Murat Kaya, Baran, et al., 2015). The chitosan from the two species were reported to have a DDA between 70-75%, although the specific DDA of each chitosan was not reported. It is possible the differences in MBC are due to slight differences in DDA. As shown in this current study (Figure 7), a 4% change in the cricket chitosan DDA (i.e., from 72 to 76 and 80%) significantly improved *E. coli* inhibition (Table 7). No difference in chitosan antimicrobial efficacy was seen between the two cricket species, likely a result of standardizing DDA prior to antimicrobial testing.

The results of this study in conjunction with others, elucidate the importance to evaluate each newly derived chitosan against specific bacterial strains to determine its anticipated antimicrobial activity. Overall, cricket chitosan derived from *A. domesticus* and *G. sigillatus* were as effective at inhibiting microbial growth of surrogate foodborne pathogens, as the traditional and commercial crustacean (shrimp) chitosan. A concentration of 0.50 mg/mL was an effective concentration for all chitosan samples against *L. innocua*, however higher concentrations of the lower DDA chitosan samples may be required to enhance the antimicrobial effect against *E. coli*. Further studies on the effect of edible cricket chitosan on bacterial cell wall material are required to understand the mechanisms of inhibition by these chitosan samples.

2.5 Conclusions

Chitin from two edible cricket species commonly reared in the United States were successfully collected as a by-product of cricket protein hydrolysis, and adequately isolated through demineralization and deproteinization processing steps. *A. domesticus* crickets were found to yield slightly higher amounts of purified chitin, compared to *G. sigillatus* crickets. FTIR results confirmed that cricket chitin was in its alpha-crystalline form, similar to that isolated from most crustacean and insect species. Cricket chitin was successfully converted to chitosan with approximately 72%, 76%, and 80% degree of deacetylation, achieved by varying deacetylation times using concentrated alkaline treatments. Structural analysis of cricket chitosan samples was chemically similar to that of commercial shrimp chitosan. Finally, the average molecular weight of chitosan derived from *A. domesticus* crickets was determined to be 344 kDa, while that from *G. sigillatus* had a larger molecular weight of 524 kDa. In contrast to the findings of other insect derived chitosan studies, the results of this study suggest that cricket chitosan can be recognized as a high molecular weight polymer, similar to commercially available chitosan with potential bioactive properties.

Lipid binding capacity of all chitosan samples were determined to be between 160 g and 220 g oil per 1 g chitosan, although physicochemical properties did not have any significant effect on lipid binding. Additionally, the edible cricket chitosan samples effectively inhibited *E. coli* and *L. innocua*. The degree of deacetylation did not have an effect on the antibacterial properties of cricket chitosan against *L. innocua*. Whereas, the antimicrobial activity of *A. domesticus* and *G. sigillatus* chitosan was more effective against *E. coli* at higher DDA values, compared to shrimp chitosan. Thus, edible cricket chitosan has the potential to inhibit the growth of foodborne pathogens, as a natural derived antimicrobial agent.

As the consumption of insects and insect-containing food products is rapidly growing, an increase of insect protein production and thereof chitin by-products will become more available. This study concludes that chitosan derived from U.S. reared edible crickets have similar physicochemical and bioactive properties as commercial crustacean (e.g. shrimp) chitosan. As a result, there is potential for the mass production of cricket-based chitosan as the consumer acceptability for arthropods widens outside the traditional source (crustaceans).

2.6 References

Aguilar-Toalá, J. E., Deering, A. J., & Liceaga, A. M. (2020). New Insights into the Antimicrobial Properties of Hydrolysates and Peptide Fractions Derived from Chia Seed (Salvia hispanica L.). *Probiotics and Antimicrobial Proteins, 12*(4), 1571-1581. doi:10.1007/s12602-020-09653-8

Ai, H., Wang, F., Yang, Q., Zhu, F., & Lei, C. (2008). Preparation and biological activities of chitosan from the larvae of housefly, Musca domestica. *Carbohydrate Polymers, 72*(3), 419-423. doi:https://doi.org/10.1016/j.carbpol.2007.09.010

Berezina, N., & Hubert, A. (2019). Marketing and Regulations of Chitin and Chitosan from Insects. *Chitin and Chitosan: Properties and Applications*, 477-489.

Berezina, N., Hubert, A., Berro, F., Levon, J.-G., Le Roux, K., Socolsky, C., . . . Laurent, S. (2018a). Chitin, hydrolysate and method for the production of one or more desired products by means of enzymatic hydrolysis, including pre-treatment with an oxidising agent. In: Google Patents.

Berezina, N., Hubert, A., Berro, F., Levon, J.-G., Le Roux, K., Socolsky, C., . . . Laurent, S. (2018b). Chitin, hydrolysate and method for the production of one or more desired products from insects by means of enzymatic hydrolysis. In: Google Patents.

Berezina, N., Hubert, A., Berro, F., Levon, J.-G., Le Roux, K., Socolsky, C., . . . Laurent, S. (2019). Chitin, hydrolysate and production of at least one desired product from insects by means of enzymatic hydrolysis, comprising a combination of steps performed prior to the enzymatic hydrolysis. In: Google Patents.

Chae, K. S., Shin, C. S., & Shin, W. S. (2018). Characteristics of cricket (Gryllus bimaculatus) chitosan and chitosan-based nanoparticles. *Food Science and Biotechnology, 27*(3), 631-639. doi:10.1007/s10068-018-0314-4

Chen, R. H., & Tsaih, M. L. (1998). Effect of temperature on the intrinsic viscosity and conformation of chitosans in dilute HCl solution. *International Journal of Biological Macromolecules, 23*(2), 135-141. doi:https://doi.org/10.1016/S0141-8130(98)00036-1

Czechowska-Biskup, R., Jarosińska, D., Rokita, B., Ulański, P., & Rosiak, J. M. (2012). Determination of degree of deacetylation of chitosan-comparision of methods. *Progress on Chemistry and Application of Chitin and its Derivatives, 17*, 5-20.

Czechowska-Biskup, R., Rokita, B., Ulanski, P., & Rosiak, J. M. (2005). Radiation-induced and sonochemical degradation of chitosan as a way to increase its fat-binding capacity. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 236*(1-4), 383-390.

Czechowska-Biskup, R., Wach, R. A., Rosiak, J. M., & Ulański, P. (2018). Procedure for determination of the molecular weight of chitosan by viscometry. *Progress on Chemistry and Application of Chitin and its Derivatives, 23*, 45-54.

Dimzon, I. K. D., Ebert, J., & Knepper, T. P. (2013). The interaction of chitosan and olive oil: Effects of degree of deacetylation and degree of polymerization. *Carbohydrate Polymers, 92*(1), 564-570. doi:https://doi.org/10.1016/j.carbpol.2012.09.035

Domard, A., & Rinaudo, M. (1983). Preparation and characterization of fully deacetylated chitosan. *International Journal of Biological Macromolecules, 5*(1), 49-52. doi:https://doi.org/10.1016/0141-8130(83)90078-8

Elshaarawy, R. F. M., Mustafa, F. H. A., Herbst, A., Farag, A. E. M., & Janiak, C. (2016). Surface functionalization of chitosan isolated from shrimp shells, using salicylaldehyde ionic liquids in exploration for novel economic and ecofriendly antibiofoulants. *RSC Advances, 6*(25), 20901-20915. doi:10.1039/C5RA27489C

Fraise, A. P., Wilkinson, M. A. C., Bradley, C. R., Oppenheim, B., & Moiemen, N. (2013). The antibacterial activity and stability of acetic acid. *Journal of Hospital Infection, 84*(4), 329-331. doi:https://doi.org/10.1016/j.jhin.2013.05.001

Greven, H., Kaya, M., Sargin, I., Baran, T., Kristensen, R. M., & Sorensen, M. V. (2019). Characterisation of chitin in the cuticle of a velvet worm (Onychophora). *Turkish Journal of Zoology, 43*(5), 416-424. doi:10.3906/zoo-1903-37

Hall, F., Johnson, P. E., & Liceaga, A. (2018). Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food Chemistry, 262*, 39-47. doi:https://doi.org/10.1016/j.foodchem.2018.04.058

Hall, F., & Liceaga, A. (2019). Effect of microwave-assisted enzymatic hydrolysis of cricket (Gryllodes sigillatus) protein on ACE and DPP-IV inhibition and tropomyosin-IgG binding. *Journal of Functional Foods*, 103634. doi:https://doi.org/10.1016/j.jff.2019.103634

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food Chemistry, 224*, 414-422. doi:https://doi.org/10.1016/j.foodchem.2016.11.138

Halstead, F. D., Rauf, M., Moiemen, N. S., Bamford, A., Wearn, C. M., Fraise, A. P., . . . Webber, M. A. (2015). The Antibacterial Activity of Acetic Acid against Biofilm-Producing Pathogens of Relevance to Burns Patients. *PLOS ONE, 10*(9), e0136190-e0136190. doi:10.1371/journal.pone.0136190

Hamed, I., Özogul, F., & Regenstein, J. M. (2016). Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. *Trends in food science & technology, 48*, 40-50.

Hu, M., & Gurtler, J. B. (2017). Selection of surrogate bacteria for use in food safety challenge studies: a review. *Journal of Food Protection, 80*(9), 1506-1536.

Ibañez-Peinado, D., Ubeda-Manzanaro, M., Martínez, A., & Rodrigo, D. (2020). Antimicrobial effect of insect chitosan on Salmonella Typhimurium, Escherichia coli O157: H7 and Listeria monocytogenes survival. *PLOS ONE, 15*(12), e0244153.

Ibitoye, E. B., Lokman, I. H., Hezmee, M. N. M., Goh, Y. M., Zuki, A. B. Z., & Jimoh, A. A. (2018). Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. *Biomed Mater, 13*(2), 025009. doi:10.1088/1748-605X/aa9dde

Jang, M.-K., Kong, B.-G., Jeong, Y.-I., Lee, C. H., & Nah, J.-W. (2004). Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. *Journal of Polymer Science Part A: Polymer Chemistry, 42*(14), 3423-3432. doi:10.1002/pola.20176

Jin, Q., Yu, H., Wang, X., Li, K., & Li, P. (2017). Effect of the molecular weight of water-soluble chitosan on its fat-/cholesterol-binding capacities and inhibitory activities to pancreatic lipase. *PeerJ, 5*, e3279.

Kaya, M., & Baran, T. (2015). Description of a new surface morphology for chitin extracted from wings of cockroach (Periplaneta americana). *International Journal of Biological Macromolecules, 75*, 7-12. doi:https://doi.org/10.1016/j.ijbiomac.2015.01.015

Kaya, M., Baran, T., Asan-Ozusaglam, M., Cakmak, Y. S., Tozak, K. O., Mol, A., . . . Sezen, G. (2015). Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnology and bioprocess engineering, 20*(1), 168-179.

Kaya, M., Baran, T., Erdoğan, S., Menteş, A., Aşan Özüsağlam, M., & Çakmak, Y. S. (2014). Physicochemical comparison of chitin and chitosan obtained from larvae and adult Colorado potato beetle (Leptinotarsa decemlineata). *Materials Science and Engineering: C, 45*, 72-81. doi:https://doi.org/10.1016/j.msec.2014.09.004

Kaya, M., Lelešius, E., Nagrockaitė, R., Sargin, I., Arslan, G., Mol, A., . . . Bitim, B. (2015). Differentiations of Chitin Content and Surface Morphologies of Chitins Extracted from Male and Female Grasshopper Species. *PLOS ONE, 10*(1), e0115531. doi:10.1371/journal.pone.0115531

Kaya, M., Sargin, I., Mulercikas, P., Labidi, J., Salaberria, A. M., Cakmak, Y. S., . . . Baublys, V. (2019). Conversion of Waste Parasitic Insect (Hylobius abietis L.) into Antioxidative, Antimicrobial and Biodegradable Films. *Journal of Renewable Materials, 7*(3), 215-226. doi:10.32604/jrm.2019.00002

Kaya, M., Sargin, I., Sabeckis, I., Noreikaite, D., Erdonmez, D., Salaberria, A. M., . . . Tubelytė, V. (2017). Biological, mechanical, optical and physicochemical properties of natural chitin films obtained from the dorsal pronotum and the wing of cockroach. *Carbohydrate Polymers, 163*, 162-169. doi:https://doi.org/10.1016/j.carbpol.2017.01.022

Kaya, M., Sofi, K., Sargin, I., & Mujtaba, M. (2016). Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of Vespa crabro (wasp). *Carbohydrate Polymers, 145*, 64-70. doi:https://doi.org/10.1016/j.carbpol.2016.03.010

Khayrova, A., Lopatin, S., & Varlamov, V. (2019). Black Soldier Fly Hermetia illucens as a Novel Source of Chitin and Chitosan. *International Journal of Sciences, 8*(04), 81-86.

Kim, M. W., Song, Y. S., Han, Y. S., Jo, Y. H., Choi, M. H., Park, Y. K., . . . Jung, W. J. (2017). Production of chitin and chitosan from the exoskeleton of adult two-spotted field crickets (Gryllus bimaculatus). *Entomological research, 47*(5), 279-285.

Kumar, M. N. R. (2000). A review of chitin and chitosan applications. *Reactive and functional polymers, 46*(1), 1-27.

Kumirska, J., Czerwicka, M., Kaczyński, Z., Bychowska, A., Brzozowski, K., Thöming, J., & Stepnowski, P. (2010). Application of spectroscopic methods for structural analysis of chitin and chitosan. *Marine drugs, 8*(5), 1567-1636. doi:10.3390/md8051567

Kurita, K. (2006). Chitin and Chitosan: Functional Biopolymers from Marine Crustaceans. *Marine Biotechnology, 8*(3), 203. doi:10.1007/s10126-005-0097-5

Liceaga, A. M. (2019). Approaches for Utilizing Insect Protein for Human Consumption: Effect of Enzymatic Hydrolysis on Protein Quality and Functionality. *Annals of the Entomological Society of America, 112*(6), 529-532.

Liu, S. F., Sun, J., Yu, L. N., Zhang, C. S., Bi, J., Zhu, F., . . . Yang, Q. L. (2012). Extraction and Characterization of Chitin from the Beetle Holotrichia parallela Motschulsky. *Molecules, 17*(4), 4604-4611. doi:10.3390/molecules17044604

Luna, G. C., Martin-Gonzalez, F. S., Mauer, L., & Liceaga, A. (2021). Cricket (Acheta domesticus) protein hydrolysates' impact on the physicochemical, structural and sensory properties of tortillas and tortilla chips. *Journal of Insects as Food and Feed, 7*(1), 109-120. doi:https://doi.org/10.3920/JIFF2020.0010

Luo, Q., Wang, Y., Han, Q., Ji, L., Zhang, H., Fei, Z., & Wang, Y. (2019). Comparison of the physicochemical, rheological, and morphologic properties of chitosan from four insects. *Carbohydrate Polymers, 209*, 266-275. doi:https://doi.org/10.1016/j.carbpol.2019.01.030

Majtán, J., Bíliková, K., Markovič, O., Gróf, J., Kogan, G., & Šimúth, J. (2007). Isolation and characterization of chitin from bumblebee (Bombus terrestris). *International Journal of Biological Macromolecules, 40*(3), 237-241. doi:https://doi.org/10.1016/j.ijbiomac.2006.07.010

Marei, N. H., El-Samie, E. A., Salah, T., Saad, G. R., & Elwahy, A. H. (2016). Isolation and characterization of chitosan from different local insects in Egypt. *International Journal of Biological Macromolecules, 82*, 871-877.

Mohan, K., Ganesan, A. R., Muralisankar, T., Jayakumar, R., Sathishkumar, P., Uthayakumar, V., . . . Revathi, N. (2020). Recent insights into the extraction, characterization, and bioactivities of chitin and chitosan from insects. *Trends in food science & technology, 105*, 17-42. doi:https://doi.org/10.1016/j.tifs.2020.08.016

No, H. K., & Meyers, S. P. (1995). Preparation and Characterization of Chitin and Chitosan—A Review. *Journal of aquatic food product technology, 4*(2), 27-52. doi:10.1300/J030v04n02_03

Panith, N., Wichaphon, J., Lertsiri, S., & Niamsiri, N. (2016). Effect of physical and physicochemical characteristics of chitosan on fat-binding capacities under in vitro gastrointestinal conditions. *LWT - Food Science and Technology, 71*, 25-32. doi:https://doi.org/10.1016/j.lwt.2016.03.013

Percot, A., Viton, C., & Domard, A. (2003). Optimization of Chitin Extraction from Shrimp Shells. *Biomacromolecules, 4*(1), 12-18. doi:10.1021/bm025602k

Purkayastha, D., & Sarkar, S. (2020). Physicochemical Structure Analysis of Chitin Extracted from Pupa Exuviae and Dead Imago of Wild Black Soldier Fly (Hermetia illucens). *Journal of Polymers and the Environment, 28*(2), 445-457.

Raafat, D., & Sahl, H.-G. (2009). Chitosan and its antimicrobial potential--a critical literature survey. *Microbial biotechnology, 2*(2), 186-201. doi:10.1111/j.1751-7915.2008.00080.x

Raafat, D., & Sahl, H. G. (2009). Chitosan and its antimicrobial potential–a critical literature survey. *Microbial biotechnology, 2*(2), 186-201.

Rabea, E. I., Badawy, M. E. T., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as Antimicrobial Agent: Applications and Mode of Action. *Biomacromolecules, 4*(6), 1457-1465. doi:10.1021/bm034130m

Roberts, G. A., & Domszy, J. G. (1982). Determination of the viscometric constants for chitosan. *International Journal of Biological Macromolecules, 4*(6), 374-377.

Sajomsang, W., & Gonil, P. (2010). Preparation and characterization of α-chitin from cicada sloughs. *Materials Science and Engineering: C, 30*(3), 357-363. doi:https://doi.org/10.1016/j.msec.2009.11.014

Sivashankari, P. R., & Prabaharan, M. (2017). 5 - Deacetylation modification techniques of chitin and chitosan. In J. A. Jennings & J. D. Bumgardner (Eds.), *Chitosan Based Biomaterials Volume 1* (pp. 117-133): Woodhead Publishing.

Song, C., Yu, H., Zhang, M., Yang, Y., & Zhang, G. (2013). Physicochemical properties and antioxidant activity of chitosan from the blowfly Chrysomya megacephala larvae. *International Journal of Biological Macromolecules, 60*, 347-354. doi:https://doi.org/10.1016/j.ijbiomac.2013.05.039

Varun, T. K., Senani, S., Jayapal, N., Chikkerur, J., Roy, S., Tekulapally, V. B., . . . Kumar, N. (2017). Extraction of chitosan and its oligomers from shrimp shell waste, their characterization and antimicrobial effect. *Veterinary world, 10*(2), 170-175. doi:10.14202/vetworld.2017.170-175

Xia, W., Liu, P., Zhang, J., & Chen, J. (2011). Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloids, 25*(2), 170-179. doi:https://doi.org/10.1016/j.foodhyd.2010.03.003

Zargar, V., Asghari, M., & Dashti, A. (2015). A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications. *Chembioeng Reviews, 2*(3), 204-226. doi:10.1002/cben.201400025

Zelencova, L., Erdoğan, S., Baran, T., & Kaya, M. (2015). Chitin extraction and chitosan production from Chilopoda (Scolopendra cingulata) with identification of physicochemical properties. *Polymer Science Series A, 57*(4), 437-444. doi:10.1134/S0965545X15040161

Zhang, M., Haga, A., Sekiguchi, H., & Hirano, S. (2000). Structure of insect chitin isolated from beetle larva cuticle and silkworm (Bombyx mori) pupa exuvia. *International Journal of Biological Macromolecules, 27*(1), 99-105. doi:https://doi.org/10.1016/S0141-8130(99)00123-3

Zhou, K., Xia, W., Zhang, C., & Yu, L. (2006). In vitro binding of bile acids and triglycerides by selected chitosan preparations and their physico-chemical properties. *LWT - Food Science and Technology, 39*(10), 1087-1092. doi:https://doi.org/10.1016/j.lwt.2005.07.009

CHAPTER 3. DEVELOPMENT OF CHITOSAN FILMS FROM REARED EDIBLE CRICKETS AND ITS PERFORMANCE AS A BIO-BASED FOOD PACKAGING MATERIAL

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3.1 Abstract

Edible insects have gained attention due to their impressive nutritional composition, as well as their efficient use of natural resources. However, a research gap remains on the applications of insect chitosan, especially as it relates to their potential use as food packaging material. Chitosan from two reared cricket species (*Acheta domesticus* and *Gryllodes sigillatus*) was evaluated for use as food packaging material. Cricket chitosan films (CCF) were structurally similar to commercial shrimp chitosan films (SCF) at controlled glycerol levels, as seen by shared spectral peaks in FT-IR analyses. Mechanical properties of CCF showed they had equal or greater tensile strength when compared to commercial SCF, although flexibility was lower. Scanning electron microscopy showed increased roughness of microstructure, likely increasing the tortuosity. As a result, CCF had improved water vapor permeability compared to commercial SCF. Melanin complexes present in cricket chitin and chitosan increased hydrophobicity and decreased light transmittance. This study also revealed that intrinsic species differences, which occur during insect and crustacean exoskeleton development, could have effects on the functionality of chitosan packaging materials. Overall, CCF were found to be as effective as commercial SCF, while providing additional advantages. CCF derived from reared crickets have good mechanical and barrier properties, and improved water resistance and light barrier characteristics. Edible cricket chitosan has the potential to be used as bio-based packaging material for food and pharmaceutical applications.

3.2 Introduction

Currently used in the food industry, petroleum-based food packaging has excellent mechanical and barrier properties to extend food quality and shelf life while providing safe food and convenience to consumers. However, these non-renewable plastics also significantly contribute to the accumulation of waste. This waste has a detrimental effect on the environment by generating a high amount of waste that is destined for landfills or enters ecological systems. Currently, the load of these petroleum-based polymers on the environment is approximately 335 million tons of plastic per year (Guillard et al., 2018). Therefore, research and production of bio-based packaging materials to replace traditional packaging materials remains a major focus of many studies. Government programs, such as BioPreferred® in the U.S., support and increase the use of bio-based products, including bio-based packaging materials, from renewable sources. Simultaneously, the world is challenged with providing sustainable and resource efficient solutions to reduce food waste and spoilage, which is expected to account for 200 million tons by the year 2050 (Guillard et al., 2018). The ability to utilize process waste streams for the production of biobased and biodegradable packaging materials is also of great interest since it supports both renewable bio-based packaging while simultaneously reducing waste otherwise destined for landfills (Babu, O'Connor, & Seeram, 2013). Scientific literature on chitosan biobased polymers shows extensive research performed on the preparation, characterization, and applications of chitosan-based polymer films over the past three decades. This research supports all of these resolutions for decreased food waste and potential substitution of synthetic food packaging.

Chitosan films have been shown to have excellent performance as food packaging materials allowing for shelf-life extension of foods, including antimicrobial, barrier, and sensing films (H. Wang, Qian, & Ding, 2018). Commercial chitin and chitosan, sourced from crustacean (e.g., shrimp) food-waste streams, have been used to create food packaging that serves as a physical barrier to food, as well as blended with other active ingredients such as antimicrobials to create active and biodegradable food packaging with shelf-life extension abilities (Ashrafi, Jokar, & Nafchi, 2018; de Moraes Crizel et al., 2018; Kaya et al., 2018; Rai, Dutta, & Mehrotra, 2017; Râpă et al., 2016; Riaz et al., 2018; Shahbazi, 2017; Siripatrawan & Vitchayakitti, 2016; H. Wang et al., 2018; Q. Wang et al., 2015; Wu et al., 2016). As consumers are beginning to shift to more sustainable protein sources, such as edible insects, new waste streams are becoming available. Like crustaceans, insects are arthropods and have similar chitin rich exoskeletons. As a result, a focus

of recent chitosan research sourced from insects was recently reviewed (Mohan et al., 2020; Zainol Abidin, Kormin, Zainol Abidin, Mohamed Anuar, & Abu Bakar, 2020). The reviews highlight extraction and purification of insect chitin, and characterize the physicochemical properties of chitin and its modification to chitosan.

Although there is now a foundation of insect chitin and chitosan literature available showing feasibility of alternative sourcing, there is little information on the application and feasibility of insect chitosan for use as food packaging materials. Therefore, this research study focuses on the manufacture of chitosan films sourced from two edible cricket species commonly reared in the United States and their application as food packaging materials. Important properties of food packaging polymers, including mechanical, vapor permeability, optical, light barrier, and hydrophobicity/hydrophilicity properties, were studied on cricket chitosan films (CCF). Shrimp chitosan films (SCF) served as the commercial reference material, to determine if alternative sourcing would improve or worsen the desirable functional properties.

3.3 Materials and Methods

3.3.1 Materials

All materials and chemical reagents were purchased from Fisher Scientific (Waltham, MA, USA) and Sigma Aldrich (St. Louis, MO, USA), unless specified. The two cricket species, Acheta domesticus (house cricket) and Gryllodes sigillatus (tropical banded cricket), were obtained from two edible cricket rearing facilities, Ovipost, Inc. (Labelle, FL, USA) and Three Cricketeers, LLC (St. Louis Park, MN, USA), respectively. Commercial shrimp chitosan (~70% deacetylated) was purchased from Sigma Aldrich (St. Louis, MO, USA).

3.3.2 Methods

3.3.3 Cricket Chitosan

Cricket chitin was extracted from a chitin-rich by-product produced during the enzymatic proteolysis of cricket proteins for food formulation (Hall, Jones, O'Haire, & Liceaga, 2017; Luna, Martin-Gonzalez, Mauer, & Liceaga, 2021). In our previous study, cricket chitin from each species was effectively demineralized and deproteinized with acidic and alkali treatments (M. Malm &

Liceaga, 2021). Furthermore, cricket chitosan was deacetylated with concentrated and hot alkali treatment for varying durations. As previously optimized, cricket chitosans were produced with various DDA values as determined by FTIR, and within each cricket species the DDAs produced were found to be significantly different (M. Malm & Liceaga, 2021). Deacetylation parameters for each cricket species were then chosen to produce a similar range of DDA values: 72, 76, and 80%. For more specific details on the extraction, deacetylation, optimization process, and molecular weight determination of these samples, the reader is referred to M. Malm and Liceaga (2021).

3.3.4 Solution casting films

For each cricket species, *Acheta domesticus* and *Gryllodes sigillatus*, chitosan films were prepared with varying degrees of deacetylation: 72, 76, and 80%. Chitosan solutions (1% *w*/*v*) were prepared by dissolving cricket chitosan in 1% acetic acid (*v*/*v*) solution and stirred for 60 min on a hotplate (100 °C). Evaporation of solutions were minimized by placing a foil lid across the beaker openings, which were then secured with parafilm around the circumference of the beaker. The plasticizing agent, glycerol, was added at 37.5% (*w*/*w* chitosan), covered, and mixed without heat for an additional 10 min. Plasticized solutions were sonicated for 5 min (Model CL- 334, QSONICA Sonicators, Newtown, USA) at 30% amplitude followed by centrifugation (Avanti J-26S, Beckman-Coulter, Brea, USA) for 15 min at 17,636× *g* to degas and remove undissolved particles. For mechanical and water contact analyses, 40 g of supernatant was dispensed in 100 × 15 mm polystyrene dishes and dried for 36 h at 50 °C. For the remaining analyses, 15 g of film solutions were dispensed in 60 × 15 mm polystyrene dishes and dried for 24 h at 50 °C. Afterwards, all films were placed in desiccators until needed. These processes were repeated using commercial shrimp chitosan (~70% deacetylated) and served as the chitosan film reference.

3.3.5 Molecular characterization

The molecular characterization of all chitosan films was analyzed as previously described (M. Malm & Liceaga, 2021) using Fourier Transform Infrared spectroscopy (FT-IR) (Thermo Scientific, Waltham, USA) from 3500 to 800 cm^{-1} with a resolution of 8 cm^{-1} (n = 4). Spectra were obtained via film transmission using ATR with a diamond prism, and automatically baselined and averaged using OMNIC software (ThermoFisher, Waltham, MA, USA).

3.3.6 Scanning electron microscopy (SEM)

Scanning electron microscopy of film surfaces and cross-sections were evaluated for CCF and SCF. For cross-sections, specimens were cryo-fractured prior to analysis. All specimens were sputter coated with platinum for 60 s, and then analyzed (FEI NOVA nanoSEM Field Emission SEM (FEI Company, Hillsboro, USA) with an accelerating voltage of 5 kV and under high vacuum. Film surface images are reported at 500× magnification, and film cross-sections are reported at 2000× and 20,000× magnification.

3.3.7 Water contact angle analysis

The surface hydrophobicity/hydrophilicity of all CCF and SCF was evaluated by water contact angle analysis (Theta Lite Tensiometer, Biolin Scientific, Gothenburg, SE) (M. J. Malm, Narsimhan, & Kokini, 2019). Duplicate film strips, approximately 5 cm2, were secured onto microscope glass slides with double sided tape. Deionized water (2 µL) was manually placed onto each film surface. Contact angles were recorded by the instrument's camera, manually baselined during analysis using the OneAttension software (Biolin Scientific) and averaged (n > 7).

3.3.8 Water vapor permeability

The water vapor permeability of CCF was measured and compared to the SCF using the procedure established by ASTM standard E96, as previously described (Urbizo-Reyes, San Martin-González, Garcia-Bravo, & Liceaga, 2020). Desiccant (CaCl2), previously dried, was placed in the bottom of the permeability cups. Cups' openings were sealed with chitosan films and secured by metal clamps. Initial weight of each cup was immediately documented and placed in a 75% RH chamber (saturated NaCl solution) at 25 °C. Weight measurements were recorded twice a day for ~4 days for each permeability cup, resulting in six measurements per cup. Water vapor transmission rate (WVTR) was calculated by dividing the slope of the linear regression of weight measurements (Δ m/Δ t) by the permeation area of the cup (m^2). Water vapor permeability of films was determined as follows:

$$WVP\ (10^{-10} g/s \cdot m \cdot Pa) = \frac{WVTR \times d}{\Delta p} \qquad (1)$$

Where d represents the thickness of chitosan films (m), and Δp is the partial pressure difference previously determined to be 1753.55 (Pa) (García, Pinotti, Martino, & Zaritzky, 2004; Urbizo-Reyes et al., 2020).

3.3.9 Mechanical properties

Tensile strength and elongation percentage at break measurements were performed on CCF and SCF following ASTM D882 standards with modifications (Kalaycıoğlu, Torlak, Akın-Evingür, Özen, & Erim, 2017; Urbizo-Reyes et al., 2020). Chitosan films were conditioned at least 48 h in 50% RH desiccators (saturated $MgNO_3$ solution) at 25 °C, and then cut into strips (6 cm × 1 cm). Thickness of each strip was determined in triplicate prior to analysis. A texture analyzer (TA.XT, Stable Microsystems, Surrey, UK) equipped with rubber faced tensile grips was calibrated and experiments were performed using a grip separation of 30 mm with a rate of separation of 0.80 mm/s ($n \geq 8$). Analysis of data was performed (Exponent Software), and TS and E% were calculated for each replicate.

3.3.10 Color measurement

Chitosan films were evaluated for color using a Hunterlab ColorFlex (HunterLab, Reston, USA) (n = 4) following the previously described protocol (Urbizo-Reyes et al., 2020). Using CIELAB scale, color measurements were reported as lightness, L^*, and chromaticity parameters a^* and b^*. Lightness values were reported between 0 (black) and 100 (white). Chromaticity parameter a^* indicates green colors when negative, while positive values represent red colors. Finally, positive chromaticity b^* values correspond to blue colors, while negative values correspond to yellow colors. Four films were evaluated for each type of chitosan film, measured against a white tile (L^*= 92.22, a^*= −0.99, *and* b^*= 0.92).

3.3.11 Light barrier properties

To evaluate the light barrier protective properties of chitosan films for food packaging applications, transmittance (%) of each film was scanned from 200 to 800 nm using a spectrophotometer (DU®720, Beckman-Coulter, CA, USA), as previously described (Kalaycıoğlu

et al., 2017). Chitosan films were physically attached to sample holders, where air served as the reference. Scans were repeated on four films for each film formulation.

3.3.12 Opacity

Utilizing the transmittance data obtained from the light barrier property experiments, the opacity of the chitosan films was calculated using the following equation:

$$Opacity\ (\mathrm{mm^{-1}}) = \frac{Absorbance_{600\ \mathrm{nm}}}{thickness\ (\mathrm{mm})} \quad (1)$$

where were $Absorbance_{600\mathrm{nm}}$ = 2- log (T $\%_{600\mathrm{nm}}$) (Souza et al., 2017).

3.3.13 Statistical analysis

All experimental data were replicated at least three times, unless otherwise indicated. Data are reported as average ± standard deviation. Where applicable, statistical analysis of observed differences among means was performed using analysis of variance (ANOVA), followed by Tukey's pairwise comparison of means at a 5% significance level with the statistical software Minitab 18® (State College, PA, USA).

3.4 Results and Discussion

3.4.1 Molecular characterization

Molecular characterization of CCF (Figure 7) aligned well with spectra reported in previous chitosan-glycerol plasticized films characterized by FT-IR (Cerqueira, Souza, Teixeira, & Vicente, 2012; Debandi, Bernal, & Francois, 2016; M. Malm & Liceaga, 2021). Additionally, CCF were not structurally different that SCF, as shown by the absence of spectral differences between all spectra. Peaks between 3500 and 3000 cm^{-1} represent N-H and O-H chemistries of both chitosan and glycerol, as well as the hydrogen bonding between the two functional groups due to plasticization (Chen et al., 2021). Furthermore, increased intensities of aliphatic stretching moieties (2970 cm^{-1} to 2750 cm^{-1}), and C–O, C–O–C asymmetrical, and C-O-C symmetrical groups (1120 to 800 cm^{-1}), are also a result of the glycerol's intrinsic chemistry (Cerqueira et al., 2012). Comparison of pristine chitosan powder, previously published (M. Malm & Liceaga, 2021),

and the corresponding plasticized chitosan films, highlighted the increased intensity of two spectral peaks. Specifically, the broad 3500–3000 cm^{-1} peak mentioned above and the amide II band centered ca. ~1530 cm^{-1}. The increase of these peaks may be associated with the chitosan-glycerol plasticization mechanism. As shown previously by NMR, glycerol's –OH functional group participates in hydrogen bonding with the acetoamide group of chitosan (Domjan, Bajdik, & Pintye-Hodi, 2009). Ultimately, these hydrogen bonds allow plasticization of films and may be the cause for the increased 3500–3000 cm^{-} peak intensity. Additionally, glycerol interrupts intramolecular hydrogen bonds (N–H functional groups) on chitosan's polymer structure (Domjan et al., 2009; Rodríguez-Núñez, Madera-Santana, Sánchez-Machado, López-Cervantes, & Valdez, 2014). Therefore, it is possible the disruption of these intramolecular bonds increases their availability and stretching of N–H functional groups. Ultimately, this could lead to an increase of the amide II band of plasticized chitosan films, as the chemical structure of glycerol does not directly contribute to this peak. Additional future experiments with pristine unplasticized chitosan films would be useful to further deconvolute the roles of solution casting and plasticization on these two functional groups of chitosan.

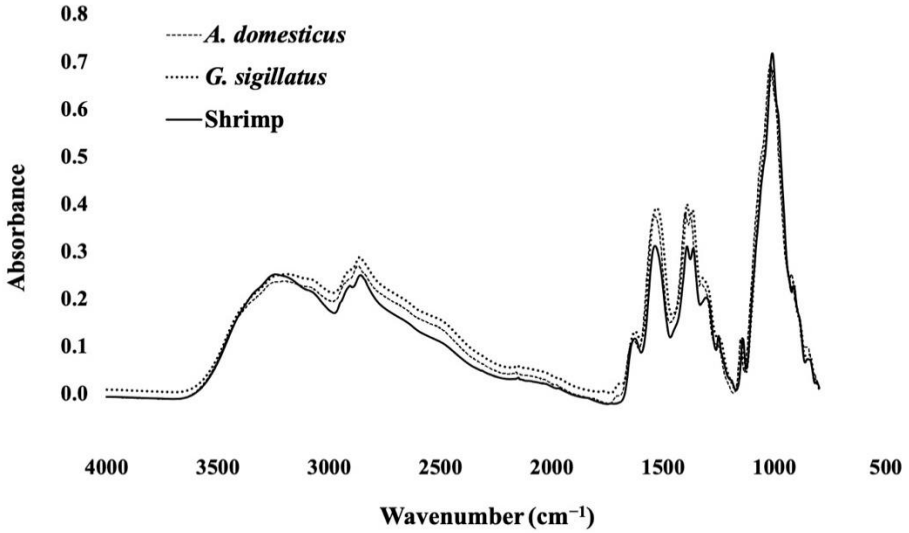

Figure 8. FTIR of ~70% deacetylated chitosan films, derived from *A. domesticus* (dashed lines), *G. sigillatus* (dashed circles) and commercial shrimp (solid line).

Finally, one difference between CCF and SCF may be a result of the intrinsic differences between the molecular conformation of insect and crustacean exoskeletons. In insects, hardening of the exoskeleton is achieved through a process noted as sclerotization. The exact mechanisms of sclerotization of different insects is yet to be discovered; however, research to date generally accepts that insect exoskeletons are formed through chitin and protein matrices that are crosslinked by catechols to stabilize the cuticle (Schaefer et al., 1987). During sclerotization, catechols undergo a tanning process leading to brown and black pigments, complexed with chitin in the exoskeleton structure (Сергей Александрович Лопатин, 2020). As shown visually in the films' optical properties, some pigments are still bound to chitin/chitosan structure. The pigmentation is documented in most insect chitin/chitosan studies; however, pigmentation has not been detectable by FT-IR. For example, in one study, chitosan films derived from yellow mealworms (*Tenebrio molitor*) also showed similar pigmentation as our CCF, but characteristic peaks of such pigments could not be distinguished from the FT-IR spectral peaks of chitosan films plasticized with glycerol (Saenz-Mendoza et al., 2020). The authors attributed these results to overlapping chemistry with chitosan and glycerol, as well as the fact it is present in low quantities.

3.4.2 Film microstructure

Surfaces and cross-sections of CCF imaged using scanning electron microscopy are shown in Figures 9 though 11 and compared to SCF (Figure 12). Overall, images of all CCF surfaces (Figure 9) and cross-sections (Figure 10 and 11) of films showed good homogeneity, were smooth, and free of cracks and pores.

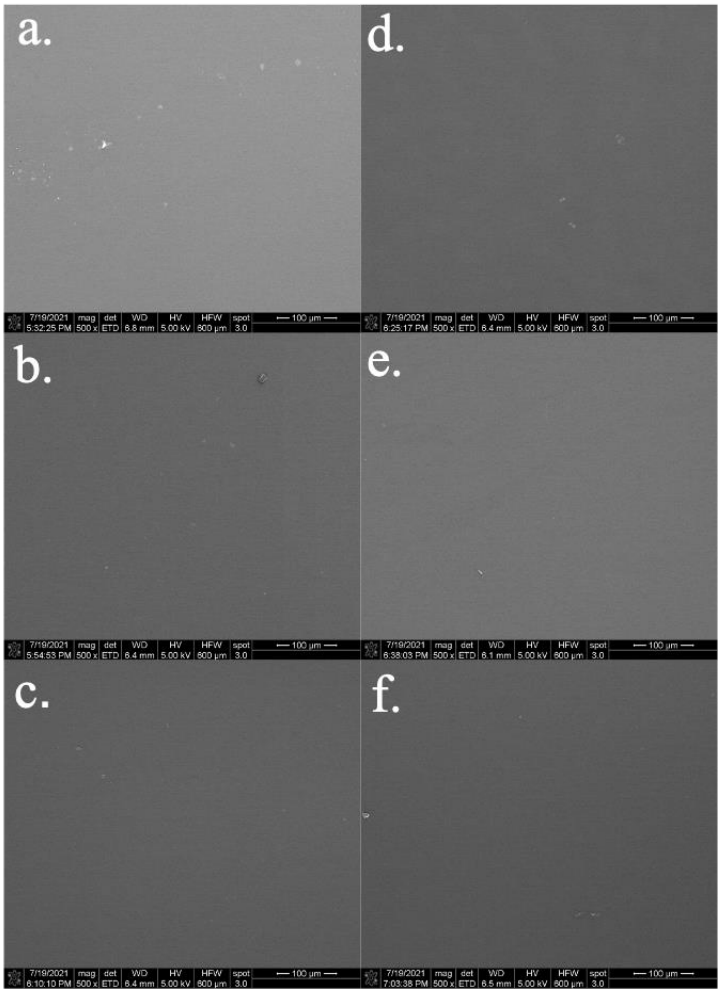

Figure 9. SEM analysis of chitosan film surfaces derived from crickets, *A. domesticus* (a-c) and *G. sigillatus* (d-f), shown at 500x magnification. Chitosan films have varying degrees of deacetylation, including 72% (a and d), 76% (b and e), and 80% (c and f).

These results are in alignment with previously studied chitosan films plasticized with glycerol and sorbitol (Flores, San-Martin, Beldarraín-Iznaga, Leiva-Vega, & Villalobos-Carvajal, 2021; M. Liu, Zhou, Zhang, Yu, & Cao, 2013). Imaging of cross-sections at 20,000x (Figure 11) elucidated differences in the microstructures of CCF compared to the commercial SCF (Figure 12). Generally, CCF had larger chitosan particles, whereas SCF had smaller and smoother-looking particles. The SEM cross-sections of CCF are visually similar to chitosan-gelatin crosslinked films (Z. Liu et al., 2012). The authors described the crosslinked film cross-sections to be more aggregated and irregular compared to the SCF control. The aggregation of the micrographs were

attributed to crosslinks formed between chitosan and gelatin in the film's 3D network (Z. Liu et al., 2012).

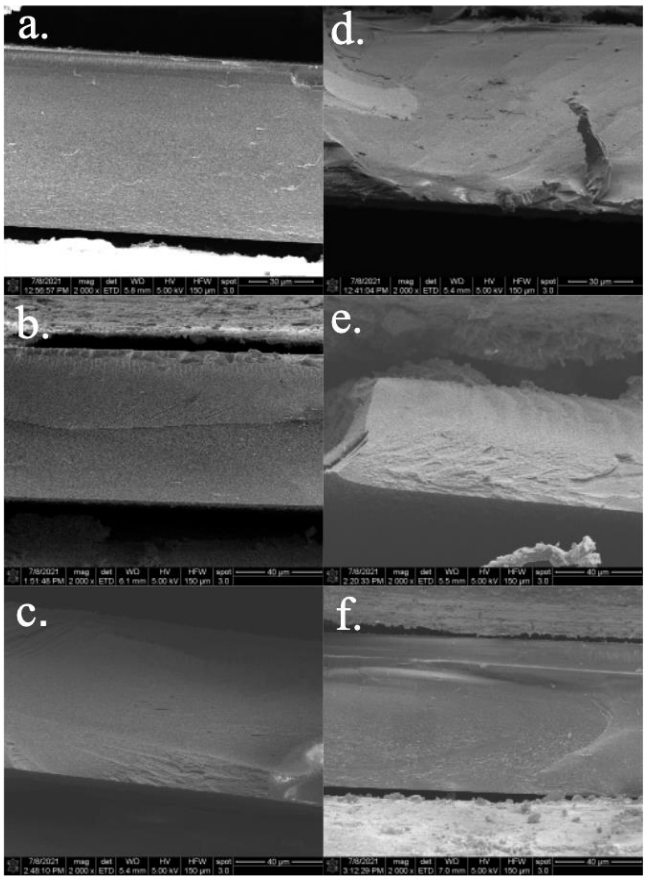

Figure 11. Cross-sections of chitosan films derived from crickets, *A. domesticus* (a-c) and *G. sigillatus* (d-f), shown at 2,000x magnification. Chitosan films have varying degrees of deacetylation, including 72% (a and d), 76% (b and e), and 80% (c and f).

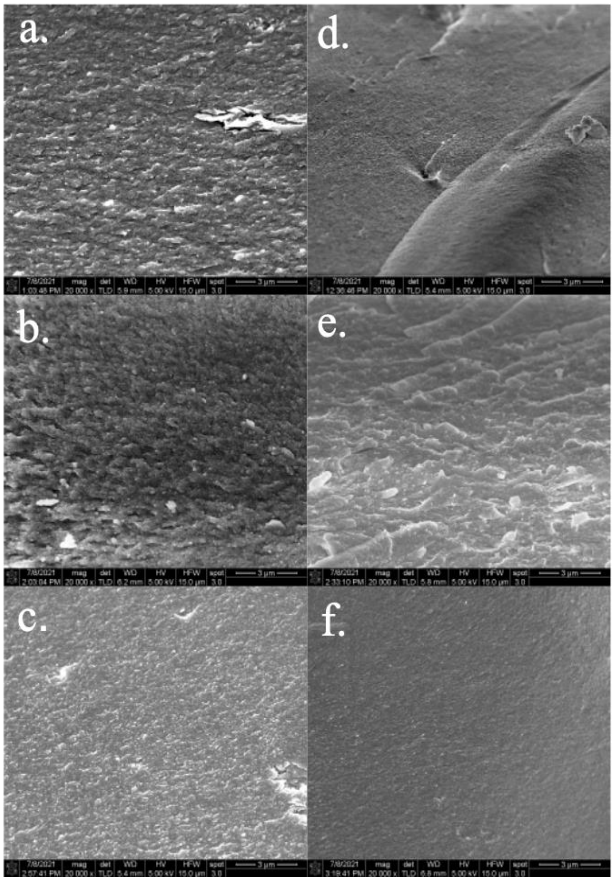

Figure 10. Cross-sections of chitosan films derived from crickets, *A. domesticus* (a-c) and *G. sigillatus* (d-f), shown at 20,000x magnification. Chitosan films have varying degrees of deacetylation, including 72% (a and d), 76% (b and e), and 80% (c and f).

Considering the chitosan–gelatin crosslinking study, the CCF reported here are believed to be complexed with residual melanin, intrinsic to cricket exoskeletons, that could also be more aggregated when compared to the SCF (Figure 12). Furthermore, the differences in structure may also be due to cricket chitosan's lower molecular weight (344 and 524 kDa) (M. Malm & Liceaga, 2021). Y. Liu et al. (2020) reported that higher molecular weight chitosan (110 kDa) promoted a more compact glycerol-chitosan film structure, seen by their scanning electron microscopy images

of smoother cross-sections. Additionally, as the degree of deacetylation of cricket chitosan increased, the cross-section of films became smoother and less aggregated (Figures 11c and f). Previous reports indicate that as the degree of deacetylation of chitosan increases, its polymer chains can pack more closely due to the decreased presence of bulky acetyl groups (Bumgardner et al., 2017). Therefore, increasing the degree of deacetylation of CCF may allow greater packing of chitosan polymer chains leading to the compact chitosan film structure at higher deacetylation.

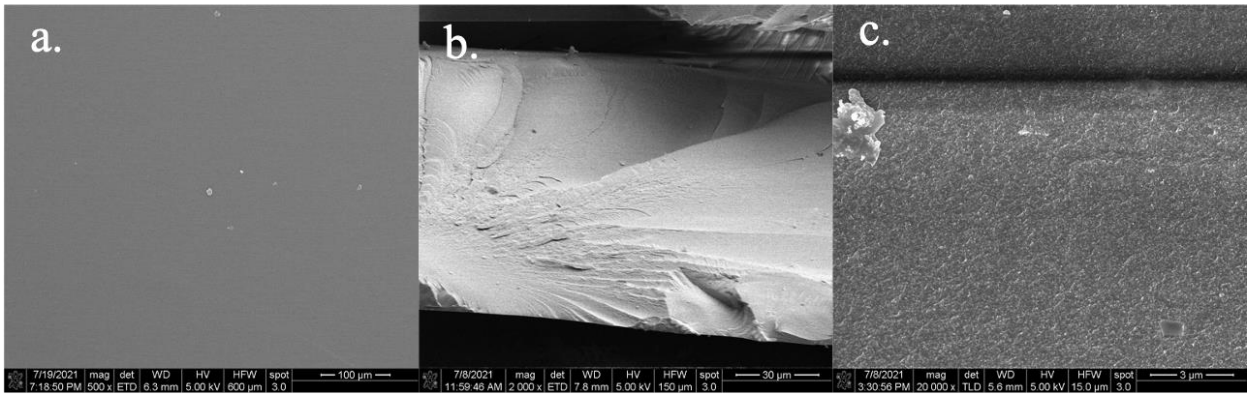

Figure 12. Commercial shrimp chitosan film surface (a) at 500x, and cross sections at 2,000x (b) and 20,000x (c) magnification.

3.4.3 Mechanical properties

In this study, glycerol was used as a plasticizing agent at a constant concentration to compare the mechanical effects (TS and E%) of the different sources of the chitosan polymer, as well as identify any effects caused by the chitosan's molecular weight and the degree of deacetylation. In all cases, CCF had similar, or greater, tensile strengths (TS) when compared to SCF (Figure 13a). These results suggest good intramolecular bonding between cricket chitosan, as previously described by microstructure analysis. Additionally, the degree of deacetylation of cricket chitosan had no clear effect on the corresponding film's TS. Although all CCF had TS values that were similar, or greater than SCF, there were significant differences in elongation percentages (E%) (Figure 13b). All CCF, with the exception of 72% deacetylated *G. sigillatus* chitosan films, had significantly lower E% values ($P < 0.05$). The mechanical results of this study were comparable to other commercial chitosan films prepared similarly (Leceta, Guerrero, & de la Caba, 2013; Souza et al., 2017; Suyatma, Tighzert, Copinet, & Coma, 2005). For example,

commercial chitosan plasticized films (30% glycerol w/v), with varying molecular weights, had TS values between 24-32 MPa and E% values of 30-37% (Leceta et al., 2013). Furthermore, CCF in this study had improved properties compared to chia seed mucilage films we have previously studied, which were plasticized with sorbitol and glycerol and had TS values of 0.38-2.7 MPa and 21-68% E% (Urbizo-Reyes et al., 2020). The molecular weight of chitosan is also thought to play an important role in the mechanical properties of films. Specifically, an increase in molecular weight typically results in an increased TS and E% due to the increased entanglements of the polymer network (Rong Huei & Hwa, 1996; Ryan, Bardosova, & Pemble, 2017). As previously reported, *A. domesticus* and *G. sigillatus* chitosan have molecular weights of 344 and 524 kDa (M. Malm & Liceaga, 2021). However, even though their molecular weights are smaller than commercial shrimp chitosan, the CCF showed excellent mechanical properties as seen by equal or improved TS compared to SCF (Figure 13a).

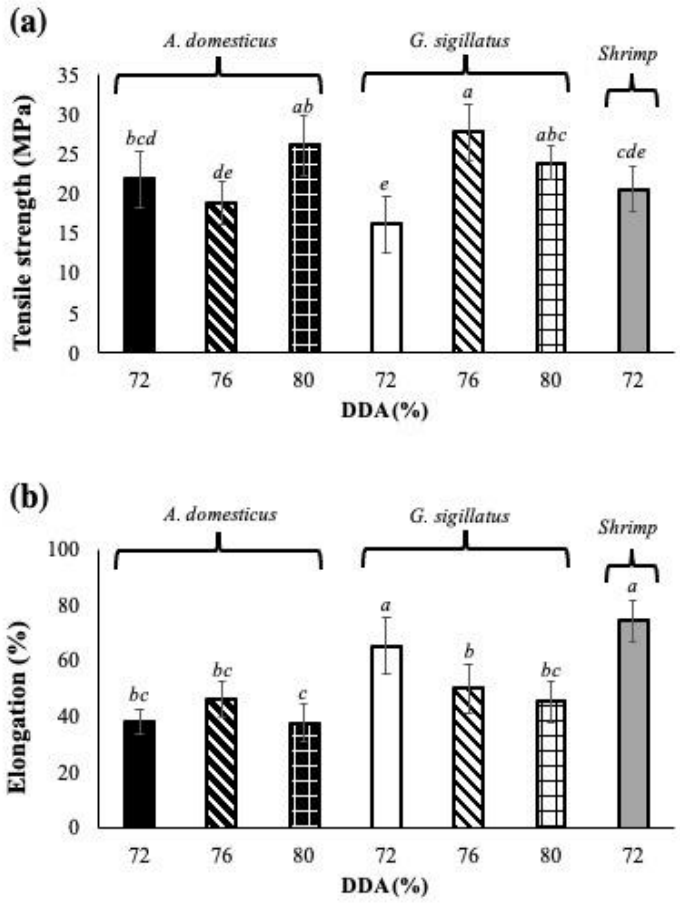

Figure 13. Tensile strength (a), and elongation % at break (b) of cricket chitosan films compared to commercial shrimp chitosan films.

Those in a graph which do not share a letter are considered significantly different.

Currently, only two studies on insect chitosan films that characterize mechanical properties can be found in literature. The first study, chitosan films from mealworms (*Tenebrio molitor*) (812 kDa) and grasshoppers (*Brachystola magna*) (696 kDa) were found to have statistically similar TS and E%, when compared to the study's low (759 kDa) and medium (870 kDa) molecular weight reference chitosan films (Saenz-Mendoza et al., 2020). In another study, grasshopper (*B. magna*) chitosan (322 kDa) films were compared to high, medium, and low molecular weight crustacean chitosan films (Tirado-Gallegos et al., 2021). They showed that grasshopper films had significantly lower TS values than high and medium molecular weight commercial chitosan, but films were statistically similar to low molecular weight crustacean chitosan. Additionally, the authors found that grasshopper chitosan films had lower E% than low and medium molecular weight crustacean chitosan.

One explanation for varied results on mechanical properties of insect chitosan films, compared to crustaceans, may originate from the intrinsic properties of insect exoskeletons. Although not completely understood, it is thought that the matrices of insect cuticles are comprised of proteins and chitin, as well as other components such as melanin. The sclerotization process, responsible for the hardening of insect exoskeleton cuticles, is often associated with the "tanning" of the exoskeleton (Andersen, 2010). Another process, melanogenesis, which occurs during insect cuticle formation, is responsible for the formation of melanin (Sugumaran & Barek, 2016). Although these two processes are separate, they are believed to be closely related and occur simultaneously, with melanin likely to be a product of both processes (Andersen, 2010; Sugumaran & Barek, 2016). Research to understand the interactions among of all these components in the exoskeleton of arthropods is currently ongoing; however, studies on chitin extraction from these sources suggest that melanin could covalently bind to chitin (Khayrova, Lopatin, & Varlamov, 2020; Khayrova, Lopatin, & Varlamov, 2021; Oh & Hwang, 2013). These assumptions are based on the inability to chemically remove all pigments, presumably melanin, from insect-extracted chitin (Khayrova, Lopatin, & Varlamov, 2019; Khayrova et al., 2021). One research group recently filed a patent, where extractable chitin from the black soldier fly, was chemically converted into chitosan with melanin still covalently bound (Khayrova et al., 2020; Сергей Александрович Лопатин, 2020). In our results, similar pigmentation of chitin, chitosan, and corresponding chitosan films can be observed, suggesting that crickets could also have melanin covalently bonded

to its chitin, making it unable to be removed during the purification and conversion processing steps (M. Malm & Liceaga, 2021).

When considering the structural complexity of insect chitin/chitosan, residual melanin may also be a contributing factor to its final properties, including its mechanical characteristics. In an *in vitro* biomimetic study of insect cuticles, chitosan was found to be covalently crosslinked to melanin, leading to corresponding films (unplasticized) to be stronger and stiffer than commercial crustacean chitosan films (Oh & Hwang, 2013). In another study, chitin-melanin complexes were extracted from dung beetles (*Catharsius molossus,* (Linnaeus)), converted to chitosan-melanin complexes, and formed into unplasticized films (Ma, Xin, & Tan, 2015). Authors attribute the increased TS of the beetle chitosan films, compared to commercial shrimp chitosan, as a result of the intramolecular interaction between chitosan and melanin. Therefore, the increased TS of CCF may too be a result of the covalent melanin crosslinks, and increased intramolecular bonding that is not present in SCF. Additionally, these chitin-melanin interactions, paired with their lower molecular weights, may explain the decreased elongation of CCF.

Overall, taking into consideration the research available on both insect and crustacean chitosan films plasticized with glycerol, CCF have similar mechanical properties to crustacean films. These findings suggest that CCF may produce packaging materials that are as strong as SCF, but may not be as flexible. However, if high flexibility is required, SCF could be further manipulated to meet performance expectations. For example, additional plasticizing agents could be used and/or their concentrations optimized. As research progresses on insect exoskeleton sclerotization, insect chitin/chitosan and their complexes, greater mechanistic understanding of insect chitosan films is expected.

3.4.4 Water vapor permeability

The water vapor permeability is an important parameter when evaluating food packaging materials. Food quality, and safety in some circumstances, require the maintenance of water activity. Therefore, the ability of a food packaging polymer to decrease water vapor migration is an important property to understand and characterize. In this study, the water vapor permeability of chitosan films from crickets was determined and compared to commercial SCF (Table 8). All films derived from cricket chitosan had decreased WVP values compared to SCF. Furthermore, chitosan films from *A. domesticus* were slightly less water permeable than *G. sigillatus* films. It is

reported that the WVP of chitosan films is directly affected by glycerol, where glycerol allows the permeation of water through the chitosan-glycerol matrix (Rivero, Damonte, García, & Pinotti, 2016).

Table 8. Permeability, surface wettability, color, and opacity properties of cricket chitosan films

DDA (%)	**Water vapor permeability (10^{-10} g/ m·s·Pa)**	**Water contact angle (°)**	**Instrumental Color and Opacity Properties**			
A. domesticus chitosan			Opacity (mm^{-1})	*L**	*a**	*b**
72	2.14 ± 0.07^{ab}	101.18 ± 4^{bc}	1.6 ± 0.10^{b}	71.79 ± 1.04a	0.8 ± 0.42^{c}	26.49 ± 3.02^{b}
76	2.29 ± 0.06^{ab}	97.21 ± 2.4^{abc}	1.6 ± 0.04^{b}	74.71 ± 0.43^{b}	-0.02 ± 0.18^{b}	24.64 ± 1.39^{b}
80	2.09 ± 0.08^{a}	102.99 ± 5.4^{c}	1.5 ± 0.14^{b}	70.6 ± 0.60^{a}	1.44 ± 0.17^{de}	30.16 ± 0.56^{b}
G. sigillatus chitosan						
72	2.34 ± 0.06^{ab}	102.58 ± 4.1^{c}	1.6 ± 0.04^{b}	72.23 ± 1.85^{ab}	1.23 ± 0.2^{cd}	25.41 ± 4.75^{b}
76	2.40 ± 0.13^{b}	95.48 ± 4.9^{ab}	1.7 ± 0.07^{b}	69.9 ± 1.42^{a}	1.86 ± 0.14^{e}	26.4 ± 3.63^{b}
80	2.43 ± 0.17^{b}	101.06 ± 5.2^{bc}	1.7 ± 0.04^{b}	71.96 ± 1^{ab}	1.56 ± 0.19^{de}	23.65 ± 0.91^{b}
Shrimp chitosan						
	2.91 ± 0.16^{c}	93.02 ± 2.3^{a}	0.5 ± 0.01^{a}	87.97 ± 0.44^{c}	-2.38 ± 0.23^{a}	8.09 ± 0.4^{a}

Little research is available on the water vapor permeability on insect chitosan films, making it difficult to understand the molecular differences responsible for the decreased film WVP. In one study, the WVP of glycerol-chitosan films from grasshoppers was evaluated and compared to low, medium, and high molecular weight commercial chitosan (Tirado-Gallegos et al., 2021). The authors reported grasshopper chitosan films had the lowest WVP (1 x 10^{-10} g/m·s·Pa) compared to all shrimp chitosan films (1.6 to 6.43 x 10^{-10} g/ m·s·Pa). The authors attribute the improved WVP of the grasshopper films to be a result of its greater compacted matrix; however, they did not support these conclusions with SEM microstructure analysis. In our study, considering SEM microstructure analysis, CCF film microstructures appear to be more rough and aggregated, leading to an increased tortuous path length for water vapor to diffuse across the membrane (Qin et al., 2015; Rhim, Hong, Park, & Ng, 2006). Ultimately, this would lead to decreased water vapor permeability of CCF, compared to the smoother and more compact microstructure of chitosan

films which would have a decreased path length for water vapor to travel. Further research on insect chitosan biopolymers, and their microstructures, is needed to make comprehensive conclusions on functional differences between insect and crustacean films.

3.4.5 Color and optical properties

Color measurements showed CCF were light brown, compared to the slight yellow color of the SCF. The color of all films was compared quantitatively using *L*, a*,* and *b** values (Table 8). Cricket films were darker than shrimp films, as shown by their lower *L** values, but overall, the cricket films from the different species had very similar lightness values. CCF also had positive *a** and larger *b** chromaticity parameter values, indicating that films were less green and more yellow in comparison to the commercial shrimp films. Melanin, as already discussed, results in a tan pigment and therefore a likely contributor to the increased darkness and yellowness of the CCF. The appearance and color of CCF films are very similar to crustacean chitosan films with melanin nanoparticles, although the study did not quantitatively study the appearance of the films (Roy, Van Hai, Kim, Zhai, & Kim, 2020). The absence of brown/yellowing pigments in shrimp chitin/chitosan is a result of the differing mechanisms crustaceans utilize during the production of their exoskeletons.

3.4.6 Light barrier properties

As stated previously, a primary function of food packaging is the protection of food materials concealed inside. This can include physical protection, as well as chemical deterioration prevention. Specifically, UV/Vis light that is transmitted through packaging into foods is known to initiate various deleterious chemical reactions, such as increase the rate of lipid oxidation, amongst others. Therefore, one mode of packaging protection is to prevent such reactions by decreasing the light transmitted through packaging.

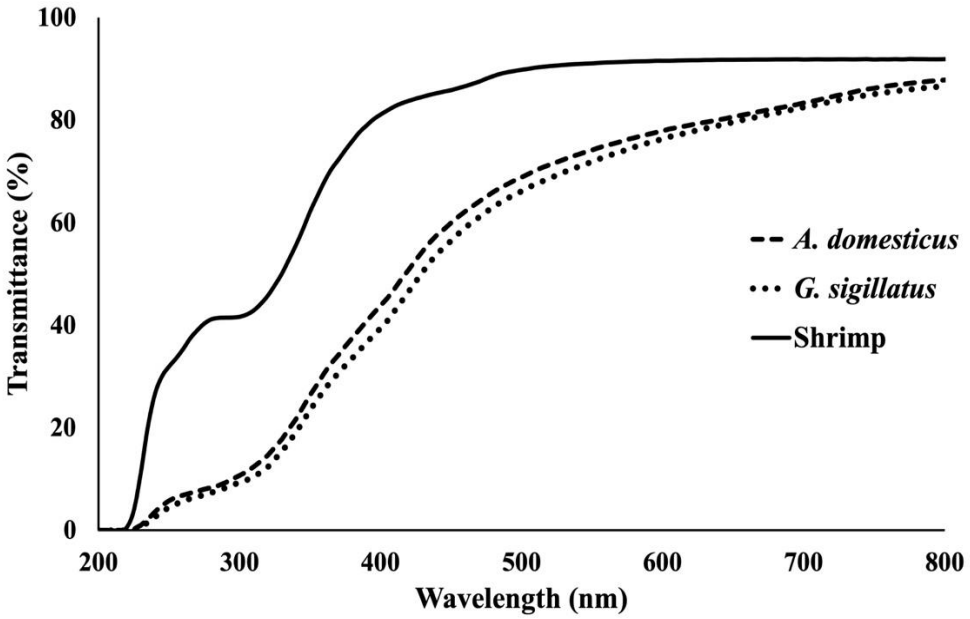

Figure 14. Transmittance (%) of UV/Vis light through chitosan films derived from *A. domesticus* crickets (dashed line), *G. sigillatus* (dotted line), and shrimp (solid line).

In this study, transmittance of light (%), from 200 to 800 nm, through chitosan films was evaluated (Figure 14). The percent of light transmitted through CCF was much less than commercial shrimp films, due to the intrinsic properties and functionality of cricket's chitin rich exoskeletons. Insect exoskeletons serve as a protectant agent from light, such as solar radiation, as well as other oxidative stresses, which is achieved through its incorporation of melanin (Cao et al., 2021; Sugumaran & Barek, 2016). Recent research studies have incorporated synthetic or naturally extracted melanin in different types of films, as an approach to mimic and achieve UV/Vis protecting materials for food packaging. This has been previously and extensively reviewed by Roy and Rhim (2021). Overall, the outcomes of these studies have shown great improvements in light barrier of packaging due to incorporation of melanin. The decreased transmission of light observed in our study is likely due to the remaining melanin present in CCF as previously hypothesized. The properties of the derivation material, crickets, may lead to chitosan films, which have greater light shielding properties than crustacean chitosan films with a slight increase in opacity, while maintaining a transparency expected of a packaging material.

3.4.7 Water contact angles

Overall, CCF derived from both species were more hydrophobic than SCF (Table 8, Figure 15). The degree of deacetylation had no effect on the water contact angle values, although 76% deacetylated CCF were most similar to SCF water contact angles. To the best of our knowledge, this current study is the first to characterize film surface hydrophobicity/hydrophilicity of insect chitosan films.

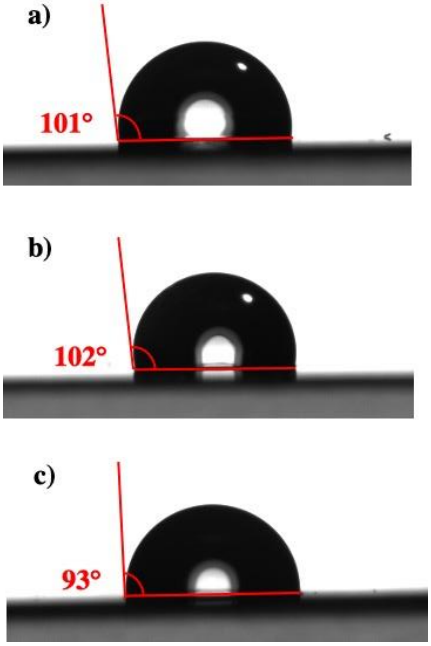

Figure 15. Sessile water droplets on the surface of (a) *A. domesticus*, (b) *G. sigillatus*, and (c) commercial shrimp chitosan films (72% DDA).

One study analyzed the functional properties of high and low molecular weight commercial chitosan films, presumably crustacean derived, plasticized with varying concentrations of glycerol (Leceta et al., 2013). The authors reported that an initial increase from 0 to 15% glycerol did not affect the water contact angle of films (~105°). However, a further increase to 30% glycerol significantly increased the wettability of the surface as seen by a decrease in water contact angle (~98°), which was attributed to the intrinsic hydrophilicity of glycerol. Furthermore, it was found that the molecular weight of chitosan did not play a significant role in the wettability of

unplasticized and plasticized films. Considering the study's results, commercial SCF produced in the present study had a water contact angle of 93°, which aligned well with the previous reported results at a similar glycerol plasticization percentage.

Overall, CCF were more hydrophobic than commercial chitosan films, which we believe could be a result of residual melanin present. In one recent review on insect chitin, chitosan, and their melanin complexes, the authors highlight increased hydrophobicity of melanin-chitosan complexes due to the hydrophobic nature of melanin (Khayrova et al., 2021). Additionally, a biomimetic chitosan film study attributed increased water contact angles due to the presence of melanin (Oh & Hwang, 2013). The increased water contact angles of CCF are likely a result of the presumable presence of melanin (hydrophobic), among possible other residual components. Therefore, if melanin crosslinks are present in CCF, intramolecular interactions would increase and lead to decreased ability of the film's surface to interact with water. In addition, the presence of hydrophobic melanin would lead to the CCF surfaces to have increased water contact angles. Ultimately the increased complexity of cricket chitosan may be an advantage for chitosan biobased food packaging, compared to traditional crustacean chitosan products.

3.5 Conclusions

In this study, chitosan derived from two cricket species showed excellent film forming properties, comparable to films from commercial shrimp chitosan. Cricket chitosan may be more complex in nature due to the differing intrinsic properties of insects; however, in some cases, this may prove an advantage for biobased food packaging. Increased water resistance, as well as light and vapor barrier properties, were achieved through chitosan films derived from crickets, compared to the shrimp chitosan film.

As edible insect rearing and consumption continues to grow, by-products from this emerging industry may provide advantageous materials for use as biobased food packaging materials. As research is currently being performed at a rapid pace, new insights on insect-based chitin/chitosan and their applications are constantly being revealed. Future research on the insect derived chitosan complexes, such as further investigation and characterization, may provide further insight on the mechanisms responsible for their differing, or similar, functionalities in comparison to that derived from commercial (crustaceans) resources. Based on the promising

results of this study, future research can be applied to evaluate insect chitosan films' effect on shelf-life and quality of food.

3.6 References

Andersen, S. O. (2010). Insect cuticular sclerotization: A review. *Insect Biochemistry and Molecular Biology, 40*(3), 166-178. doi:https://doi.org/10.1016/j.ibmb.2009.10.007

Ashrafi, A., Jokar, M., & Nafchi, A. M. (2018). Preparation and characterization of biocomposite film based on chitosan and kombucha tea as active food packaging. *International journal of biological macromolecules, 108,* 444-454.

Babu, R. P., O'Connor, K., & Seeram, R. (2013). Current progress on bio-based polymers and their future trends. *Progress in Biomaterials, 2*(1), 8. doi:https://doi.org/10.1186/2194-0517-2-8

Bumgardner, J. D., Murali, V. P., Su, H., Jenkins, O. D., Velasquez-Pulgarin, D., Jennings, J. A., . . . Jayakumar, R. (2017). 4 - Characterization of chitosan matters. In J. A. Jennings & J. D. Bumgardner (Eds.), *Chitosan Based Biomaterials Volume 1* (pp. 81-114): Woodhead Publishing.

Cao, W., Zhou, X., McCallum, N. C., Hu, Z., Ni, Q. Z., Kapoor, U., . . . Gianneschi, N. C. (2021). Unraveling the Structure and Function of Melanin through Synthesis. *Journal of the American Chemical Society, 143*(7), 2622-2637. doi:https://doi.org/10.1021/jacs.0c12322

Cerqueira, M. A., Souza, B. W. S., Teixeira, J. A., & Vicente, A. A. (2012). Effect of glycerol and corn oil on physicochemical properties of polysaccharide films – A comparative study. *Food Hydrocolloids, 27*(1), 175-184. doi:https://doi.org/10.1016/j.foodhyd.2011.07.007

Chen, S., Wei, X., Sui, Z., Guo, M., Geng, J., Xiao, J., & Huang, D. (2021). Preparation of Antioxidant and Antibacterial Chitosan Film from Periplaneta americana. *Insects, 12*(1), 53. doi:https://doi.org/10.3390/insects12010053

de Moraes Crizel, T., de Oliveira Rios, A., Alves, V. D., Bandarra, N., Moldão-Martins, M., & Flôres, S. H. (2018). Active food packaging prepared with chitosan and olive pomace. *Food Hydrocolloids, 74,* 139-150. doi:https://doi.org/10.1016/j.foodhyd.2017.08.007

Debandi, M. V., Bernal, C., & Francois, N. J. (2016). Development of biodegradable films based on chitosan/glycerol blends suitable for biomedical applications. *J. Tissue Sci. Eng, 3*(4172), 2157-7552.1000187. doi:http://dx.doi.org/10.4172/2157-7552.1000187

Domjan, A., Bajdik, J., & Pintye-Hodi, K. (2009). Understanding of the plasticizing effects of glycerol and PEG 400 on chitosan films using solid-state NMR spectroscopy. *Macromolecules, 42*(13), 4667-4673. doi:https://doi.org/10.1021/ma8021234

Flores, Z., San-Martin, D., Beldarraín-Iznaga, T., Leiva-Vega, J., & Villalobos-Carvajal, R. (2021). Effect of Homogenization Method and Carvacrol Content on Microstructural and Physical Properties of Chitosan-Based Films. *Foods, 10*(1), 141. doi:https://doi.org/10.3390/foods10010141

García, M. a. A., Pinotti, A., Martino, M. N., & Zaritzky, N. E. (2004). Characterization of composite hydrocolloid films. *Carbohydrate polymers, 56*(3), 339-345. doi:https://doi.org/10.1016/j.carbpol.2004.03.003

Guillard, V., Gaucel, S., Fornaciari, C., Angellier-Coussy, H., Buche, P., & Gontard, N. (2018). The Next Generation of Sustainable Food Packaging to Preserve Our Environment in a Circular Economy Context. *Frontiers in Nutrition, 5*(121). doi:https://doi.org/10.3389/fnut.2018.00121

Hall, F. G., Jones, O. G., O'Haire, M. E., & Liceaga, A. M. (2017). Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food Chemistry, 224*, 414-422. doi:https://doi.org/10.1016/j.foodchem.2016.11.138

Kalaycıoğlu, Z., Torlak, E., Akın-Evingür, G., Özen, İ., & Erim, F. B. (2017). Antimicrobial and physical properties of chitosan films incorporated with turmeric extract. *International Journal of Biological Macromolecules, 101*, 882-888. doi:https://doi.org/10.1016/j.ijbiomac.2017.03.174

Kaya, M., Khadem, S., Cakmak, Y. S., Mujtaba, M., Ilk, S., Akyuz, L., . . . Deligöz, E. (2018). Antioxidative and antimicrobial edible chitosan films blended with stem, leaf and seed extracts of Pistacia terebinthus for active food packaging. *RSC Advances, 8*(8), 3941-3950. doi:https://doi.org/10.1039/C7RA12070B

Khayrova, A., Lopatin, S., & Varlamov, V. (2019). Black Soldier Fly Hermetia illucens as a Novel Source of Chitin and Chitosan. *International Journal of Sciences, 8*, 81-86. doi:http://dx.doi.org/10.18483/ijSci.2015

Khayrova, A., Lopatin, S., & Varlamov, V. (2020). Obtaining Chitin/Chitosan-Melanin Complexes from Black Soldier Fly Hermetia Illucens. *IOP Conference Series: Materials Science and Engineering, 809*, 012020. doi:http://dx.doi.org/10.1088/1757-899X/809/1/012020

Khayrova, A., Lopatin, S., & Varlamov, V. (2021). Obtaining chitin, chitosan and their melanin complexes from insects. *International Journal of Biological Macromolecules, 167*, 1319-1328. doi:https://doi.org/10.1016/j.ijbiomac.2020.11.086

Leceta, I., Guerrero, P., & de la Caba, K. (2013). Functional properties of chitosan-based films. *Carbohydrate Polymers, 93*(1), 339-346. doi:https://doi.org/10.1016/j.carbpol.2012.04.031

Liu, M., Zhou, Y., Zhang, Y., Yu, C., & Cao, S. (2013). Preparation and structural analysis of chitosan films with and without sorbitol. *Food Hydrocolloids, 33*(2), 186-191. doi:https://doi.org/10.1016/j.foodhyd.2013.03.003

Liu, Y., Yuan, Y., Duan, S., Li, C., Hu, B., Liu, A., . . . Wu, W. (2020). Preparation and characterization of chitosan films with three kinds of molecular weight for food packaging. *International Journal of Biological Macromolecules, 155*, 249-259. doi:https://doi.org/10.1016/j.ijbiomac.2020.03.217

Liu, Z., Ge, X., Lu, Y., Dong, S., Zhao, Y., & Zeng, M. (2012). Effects of chitosan molecular weight and degree of deacetylation on the properties of gelatine-based films. *Food Hydrocolloids, 26*(1), 311-317. doi:https://doi.org/10.1016/j.foodhyd.2011.06.008

Luna, G. C., Martin-Gonzalez, F. S., Mauer, L., & Liceaga, A. (2021). Cricket (Acheta domesticus) protein hydrolysates' impact on the physicochemical, structural and sensory properties of tortillas and tortilla chips. *Journal of Insects as Food and Feed, 7*(1), 109-120. doi:https://doi.org/10.3920/JIFF2020.0010

Ma, J., Xin, C., & Tan, C. (2015). Preparation, physicochemical and pharmaceutical characterization of chitosan from Catharsius molossus residue. *International journal of biological macromolecules, 80*, 547-556.

Malm, M., & Liceaga, A. M. (2021). Physicochemical Properties of Chitosan from Two Commonly Reared Edible Cricket Species, and Its Application as a Hypolipidemic and Antimicrobial Agent. *Polysaccharides, 2*(2), 339-353. doi:https://doi.org/10.3390/polysaccharides2020022

Malm, M. J., Narsimhan, G., & Kokini, J. L. (2019). Effect of contact surface, plasticized and crosslinked zein films are cast on, on the distribution of dispersive and polar surface energy using the Van Oss method of deconvolution. *Journal of Food Engineering, 263*, 262-271. doi:https://doi.org/10.1016/j.jfoodeng.2019.07.001

Mohan, K., Ganesan, A. R., Muralisankar, T., Jayakumar, R., Sathishkumar, P., Uthayakumar, V., . . . Revathi, N. (2020). Recent insights into the extraction, characterization, and bioactivities of chitin and chitosan from insects. *Trends in food science & technology, 105*, 17-42. doi:https://doi.org/10.1016/j.tifs.2020.08.016

Oh, D. X., & Hwang, D. S. (2013). A biomimetic chitosan composite with improved mechanical properties in wet conditions. *Biotechnology Progress, 29*(2), 505-512. doi:https://doi.org/10.1002/btpr.1691

Qin, Y.-Y., Zhang, Z.-H., Li, L., Yuan, M.-L., Fan, J., & Zhao, T.-R. (2015). Physio-mechanical properties of an active chitosan film incorporated with montmorillonite and natural antioxidants extracted from pomegranate rind. *Journal of food science and technology, 52*(3), 1471-1479. doi:10.1007/s13197-013-1137-1

Rai, S., Dutta, P., & Mehrotra, G. (2017). Lignin incorporated antimicrobial chitosan film for food packaging application. *Journal of Polymer Materials, 34*(1), 171.

Râpă, M., Miteluţ, A. C., Tănase, E. E., Grosu, E., Popescu, P., Popa, M. E., . . . Vasile, C. (2016). Influence of chitosan on mechanical, thermal, barrier and antimicrobial properties of PLA-biocomposites for food packaging. *Composites Part B: Engineering, 102*, 112-121. doi:https://doi.org/10.1016/j.compositesb.2016.07.016

Rhim, J.-W., Hong, S.-I., Park, H.-M., & Ng, P. K. W. (2006). Preparation and Characterization of Chitosan-Based Nanocomposite Films with Antimicrobial Activity. *Journal of Agricultural and Food Chemistry, 54*(16), 5814-5822. doi:https://doi.org/10.1021/jf060658h

Riaz, A., Lei, S., Akhtar, H. M. S., Wan, P., Chen, D., Jabbar, S., . . . Zeng, X. (2018). Preparation and characterization of chitosan-based antimicrobial active food packaging film incorporated with apple peel polyphenols. *International Journal of Biological Macromolecules, 114*, 547-555. doi:https://doi.org/10.1016/j.ijbiomac.2018.03.126

Rivero, S., Damonte, L., García, M. A., & Pinotti, A. (2016). An insight into the role of glycerol in chitosan films. *Food Biophysics, 11*(2), 117-127. doi:https://doi.org/10.1007/s11483-015-9421-4

Rodríguez-Núñez, J. R., Madera-Santana, T. J., Sánchez-Machado, D. I., López-Cervantes, J., & Valdez, H. S. (2014). Chitosan/hydrophilic plasticizer-based films: preparation, physicochemical and antimicrobial properties. *Journal of Polymers and the Environment, 22*(1), 41-51. doi:https://doi.org/10.1007/s10924-013-0621-z

Rong Huei, C., & Hwa, H.-D. (1996). Effect of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical, and permeability properties of the prepared membrane. *Carbohydrate Polymers, 29*(4), 353-358. doi:https://doi.org/10.1016/S0144-8617(96)00007-0

Roy, S., & Rhim, J.-W. (2021). New insight into melanin for food packaging and biotechnology applications. *Critical Reviews in Food Science and Nutrition*, 1-27. doi:https://doi.org/10.1080/10408398.2021.1878097

Roy, S., Van Hai, L., Kim, H. C., Zhai, L., & Kim, J. (2020). Preparation and characterization of synthetic melanin-like nanoparticles reinforced chitosan nanocomposite films. *Carbohydrate Polymers, 231*, 115729. doi:https://doi.org/10.1016/j.carbpol.2019.115729

Ryan, C., Bardosova, M., & Pemble, M. (2017). Structural and mechanical properties of a range of chitosan-based hybrid networks loaded with colloidal silica and polystyrene particles. *Journal of Materials Science, 52*(13), 8338-8347. doi:https://doi.org/10.1007/s10853-017-1051-4

Saenz-Mendoza, A. I., Zamudio-Flores, P. B., García-Anaya, M. C., Velasco, C. R., Acosta-Muñiz, C. H., de Jesús Ornelas-Paz, J., . . . Salgado-Delgado, R. (2020). Characterization of insect chitosan films from Tenebrio molitor and Brachystola magna and its comparison with commercial chitosan of different molecular weights. *International Journal of Biological Macromolecules, 160*, 953-963. doi:https://doi.org/10.1016/j.ijbiomac.2020.05.255

Schaefer, J., Kramer, K. J., Garbow, J. R., Jacob, G. S., Stejskal, E. O., Hopkins, T. L., & Speirs, R. D. (1987). Aromatic cross-links in insect cuticle: detection by solid-state 13C and 15N NMR. *Science, 235*(4793), 1200-1204. doi:https://doi.org/10.1126/science.3823880

Shahbazi, Y. (2017). The properties of chitosan and gelatin films incorporated with ethanolic red grape seed extract and Ziziphora clinopodioides essential oil as biodegradable materials for active food packaging. *International journal of biological macromolecules, 99*, 746-753.

Siripatrawan, U., & Vitchayakitti, W. (2016). Improving functional properties of chitosan films as active food packaging by incorporating with propolis. *Food Hydrocolloids, 61*, 695-702. doi:https://doi.org/10.1016/j.foodhyd.2016.06.001

Souza, V. G. L., Fernando, A. L., Pires, J. R. A., Rodrigues, P. F., Lopes, A. A. S., & Fernandes, F. M. B. (2017). Physical properties of chitosan films incorporated with natural antioxidants. *Industrial Crops and Products, 107*, 565-572. doi:https://doi.org/10.1016/j.indcrop.2017.04.056

Sugumaran, M., & Barek, H. (2016). Critical Analysis of the Melanogenic Pathway in Insects and Higher Animals. *International Journal of Molecular Sciences, 17*(10), 1753. doi:10.3390/ijms17101753

Suyatma, N. E., Tighzert, L., Copinet, A., & Coma, V. (2005). Effects of Hydrophilic Plasticizers on Mechanical, Thermal, and Surface Properties of Chitosan Films. *Journal of Agricultural and Food Chemistry, 53*(10), 3950-3957. doi:https://doi.org/10.1021/jf048790+

Tirado-Gallegos, J. M., Zamudio-Flores, P. B., Espino-Díaz, M., Salgado-Delgado, R., Vela-Gutiérrez, G., Hernández-Centeno, F., . . . Ortega-Ortega, A. (2021). Chitosan Films Obtained from Brachystola magna (Girard) and Its Evaluation on Quality Attributes in Sausages during Storage. *Molecules, 26*(6), 1782. doi:https://doi.org/10.3390/molecules26061782

Urbizo-Reyes, U., San Martin-González, M. F., Garcia-Bravo, J., & Liceaga, A. M. (2020). Development of chia seed (Salvia hispanica) mucilage films plasticized with polyol mixtures: Mechanical and barrier properties. *International Journal of Biological Macromolecules, 163*, 854-864. doi:https://doi.org/10.1016/j.ijbiomac.2020.07.023

Wang, H., Qian, J., & Ding, F. (2018). Emerging Chitosan-Based Films for Food Packaging Applications. *Journal of Agricultural and Food Chemistry, 66*(2), 395-413. doi:https://doi.org/10.1021/acs.jafc.7b04528

Wang, Q., Tian, F., Feng, Z., Fan, X., Pan, Z., & Zhou, J. (2015). Antioxidant activity and physicochemical properties of chitosan films incorporated with Lycium barbarum fruit extract for active food packaging. *International Journal of Food Science & Technology, 50*(2), 458-464. doi:https://doi.org/10.1111/ijfs.12623

Wu, C., Tian, J., Li, S., Wu, T., Hu, Y., Chen, S., . . . Ye, X. (2016). Structural properties of films and rheology of film-forming solutions of chitosan gallate for food packaging. *Carbohydrate Polymers, 146*, 10-19. doi:https://doi.org/10.1016/j.carbpol.2016.03.027

Zainol Abidin, N. A., Kormin, F., Zainol Abidin, N. A., Mohamed Anuar, N. A. F., & Abu Bakar, M. F. (2020). The Potential of Insects as Alternative Sources of Chitin: An Overview on the Chemical Method of Extraction from Various Sources. *International journal of molecular sciences, 21*(14), 4978. doi:https://doi.org/10.3390/ijms21144978

Сергей Александрович Лопатин, А. Ш. Х., Валерий Петрович Варламов, Иван Викторович Соколов. (2020). Russia Patent No. RU2728458C1. Russia.

CHAPTER 4. CONCLUSIONS

In this dissertation, cricket derived chitin and chitosan were evaluated as a potential source for commercial chitosan and impacts of species change on applications were studied. Chitin-rich pellets served as the starting material, a waste stream obtained during protein isolation from two cricket species (*Acheta domesticus* and *Gryllodes sigillatus*). Extraction and purification methods used in this study produced demineralized and deproteinized chitin, which then was chemically modified to chitosan using methods commonly used in the production of shrimp chitosan. The degree of modification, specifically the degree of deacetylation, was optimized to 72, 76, and 80% DDA. Cricket chitosan was structurally similar to commercial shrimp chitosan, as confirmed with signature FT-IR spectra peaks. As expected, the molecular weight of chitosan from crickets was similar to that previously reported for other insect species, and smaller than the molecular weight of crustacean chitosan. The molecular weight, species origination, and deacetylation of cricket chitosan had no significant effect on the *in vitro* lipid binding capacity, with shrimp chitosan serving as the reference material. Chitosan's antimicrobial activity at the concentration chosen, showed good inhibition against two surrogate food pathogens. For *L. innocua*, 100% inhibition was achieved with all cricket and commercial chitosan samples for up to 24 hours. This suggests that molecular weight, cricket species, and deacetylation did not play a significant role against *L. innocua*, or such effects were not detectable due to the confounding effects of acetic acid present. At the same concentration, *E. coli* inhibition was directly affected by cricket chitosan's degree of deacetylation, where 90% of growth was inhibited by 80% DDA cricket chitosan after 24 hours.

After successful extraction and purification of chitin, optimization of cricket chitosan, and evaluation of cricket chitosan bioactivity, cricket chitosan samples were functionalized into bio-based polymers. The results of this second study showed cricket chitosan films had similar or improved properties when compared to commercial shrimp chitosan films. The close association of residual melanin and protein, likely facilitated the greater compaction and aggregation of cricket chitosan films' microstructures. As a result, cricket chitosan films had improved water vapor permeability, surface hydrophobicity, and light barrier properties in comparison to shrimp chitosan films. Mechanical properties of all chitosan films showed similarities, although shrimp chitosan films were more flexible.

Overall, this dissertation research elucidates foundational differences between cricket chitin/chitosan and shrimp chitin/chitosan, and highlights the feasibility for using cricket chitosan as an alternative resource to commercial crustacean chitosan. Although some physicochemical properties of cricket chitosan were found to be different than crustaceans, the studies presented in this dissertation show that applications of chitosan would be unaffected, and even improved in some cases. The research presented in this dissertation also provides preliminary support for successful utilization of insect by-products in different applications, waste streams that will likely have greater impact in the near future as the use of insects for sustainable human and animal nutrition is rapidly growing. Future research on cricket chitosan films with different food products will elucidate further a targeted food packaging application.

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May 2016 – Dec. 2021 **Doctor of Philosophy in Food Science**
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REFEREED JOURNAL PUBLICATIONS

1. Development of Chitosan Films from Edible Crickets and its Performance as a Bio-based Food Packaging Material. *Polysaccharides* (under external review).
2. Malm, M., & Liceaga, A. M. 2021. Physicochemical Properties of Chitosan from Two Commonly Reared Edible Cricket Species, and Its Application as a Hypolipidemic and Antimicrobial Agent. *Polysaccharides, 2*(2), 339-353.
3. Malm MJ, Narsimhan G, Kokini JL. 2019. Effect of contact surface, plasticized and crosslinked zein films are cast on, on the distribution of dispersive and polar surface energy using the Van Oss method of deconvolution. *Journal of Food Engineering*. 2019;263:262-71.
4. Li, X., Maldonado, L., Malm, M**.**, Rouf, T., Hua, Y., and Jozef Kokini. 2019. Development of Hollow Kafirin-Based Nanoparticles Fabricated through Layer-by-Layer Assembly as Delivery Vehicles for Curcumin. *Food Hydrocolloids* 96 (November): 93–101.
5. Turasan, H., Barber, E., Malm, M., and Kokini, J. 2018. Mechanical and Spectroscopic Characterization of Crosslinked Zein Films Cast from Solutions of Acetic Acid Leading to a New Mechanism for the Crosslinking of Oleic Acid Plasticized Zein Films. *Food Research International* 108 (June): 357–67.

BOOK CHAPTERS

1. Turasan, H., Bonilla J., Jia, F., Maldonado, L., Malm, M., Rouf, T.B. and Kokini, J.L. (2020). Chapter 12 - Advances in food functionality and packaging using nanotechnology. In Food Applications of Nanotechnology, First edition, pp. 271–318. CRC Press Taylor & Francis Group.

CONFERENCE PRESENTATIONS

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