DEVELOPING A NOVEL MARINE GELATIN FROM SALTED AND DRIED CANNONBALL JELLYFISH (*STOMOLOPHUS MELEAGRIS*) AND ITS APPLICATION AS A MICROENCAPSULATING AGENT FOR THE PROBIOTIC *LACTOBACILLUS RHAMNOSUS* GG

by

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(Under the Direction of Kevin Mis Solval)

ABSTRACT

Cannonball jellyfish contain high collagen content, which has great potential as a raw material for developing novel ingredients. The objective was to determine the feasibility of hydrolyzing jellyfish collagen for producing food-grade gelatin powders from salted, dried jellyfish (SDJ). Hydrolyzed-SDJ (H-SDJ) produced gels with Bloom values (g) of 3.40±0.30; while unhydrolyzed-SDJ did not form a gel. This illustrates (for the first time) the feasibility of using a novel hydrolysis method to produce gelatin powders from U.S.-caught jellyfish. Demineralization using dialysis gave dialyzed-SDJ (D-SDJ) where pH, maturation temperature, and solid concentration adjustments produced significant differences in the Bloom strengths. Crosslinked D-SDJ with polyphenols at pH 4.4 and 6.5 led to a 49.8% and 42.8% increase in Bloom strength over non-crosslinked D-SDJ (control), respectively. Microencapsulation of *Lactobacillus rhamnosus* GG (LRGG) using D-SDJ with polyphenols or Na-Alg at pH 4.4 and 6.5 improved survival during a 28-day shelf-life study to ~8 log CFU/g.

INDEX WORDS: Jellyfish, hydrolysis, Bloom, dialysis, *Lactobacillus rhamnosus* GG

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DEDICATION

This research is dedicated to all those who were told they were not smart enough to go to college. Believe in yourself and you will succeed.

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This journey at The University of Georgia (UGA) has been one that I did not fully expect. After completing my undergraduate degree at The University of Florida (UF), I never thought I would end up going to graduate school at UGA; however, I believe everything happens for a reason and it was one of the best choices I ever made. This journey would not have been possible without the assistance of my major professor Kevin Mis Solval and his patience and willingness to always do what he can for me so I can succeed. I also want to thank my committee members Ronald Pegg and Govindaraj Dev Kumar for your willingness to always assist me in this project whenever I needed it. Apart from the professors, a special thanks goes to the wonderful employees in Griffin and Athens specifically Glenn Farrell, Bobby Goss, Karen Simmons, Donna Brown, and roommate/lab mate (Boran Yang) for all the behind the scenes work that made projects work more effectively, assisted in running equipment, making materials, providing information on coursework, deadlines, and other crucial information that without I wouldn't be here so thank you. I also want to thank my beautiful family, girlfriend, best friend, and all my former teammates from the UF Cheer team because without all their love and support I would not be who I am and never would I have made it through the stress that is graduate school. Additionally, Georgia Sea Grant Research Traineeship for providing funding for this project, Tori Stivers from Marine Extension and GA Sea Grant, Brandon Tonnis from the USDA ARS, and the USDA NIFA AFRI Foundational Program Award #2021-67017-33442. Sincerely, thank you to all that made this journey possible and I look forward to the next chapter as I continue this research and work towards my doctoral degree.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

INTRODUCTION AND LITERATURE REVIEW

The world's gelatin market is expected to be worth 6.7 billion USD in the year 2027 with a compound annual growth rate of 9.2% (Grand View Research, 2020). In Georgia, the processing of cannonball jellyfish (CBJ) has been limited to 6-12 fishers, mainly due to limited processing capacity and a small domestic market (Page, 2015). The jellyfish fisheries in Georgia bring in an annual catch of 4,000 tonnes with the possibility to expand efforts (Brotz et al., 2017). Georgia's CBJ are exported to several Asian countries mainly China and Japan in a salted and dried form. However, tariffs placed on China in recent years has led to a decrease in Georgia CBJ exports; thus, the local jellyfish industry has been exploring the possibility of expanding the domestic market using CBJ. To date, little research has been reported on the alternative uses of jellyfish and its potential as a functional food ingredient. Because jellyfish is an excellent source of collagen, our team has identified an opportunity to develop food ingredients by hydrolyzing jellyfish collagen to produce jellyfish gelatin (JG), which can potentially be used as a novel ingredient in various food applications. Therefore, a wealth of information is yet to be discovered on the possibilities of consuming jellyfish within the U.S., specifically as gelatin.

Nevertheless, the application of gelatins from other species of jellyfish in the food industry is limited due to their suboptimal properties (e.g., poor solubility, sensory properties, weak gel strength) (Zhuang, Sun, Zhao, Hou, & Li, 2010). Demineralizing then modifying the pH, maturation temperature, and solids content in addition to crosslinking JG with sodium alginate (Na-Alg) and isolated polyphenols (PPs) from Georgia-grown pomegranates will be

performed to study the effect on the physicochemical properties of the JG. Then, the feasibility of using the novel gelatins as a microencapsulating agent for *Lactobacillus rhamnosus* GG (LRGG) will be assessed. Currently, there is a lack of knowledge on jellyfish as a whole, especially in regards to demineralizing, modifying and crosslinking JG and its overall capability as a microencapsulating agent for probiotic bacteria.

The results of this research will impact both the gelatin and local jellyfish industries. Moreover, positive results of this research will demonstrate that suboptimal JG can be improved by specific modifications to the gelatin for use in various food applications as a novel ingredient. This research will also assist the probiotic industry to overcome the main challenge of developing probiotic supplements/foods to have viable counts more than 10^9 per daily ingested dose, which is the recommended amount to confer health benefits in the host (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; Prado, Parada, Pandey, & Soccol, 2008). To combat this, microencapsulating probiotics with a compatible food-grade material, like JG, may be a feasible solution to improve probiotic survival in supplements and foods.

The goal of this project is to develop a safe, high-quality modified jellyfish gelatin that can be used as a novel food ingredient and microencapsulating agent for LRGG. This project will (1) take the raw material (salted, dried jellyfish (SDJ)), hydrolyze the collagen, produce a powder and perform tests on the gelatin; then (2) perform demineralization and test the gelatin; (3) perform modifications to the gelatins' pH, maturation temperature, and solids concentration as well as crosslink the gelatins with Na-Alg and PPs then test the gelatins; (4) microencapsulate LRGG to give powders using the novel jellyfish gelatins; and (5) monitor the LRGG survival in the probiotic powders during a shelf-life study.

If successful, the JGs may be used as a novel food ingredient in replacement of

mammalian gelatins. The results of this project will help Georgia's jellyfish industry to diversify its portfolio of not only food products but to eventually expand to other industry's (e.g., cosmetics and pharmaceuticals), which will help to maximize its profits while stimulating economic development in rural, coastal communities. As the third largest commercial fishery (by landings) in Georgia, finding new ways to utilize jellyfish will allow for more jobs to be created, including fishermen/women and processing jobs within and around the coast of Georgia (Page, 2015). As the need for this species increases, it will provide an additional boost to the state economy. Niche/exotic markets can also be created for those with keen marketing strategies; for example, JG-based food products which might attract a specific market like Generation Z consumers, who are always looking for the next "cool" product. Wherever the future may lie for JG, a world of increasing gelatin demand calls for sustainable, alternative sources which can provide additional benefits to the state, industry and consumer. This is the future of the gelatin industry.

Cannonball jellyfish

In recent years, jellyfish blooms have increased in frequency and intensity due to a variety of factors including climate change (e.g., warming ocean waters and ocean acidification) as well as practices/stressors like overfishing of larger predators and competitors, accidental translocations, and eutrophication of coastal waters. These jellyfish blooms have caused negative ecological, economic and social impacts to not only humans but also to other marine species (Brotz et al., 2017; Kienberger et al., 2018). Research has found that jellyfish population trends in various locations throughout the world since the 1950s until 2012 have been increasing (Brotz, Cheung, Kleisner, Pakhomov, & Pauly, 2012). Historically, jellyfish have been infamous by fishermen/women as they commonly clog fishing trawlers nets. In Georgia, jellyfish started as an

experimental fishery in 1998, but due to its successful results, it became a commercial fishery in 2013. Usually, the jellyfish season runs from late fall through spring. This "emerging" industry is based in the rural coastal communities of Georgia where the jellyfish are caught, processed and shipped overseas in a salted and dried form (Page, 2015). Jellyfish is one of the top three commercial fisheries in Georgia by landings. Most of Georgia's salted and dried jellyfish (SDJ) is exported to Asian countries while only small quantities are sold domestically. Recently, the U.S. jellyfish industry has faced a significant decline in exports and sales of SDJ due to current political events. This creates an opportunity to develop alternative products from SDJ and diversify the portfolio of products for the U.S. jellyfish industry, which may assist in developing local markets for jellyfish products (Page, 2015; Raposo, Coimbra, Amaral, Gonçalves, & Morais, 2018).

Jellyfish consists of the body, which is a hemispherical transparent umbrella, the mouth, which is on the underside of the umbrella, the fused arms known as tentacles or 'oral arms' as well as additional parts like the gonads (sex organs), mucosal membrane, and stomach (Fluech, 2018; Hsieh, Leong, & Rudloe, 2001). Within hours of the harvest, the umbrella is hand separated from the oral arms, washed with sea water, and then removal of the mucosal membrane and gonadal layer is performed. From here, processing involves removing the excess moisture by using a 1 kg salt mixture containing 10% alum per 8-10 kg of fresh jellyfish (Hsieh et al., 2001). Over the course of 30-40 days, the brining process is repeated several times with fresh salt:alum mixtures until the jellyfish is dried to 60-70% moisture and contains 16-25% salt (Hsieh et al., 2001). The proximate composition of SDJ is 68% moisture, 25.1% ash, 5.5% protein, 1.4% fat and provides 36 kcal per 100 g (USDA, 2009). In Asian cuisine, SDJ is normally incorporated in several dishes including salads and noodles. However, consuming SDJ

may be difficult or far-fetched for a majority of Americans. A proposed solution is to use SDJ as a raw material for producing a functional food ingredient, gelatin, which may be more acceptable by American consumers.

It is important to note that SDJ is not a complete protein because of its low levels of tryptophan and being abundant in glycine, hydroxyproline, and hydroxylysine (Hsieh & Rudloe, 1994; Kimura, Miura, & Park, 1983). Amino acids like glutamine, lysine, tyrosine and cysteine residues have several reactive groups that allow for crosslinking to take place; however, in most gelatins extracted from marine species (including jellyfish), these key amino acids are in low quantities thus being a reason for poor gel strength and high melting temperatures (Buchert et al., 2010). Research that has been conducted with jellyfish from around the world is most commonly in regards to medicinal purposes like as a building material for cell tissue, wound healing, cell growth and differentiation, soft-tissue restoration and in treatment of diseases like rheumatoid arthritis, bronchitis, etc. (Hsieh & Rudloe, 1994). Still, not much research in regards to processing, consumption, and food product development is being reported.

The next sections will include similar research being done in this area in regards to gelatin, crosslinking with polyphenolic compounds as well as potential application usages, specifically JG for use as a microencapsulating agent for LRGG will be discussed.

Gelatin & Gelatin Modification

Gelatin (hydrolyzed collagen) is used in the following food applications: confections (chewiness, texture, and foam stabilization), low-fat spreads (creaminess, fat reduction, and mouthfeel), dairy (stabilization and texturization), baked goods (emulsification, gelling, and stabilization), meats (water-binding) as well as lowering caloric density (Karim & Bhat, 2009). Gelatin is also used as low-calorie thickener in gluten-free products. Commercial gelatins are

normally obtained from mammalian collagen, mainly from bovine and porcine sources. Moreover, these gelatins are under constraints and skepticism because of social and cultural concerns. Some of the negative outlook on these mammalian gelatins are related to how they are derived, which is by utilizing the non-ideal cuts, hides, etc. and other leftovers from the pork and beef industry's (Leone, Lecci, Durante, Meli, & Piraino, 2015). Therefore, developing gelatins from alternative collagen sources, including marine sources, has gained more attention in recent years; however, poor rheological and mechanical properties, variable quality, off-flavors, production costs and low yields have been observed in marine gelatins. Nevertheless, they have unique functional properties that may be of great interest to the food industry. For example, marine gelatins have lower gelling and melting temperatures compared to bovine and porcine gelatins which may allow for a quick release of encapsulated aromas and flavors. Moreover, it has been reported that marine gelatins can be excellent encapsulating agents for vitamins, bioactives, colors, flavors, and even probiotic bacteria (Borza et al., 2010; Karim & Bhat, 2009; Li, Chen, Cha, Park, & Liu, 2009). A study performed by Lueyot et al. (2020) verified Rodsuwan, Thumthanaruk, Kerdchoechuen, and Laohakunjit (2016) that JG extraction parameters like pH, temperature, time of pretreatment, and the extraction process initially performed on the jellyfish significantly affects the yield and strength of the gelatins being produced, which demonstrates the potential for JG being improved/optimized.

Marine gelatins extracted from cold-water fish have shown excellent functional and sensorial properties as a low-calorie fat substitute. Gelatin properties such as viscosity, gel strength, gelling and melting temperature are affected by factors like average molecular weight, molecular distribution, concentration of the gelatin solution, gel maturation time, gel maturation temperature, pH, and salt content (Karim & Bhat, 2009). Some gelatin properties such as gelling

and melting temperatures can be increased by crosslinking with hydrocolloids, polymers, plant phenolics, genipin, and enzymes like transglutaminase (Karim & Bhat, 2009). Currently, the food industry is in search of crosslinking agents that are non-toxic, non-specific, inexpensive, and readily available. Strauss and Gibson (2004) demonstrated that grape juice, coffee, and purified caffeic acid can be successfully crosslinked with Type A mammalian gelatin under oxidizing conditions. The study demonstrated that the three PPs crosslinked with the gelatin each improved the rigidity of the gelatin. In addition, the crosslinked gelatin showed increased mechanical strength and thermal stability but less ability to expand and absorb water (Strauss & Gibson, 2004). The gelatins created in this study also exposed fewer ionic groups making it more lipophilic, perhaps this can mimic fat globules and be used in low-fat foods as a reduced calorie fat replacer, flavor binder, or texturizer. Zhao and Sun (2017) reported successful crosslinking of bovine gelatin with PPs from *fructus chebulae*, or Helile, and found that crosslinking at 360 µg PPs/g of gelatin resulted in the highest thermal stability and the strongest mechanical properties in the gelatin hydrogels. The study also reported that polyphenolic crosslinking of gelatin was more cost-effective than genipin crosslinking, the most common natural crosslinking agent. Etxabide, Urdanpilleta, Gómez-Arriaran, De La Caba, and Guerrero (2017) demonstrated that gelatin films processed at pH 5.4 had higher decoloration and lower solubility which signified a greater extension of crosslinking compared to pH 2.0.

Additionally, other techniques may be used to improve gelatins; for example, Sow, Toh, Wong, and Yang (2019) demonstrated that the addition of Na-Alg into fish gelatin (FG) at a concentration of 0.4% (w/v) had the highest gel strength compared to various other Na-Alg concentrations, FG alone, and porcine gelatin alone. The modification of pH is another means to modify gelatins; for example, this was demonstrated by Etxabide et al. (2017) mentioned above

along with Li, Li, Li, Yang, and Jin (2020) where the team mentioned that zeta potential is critical in understanding the stability of collagen. When the zeta potential approaches zero, for most collagens, they begin to form aggregates within solution and this is considered to be the isoelectric point (pI) of that collagen. It has been noted that collagen extracted from different organisms tend to have differing pIs due to variations in their respective amino acid profile. For example, a notable difference is in organisms that tend to have more acidic amino acids like glutamic acid they tend to have lower pIs demonstrated by Li et al. (2020). Additionally, Nurul and Sarbon (2015) performed similar research and looked at the effect of pH adjustment on functional, rheological, and structural properties of eel skin gelatin. The researchers showed that eel skin treated at a higher pH, 8 instead of 5, exhibited higher emulsifying, foaming, fat binding, gel strength, gelling and melting temperature, and viscoelasticity properties (Nurul & Sarbon, 2015). In Osorio, Bilbao, Bustos, and Alvarez (2007), 3 different Bloom strengths of gelatins (180, 220, 240) were obtained and were prepared at 3 concentrations (5, 7, and 10% w/v) at various pH's ranging from 3 to 9. The melting (T_m) and gelling (T_g) temperatures increased with an increase in concentration for all gelatin strengths and pH values from 3 to 6. The T_g and T_m increased with increasing concentration but the temperatures were not affected by applied frequencies and were greatly affected by pH ranging from 3 to 6. Every gelatin demonstrated higher storage modulus values (more elasticity) than loss modulus (less viscosity) for temperatures lower than T_m. When holding concentration and pH constant but increasing gel strength, the T_m decreased and T_g increased. The study also produced a model which correlates T_m and T_g with pH and concentration at a fixed gel strength (Osorio et al., 2007).

The data collected in this study can be extremely useful in finding new and innovative ways to make suboptimal gelatins into gelatins with more ideal properties for use in products

ranging from foods to cosmetics, pharmaceuticals, or even medical applications. To date, there is no known scientific literature reporting the effect of polyphenolic crosslinking on the mechanical properties of JG. Therefore, research done in this area can add major contribution to the utilization of jellyfish for gelatin and naturally crosslinking for improvement of gelatins.

Microencapsulation & Probiotics

The feasibility of improving the survival of LRGG by microencapsulating the probiotic with JG+PPs and JG+Na-Alg will also be assessed in this study. Microencapsulation has been used for several decades to improve the stability of colors, flavors, PUFAs, vitamins, probiotics, etc. Microencapsulation of probiotics has been a hot topic recently; therefore, we propose to study the cell viability of LRGG after microencapsulating the probiotic with JG.

Microencapsulation is a process where thin films or polymer coats are applied to small solid particles, droplets of liquids or even gases (Jackson & Lee, 1991). The first microencapsulated drug was developed in 1931 and commercial microencapsulation has been around since the late 1930s. This process has evolved and can now be used to improve cell viability of bacterial cultures (Ann et al., 2007). According to Jackson and Lee (1991), microencapsulation requires the use of a wall material or microencapsulating agent (e.g., gums, lipids, hydrocolloids, carbohydrates, and even proteins like gelatin). The selection of the microencapsulating agent depends on the chemical and physical properties of the core material AKA the bioactive or probiotic bacteria, in this case. Generally, microencapsulation involves (1) forming an emulsion or suspension of the microencapsulating agent with the bioactive/probiotic; and (2) removing excess moisture that results in the production of powdered products.

Probiotics are live microorganisms which when administered in adequate amounts confer health benefits to the host, specifically, but not exclusively, in humans (Reid, 2016). The

probiotic that is of interest in this study is LRGG which is a gram positive, lactic acid-producing probiotic strain. The origin of LRGG is thought to have been isolated in 1983 from the human GI tract (Gorbach, 1990). LRGG has shown positive effects in treating various diseases/illnesses like rotavirus, atopic dermatitis, and diarrhea (Blaabjerg, Artzi, & Aabenhus, 2017; Reid, 2016). Moreover, treating children with LRGG in 125 mg dosages at a concentration of 5×10^9 viable bacteria per gram solid for two weeks has been successful against *C. difficile* and antibiotic-associated diarrhea (Biller, Katz, Flores, Buie, & Gorbach, 1995; Blaabjerg et al., 2017).

One challenge of microencapsulating probiotics is to find a compatible microencapsulating agent that can protect each specific probiotic strain against environmental stressors (e.g., oxygen, water, temperature, pH changes) which ultimately affect the viability of the bacterial cells during storage and in the gastrointestinal tract (Liu et al., 2019). It has been reported that microencapsulation by spray drying can improve the cell viability of LRGG over a period of 6-months using a double-encapsulation technique (Sohail, Turner, Coombes, & Bhandari, 2013). Our research group has successfully microencapsulated LRGG with FG by mixed-flow spray-drying (MFSD), a process where the air flow is against the injected atomized product. The resulting probiotic powders had higher biofilm forming capabilities over probiotic powders produced by concurrent spray-drying (CSD) (air flow is with the injected atomized product), freeze drying, and the non-encapsulated cells. This may indicate that MFSD can cause the probiotic strain to survive better and perhaps persist in the human gut longer over the other methods utilized (Jiang, Dev Kumar, Chen, Mishra, & Mis Solval, 2020). Additionally, extracted PPs from the pomegranate peels, the crosslinking agent, is assumed to be ~6% hydrolyzable tannins (majority ellagitannins, specifically punicalagin isomers and punicalin) and less than 1% catechin followed by quercetin, epicatechin, etc., which was determined from analysis of

individual phenolic compounds in six different cultivars of Georgia-grown pomegranates (Pande & Akoh, 2009). Due to a majority of the PPs in the peel being hydrolyzable tannins, this will act as the major binding agent during the crosslinking of JG.

Importantly, there is currently no research that compares how the cell viability of probiotics is affected by using a JG alone, JG+PPs, and JG+Na-Alg. It has been reported that using crosslinked gelatin as a microencapsulating agent for probiotics can improve cell viability by creating a more compact/denser wall material which can act to reduce oxygen and light exposure to the cells (Johnston, 2013; Zhao & Sun, 2017). Therefore, it is predicted that the PPs crosslinked with JG will reduce environmental stressors on the LRGG cells thus improving their viability during processing and storage. Overall, there have been numerous publications about the use of crosslinked mammalian gelatins as microencapsulating agents; however, there are no studies that microencapsulate probiotics using a naturally crosslinked JG.

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CHAPTER 1

ENGINEERING NOVEL GELATIN POWDERS FROM SALTED AND DRIED CANNONBALL JELLYFISH (*STOMOLOPHUS MELEAGRIS*): A FEASIBILITY STUDY[1]

[1] Chiarelli, P., & Mis Solval, K. To be submitted to *Food Hydrocolloids*.

Abstract

Cannonball jellyfish contain high collagen content, which has great potential of becoming an excellent raw material for developing novel ingredients. The aim of this study was to determine the feasibility of hydrolyzing jellyfish collagen for producing food-grade gelatin powders from salted, dried jellyfish (SDJ). SDJ was hydrolyzed using a novel acid hydrolysis method. After 4.5 h, the degree of hydrolysis was 21.22±0.51%. Hydrolyzed-SDJ (H-SDJ) powders had higher moisture than the non-hydrolyzed SDJ (U-SDJ) (control powder). The ash content (%, dry basis) and lightness of U-SDJ powders were significantly (p<0.05) higher than the H-SDJ powders. Both, H-SDJ and U-SDJ powders showed significant amounts of Na, Al, S, and K. Remarkably, H-SDJ powders were able to produce gelatin gels with Bloom values (g) of 3.40±0.30; while SDJ powders did not form gelatin gels. This illustrates (for the first time) the feasibility of using a novel hydrolysis method to produce jellyfish gelatin (JG) powders from U.S.-caught SDJ with a strong potential for improvement. Powders showed irregular morphologies and with a D_{50} particle size (μm) of H-SDJ and U-SDJ of 12.81±1.38 and 1.81±0.02, respectively. This study demonstrates the feasibility of producing JG powders from SDJ with potentially numerous food applications as a functional ingredient.

Keywords: Cannonball jellyfish, collagen, gelatin, Bloom, hydrolysis

Introduction

The world's gelatin market is expected to be worth 6.7 billion USD in the year 2027 (Grand View Research, 2020). With an increasing demand for trendy collagen and gelatin-based products not to mention a human population expected to reach 9 billion by 2050, finding alternative and sustainable sources of collagen/gelatin will be crucial over the next few years. For centuries, edible jellyfish has been considered a delicacy in countries such as China, Japan, Malaysia, and Korea not only for its sensorial attributes, but also for its nutritional composition (low in calories, fat and cholesterol, and high in vitamin B12, Mg and Fe) (Future of Food Report, 2019; Leone, Lecci, Durante, Meli, & Piraino, 2015). Several species of edible jellyfish have been identified worldwide including Cannonball jellyfish (CBJ), which is found in the coastal waters of the South Atlantic Bight, a region off the coast of Georgia, and the Gulf Coast of the U.S. (Ang, Liu, & Huang, 1999; Fluech, 2018). CBJ is commercialized as salted and dried jellyfish (SDJ) that contains ~60-70% (w/w) moisture; ~16-25% (w/w) salt, ~5.5% (w/w) crude protein, 1.4% (w/w) fat and provides 36 kcal per 100 g (Hsieh, Leong, & Rudloe, 2001; USDA, 2009). Additionally, edible jellyfish is a good source of type II collagen (insoluble in water), the raw material of gelatin (Zhu, Wang, He, & Wang, 2012).

Gelatin is a water-soluble protein hydrolysate obtained from the partial hydrolysis of collagen and is widely used by the food, cosmetic, and pharmaceutical industries because of its great functionalities given by its surface-active properties. Food-grade gelatins are used in confections (chewiness, texture, and foam stabilization), low-fat spreads (creaminess, fat reduction, and mouthfeel), dairy (stabilization and texturization), baked goods (emulsification, gelling, and stabilization), meats (water-binding, stabilization), as well as a lowering caloric density agent (Karim & Bhat, 2009). Moreover, gelatin can be used as a low-calorie thickener in

gluten-free products. Commercial gelatins are normally obtained from pig skin, bovine hide, and pork and cattle bones. However, collagen and gelatin from mammalian sources are under constraints and skepticism because of social, cultural and health-related concerns (Karim & Bhat, 2009). Thus, the production of gelatin from marine sources, like fish and jellyfish, has been constantly rising over the past few decades. Extraction and characterization of marine gelatins from fish scales, skin and bones have been reported (Leone et al., 2015). According to Karim and Bhat (2009), several challenges have been associated with fish gelatin, including poor rheological properties, insufficient availability of raw materials, variable quality, off-flavors, production costs and low yields. Consequently, fish gelatin is not a popular product in the market and its costs can be 4-5 times higher than the cost of bovine and porcine gelatins. Remarkably, marine gelatins have unique functional properties (e.g., lower gelling and melting temperatures compared to mammalian gelatins, which allows a quick release of encapsulated aromas and flavors) that may be of great interest to the food industry (Karim & Bhat, 2009). Marine gelatins can be excellent encapsulating agents for vitamins, bioactives, colors, flavors and probiotic bacteria because of their emulsifying, gelation and stabilization properties while specifically gelatins extracted from cold-water fish have shown beneficial functional and sensorial properties as a low-calorie fat substitute (Borza et al., 2010; Dickinson & Lopez, 2001; Karim & Bhat, 2009; Li, Chen, Cha, Park, & Liu, 2009).

The feasibility of producing gelatin from three species of jellyfish *Acromitus hardenbergi*, *Rhopilema hispidum* and *Rhopilema esculentum* has been reported with promising results through acidic and alkaline hydrolyses methods using harsh chemicals like NaOH, HCl, and sulfuric acid (Chancharern, Laohakunjit, Kerdchoechuen, & Thumthanaruk, 2016; Cho, Ahn, Koo, & Kim, 2014; Karim & Bhat, 2009; Khong et al., 2016). However, no studies have

reported the feasibility of producing gelatin from SDJ. According to Bonaccorsi, Garamella, Cavallo, and Lorini (2020), edible jellyfish is relatively safe in terms of the allergenic and microbial risks.

It has been suggested that global jellyfish populations have increased and jellyfish "blooms" (outbreaks of jellyfish) have been reported at a higher frequency worldwide since the 1950s (Brotz, Cheung, Kleisner, Pakhomov, & Pauly, 2012). This has affected local economies by interfering with tourist activities and coastal mariculture operations, decreasing fishing efficiencies, clogging power plants' intakes, and consuming eggs and larvae of commercially important aquatic species, which can lead to ecologically imbalances (Patwa et al., 2015). These jellyfish trends have been linked with global changes (e.g., eutrophication and global warming) driven by the increased number of human activities (Duarte et al., 2013). A number of these factors mentioned have reduced the number of natural jellyfish predators and promoted the growth and survival of jellyfish worldwide (Leone et al., 2015). Because of the gradual increase in the abundance of jellyfish, it is expected that its consumption will be adopted by Western societies interested in novel foods.

To date, SDJ (produced from traditional Chinese methods developed more a hundred years ago) is the only commercial product of the emerging U.S. jellyfish industry. Unfortunately, food product innovation has been historically limited in the U.S. jellyfish industry because of its relative newness and the lack of interest from the public and private sectors to promote this emerging fishery. While some researchers have reported on the potential for developing pharmaceutical and food applications from Mediterranean jellyfish, no studies have evaluated the feasibility of developing novel food ingredients from CBJ (De Domenico, De Rinaldis, Paulmery, Piraino, & Leone, 2019; Leone, Lecci, Durante, & Piraino, 2013). Recognizing the

new opportunities to introduce jellyfish-based products in the Western diet, our research group has hypothesized that novel food ingredients, such as gelatin, may be produced from collagen derived from SDJ. Therefore, the aim of this study was to determine the feasibility of hydrolyzing jellyfish collagen for producing food-grade gelatin powders from SDJ.

Materials/Methods

Materials

Salted, dried cannonball jellyfish (SDJ) were purchased from Golden Island International LLC (Darien, Georgia, USA). Food-grade citric acid was from Milliard (New Jersey, USA). Di-sodium-tetraborate decahydrate, sodium-dodecyl-sulfate (SDS), *o*-phthalaldehyde (OPA), dithiothreitol (DTT), and serine for determining the degree of hydrolysis were all purchased from Millipore Sigma (St. Louis, MO, USA).

Preliminary characterization of SDJ

SDJ was divided into umbrellas and oral arms for preliminary characterization. Then, SDJ samples were analyzed for moisture, ash content, and crude protein by following the AOAC Official Method 934.01 (oven drying), 938.08 (furnace combustion), and 993.13 (Nitrogen (total) dry combustion), respectively (AOAC, 2019). Crude protein was determined by following the Dumas method which quantified the total nitrogen by a dry combustion method using an automated nitrogen analyzer (Rapid N Exceed, Elementar, Langenselbold, Germany), described according to Jung et al. (2003). A conversion factor of 5.8 was used to determine crude protein (Khong et al., 2016). Water activity (a_w) values were obtained with a water activity meter (AquaLabSeries 3 TE, Decagon Devices, Inc., WA, USA). Color values (L*, a*, b*, chroma, and hue angle) were determined using a Lab Scan XE Colorimeter (Hunter Associates Laboratory, Inc., Resbon, VA, USA).

Production of gelatin powders from SDJ

The process diagram for producing gelatin powders from SDJ is presented in Figure 1.1. Approximately 1 kg of SDJ was rinsed and soaked in 8 L of tap water overnight for rehydration and removal of excess salt. Rehydrated SDJ was rinsed with clean tap water, chopped, and then soaked in a 3 L citric acid solution (1.5% w/v) for 10 min. Afterwards, SDJ was drained of excess citric acid solution, blended (Model BL610, NINJA, SharkNinja Operating LLC, Needham, MA, USA) together at medium power for 8 min and then at high power for 4 min. This procedure provided a composite sampling for the jellyfish. The jellyfish solution was then processed with an ultra-shearing homogenizer (Homogenizer 850, Fisherbrand, Fisher Scientific UK Ltd, Loughborough, UK) at 8000 rpm for 6 min then at 10000 rpm for 8 min, until a visibly thin liquid jellyfish product was observed. Then, about 350 mL of liquified jellyfish were placed into 600 mL beakers, covered with aluminum foil and incubated at 60°C for 4.5 h in a water bath (Model 2872, Precision, Thermo Electron Corporation, Waltham, MA, USA) to allow the hydrolyzation of the jellyfish collagen. Afterwards, the hydrolyzed SDJ was frozen at -4°C for 12 h and freeze-dried at -55°C for 2 days. Then, gradual increasing of the chamber temperature to 20°C over 3 days was performed using a pilot-scale lyophilizer (Virtis, The Virtis Company, Gardiner, NY, USA). Freeze-dried samples were pulverized using an electric grain grinder mill (Model SUS304, SLSY & MOONCOOL, Shanghai, China) to obtain the hydrolyzed SDJ powders (H-SDJ). Concurrently, un-hydrolyzed SDJ powders (U-SDJ) were prepared using the procedure described above (except the hydrolysis) and used as the control. Both, H-SDJ and U-SDJ powders were immediately stored in desiccators at 20°C until ready for analysis.

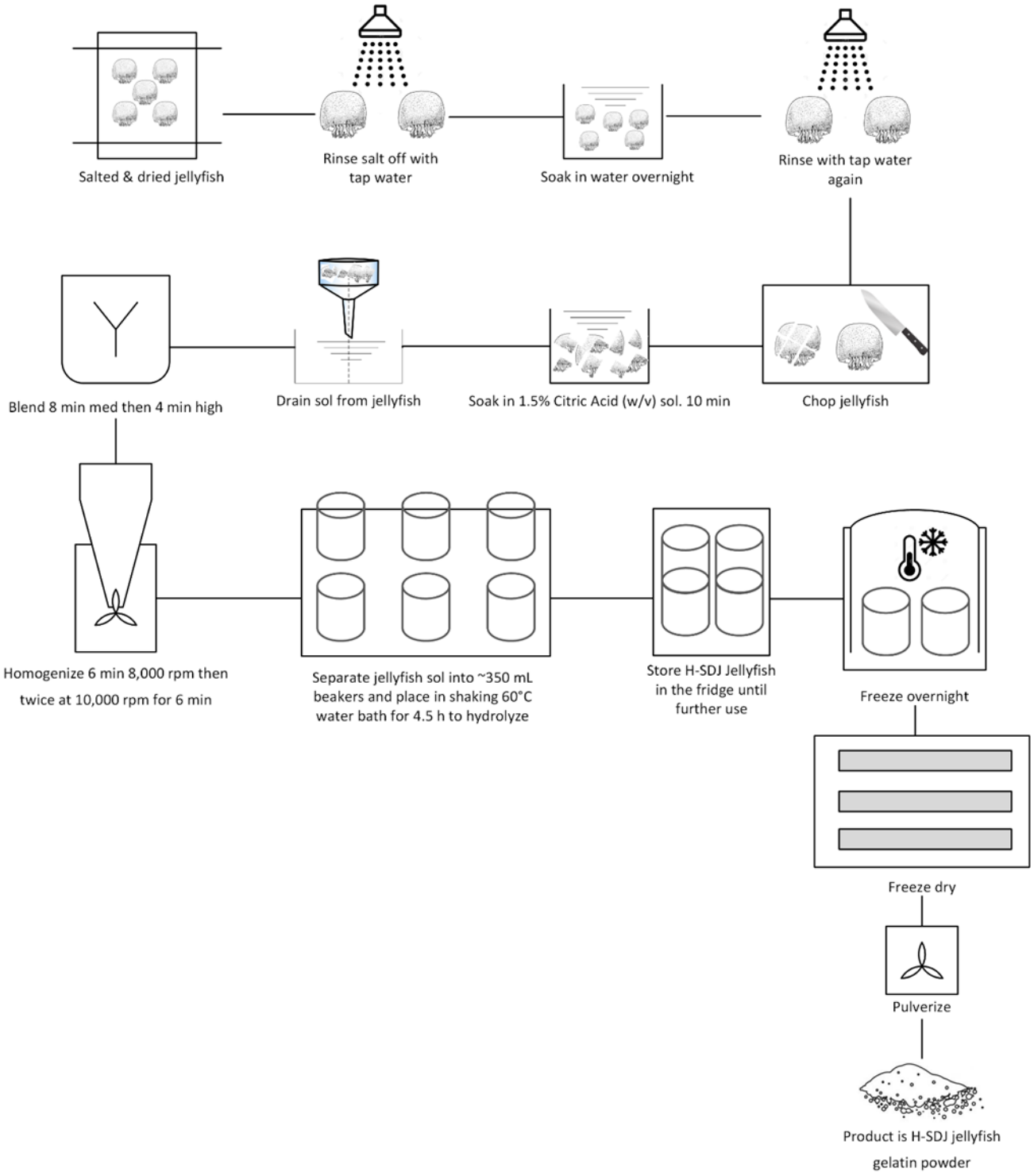

Fig. 1.1. – Process diagram to turn salted, dried jellyfish (SDJ) into hydrolyzed (H-SDJ) gelatin powder.

Degree of hydrolysis (DH)

The DH, or percentage of cleaved peptide bonds, is the factor being monitored when trying to determine the extent to which hydrolysis is occurring in a protein. DH was measured by following the OPA method described by Nielsen, Petersen, and Dambmann (2001). Briefly, samples of liquified jellyfish were collected during the hydrolysis procedure previously described in section 2.3. Aliquots of 9 mL of sample were taken every 0.5 h for the first 5 h then sampling continued every 1 h for an additional 5 h, and one last sample was taken 24 h after the incubation process had started. Liquified jellyfish samples were immediately placed into an ice-water bath to stop hydrolysis and then stored at 4°C until further analysis. An OPA reagent, serine, and sample solution were prepared according to Nielsen et al. (2001), then 3 mL of the OPA reagent was put into three sampling test tubes: standard, blank, and sample. A Genesys 30™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was blanked (OD of -0.001), then the serine solution (0.9516 meqv/L) was read which gave an OD of 0.675. Each sample was run in triplicate then the DH was calculated using Eqs. (1) and (2).

$$Serine - NH_2 = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} * 0.9516 \frac{meqv}{L} * 0.1 * \frac{100}{X*P} \qquad (1)$$

Where, serine-NH_2 = meqv serine NH_2/g protein; X = g sample (in this study, 1.0 g); P = protein % in sample (assumed to be 1); 0.1 is the sample volume in liters (L). h = (Serine-NH_2 – β) / α meqv/g protein, where α is given as 0.796, β is given as 0.457, and h_{tot} is given as 11.1, according to Nielsen et al. (2001).

$$Degree\ of\ Hydrolysis = \frac{h}{h_{tot}} * 100\% \qquad (2)$$

Physico-chemical properties of H-SDJ powders

Water activity (a_w) and moisture content

Moisture content of the powders was determined by the AOAC Official Method 934.01 (AOAC, 2019) using an Isotemp® Vacuum Oven (Model 281A, Thermo Fisher Scientific, Waltham, MA, USA). Water activity was determined using an Aqualab water activity meter (Model Series 3 TE, Decagon Devices, Inc., Pullman, WA, USA).

Ash content

The ash content of the powders was determined by following the general AOAC Official Method for ash analysis, performed in triplicate (Marshall, 2010). In short, the samples were weighed then placed into a muffle furnace (Model F-A170, Thermolyne, Dubuque, IA, USA) programed at 550°C for 12-18 h. The samples were then taken out, weighed, and the ash content was calculated using Eq. (3).

$$Percent\ Ash = \frac{Sample\ wt.\ after\ ashing\ (g)}{Original\ Sample\ wt.\ (g)} * 100 \qquad (3)$$

Color (L*, a*, b*)

Color of the powders was measured using a Lab Scan XE Colorimeter (Hunter Associates Laboratory, Inc., Resbon, VA, USA) and reported in CIE L.A.B color scales (L*, a*, and b* values). Petri dishes (100mm x 15mm) were filled with powder until it completely covered the bottom, the color was tested in triplicate then these values were averaged. Afterwards, calculating chroma and hue angle values were done using Eqs. (4) and (5).

$$Chroma = [(a^*)^2 + (b^*)^2]^{\frac{1}{2}} \qquad (4)$$

$$Hue = tan^{-1}(\frac{b^*}{a^*}) \qquad (5)$$

Mineral Analysis

The mineral analysis of H-SDJ and U-SDJ powders was determined by a Spectro Arcos Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Model FHS16, Ametek Inc., Berwyn, PA, USA) method and conducted at the Soil, Plant, and Water Laboratory of the University of Georgia (UGA). The minerals P, K, Ca, Mg, Mn, Fe, Al, B, Cu, Zn, Na, Pb, Cd, Ni, Cr and Mo were quantified using a microwave-acid (HNO_3) digestion, ICP method using CEM Mars5 microwave digestion system (Model 61E ICP, Thermo Jarrell-Ash, Franklin, MA, USA).

Particle size distribution

Pulverization of freeze-dried samples was standardized by blending in a SLSY 2500g Electric Grain Grinder Mill Machine (Model SUS304, SLSY & MOONCOOL, Shanghai, China) for 1 min then put through a fine sieve (mesh size 1/32 inch) before testing in the particle size analyzer. Powdered samples were tested for their particle size distribution using a Particle Size Analyzer (Model PSA 1190, Anton Paar, Austria) equipped with laser diffraction. H-DSJ and U-DSJ powders were placed in the unit's hopper and transported via Venturi/free fall to the analytical area where they were illuminated with three lasers from low to high angles and the diffracting light was then analyzed. Each sample utilized a 10 s run time with dispersion parameters of 40% vibrator duty cycle, 40 Hz vibrator frequency, and 1200 mBar of air pressure. The whole light scatter pattern was recorded and used to quantify the particle size distribution by the Fraunhofer reconstruction method, which quantifies the angular distribution of backscattered light. The data was reported as D_{10}, D_{50}, and D_{90}, which is the average volume diameter of the particles at 10%, 50%, and 90% cumulative volume, respectively. The span value (spread of

particles) was calculated by following the method referred to Mis Solval, Bankston, Bechtel, and Sathivel (2016).

Scanning Electron Microscopy (SEM)

SEM images of the powders was collected using a SEM (1450 EP, Carl Zeiss MicroImaging, Thornwood, NY, USA) in Athens, GA at the UGA - Georgia Electron Microscopy facility. The powdered samples were first sputtered-coated with gold and then images were collected using an acceleration potential of 2 kV, which provided the best sample morphologies. This process was previously used in our laboratory and described by Jiang, Dev Kumar, Chen, Mishra, and Mis Solval (2020).

Bloom value

The official method of the Gelatin Manufacturers Institute of America, Inc. (GMIA) for gel strength (Bloom) was used to determine the Bloom values of the H-SDJ and U-SDJ powders (GMIA, 2019). In short, 6.67% (w/w) (7.50±0.01 g dry wt. powders + 105.0±0.2 g deionized water) gelatin samples were weighed into 120 mL glass gelatin Bloom jars (Height = 59+1mm H. ∅ (diameter) = 85mm) (Brookfield Engineering, Middleboro, MA, USA). The samples were allowed to completely swell for 1-2 hours at room temperature. Next, dissolution was achieved by heating at 65°C for 15 min, swirling periodically. Afterwards, the gelatins were allowed to temper at room temperature for ~20 min, then the lids were wiped off and any foam/bubbles on the top of the samples were skimmed off. Maturation of the samples at 10°C was allowed for 17±1 h to form the gelatin matrix. After maturation, a texture analyzer (TA.XT Plus, Stable Micro Systems Ltd, Godalming, UK) was used to determine the Bloom strength of the gelatins at 4 mm penetration depth. The parameters used were a 12.7 mm diameter probe, which depressed

the surface of the gel by 4 mm at a speed of 1 mm/sec. The peak force in grams was recorded and this is known as the Bloom strength.

Statistical Analysis

Each of the experiments and analysis performed were conducted in triplicate. Mean values and standard deviations (SD) were calculated from the collected data. Analysis of variance (ANOVA) and post-hoc Tukey's studentized range tests ($\alpha = 0.05$) were conducted to determine the statistical significance of the observed differences among the means. This was performed using RStudio statistical software version 1.2.5033 (RStudio, Inc. Boston, MA, USA).

Results/Discussion

Preliminary characterization of SDJ as a raw material

Withing hours of the harvest, fresh jellyfish (highly perishable at room temperature) is hand separated into umbrellas and oral arms, cleaned, cured and dehydrated with a mix of table salt (NaCl) and alum ($KAl(SO_4)_2$) at room temperature. This ~20-40 day process reduces the moisture content of fresh jellyfish from 95-98 to about 68-70 (% w/w, wet basis) and allows for the conversion of a gel-like characteristic into a rubber-like texture with a crunchy feel common to SDJ, which has a shelf life up to two years (Hsieh & Rudloe, 1994; Hsieh et al., 2001). The results obtained in this study indicated that oral arms of SDJ had a slightly higher moisture content (71.28 g/100g) than the umbrellas (70.28 g/100 g) (Table 1.1). According to Zhu et al. (2012), table salt reduces moisture and helps maintain the microbial stability of jellyfish; whereas alum precipitates collagen, disinfects and hardens jellyfish tissues. Similar results are reported by Khong et al. (2016) for other species of edible jellyfish. Higher crude protein content (g/100g, dry basis (d.b.)) and ash content (g/100g, d.b.) were found in oral arms than in the umbrellas (Table 1.1). It has been reported that SDJ contains ~60-70% (w/w, wet basis (w.b.))

Table 1.1 – Preliminary characterization of salted, dried jellyfish (SDJ).

		Umbrellas	Oral arms
Moisture (g/100g, wet basis)		70.28 ± 0.59	71.28 ± 0.07[1]
Crude protein (g/100g, dry basis)		6.43 ± 0.67	7.96 ± 0.55[1]
Ash (g/100g, dry basis)		88.40 ± 0.04	88.67 ± 0.01[1]
Water activity (a_w)		0.76 ± 0.01	0.76 ± 0.02
Color	**L***	23.88 ± 1.41	34.48 ± 0.29[1]
	a*	5.56 ± 0.83	1.06 ± 0.40[1]
	b*	19.21 ± 2.96	13.18 ± 0.80[1]
	Hue	73.87 ± 0.71	85.39 ± 2.05[1]
	Chroma	20.00 ± 3.07	13.22 ± 0.77[1]

*Values are means ± SD of triplicate determinations.

*Means with [1] in the same row signify significant difference ($p<0.05$).

moisture; ~16-25% (w/w, w.b.) salt, ~5.5% (w/w, w.b.) crude protein, 1.4% (w/w, w.b.) fat and provides 36 kcal per 100 g (Hsieh et al., 2001; USDA, 2009). Furthermore, the a_w values of both oral arms and umbrellas were ~0.76, which indicates that the products are microbiologically stable at room temperature. A lower chroma (saturation) was observed in the oral arms compared to the umbrellas, meaning that the oral arms were more grey or less bright of a color than the umbrellas; while the umbrellas were more yellowish and oral arms were less reddish than the umbrellas (b* and a*, respectively) (Table 1.1). The change in color observed in SDJ samples may be due to the release of polyphenols that may been trapped in different tissues of the jellyfish (Leone, Lecci, Milisenda, & Piraino, 2019). Understanding the composition of SDJ is critical for developing customized food ingredients with tailored functional properties. Before consumption, SDJ is typically soaked in tap water overnight, sliced into strips, and consumed either raw or flash boiled. It can also be incorporated in several dishes including salads and noodles.

Degree of hydrolysis

The DH was monitored every 30 min for 5 h, every 1 h for an additional 5 h, and again at 24 h. The initial DH was 3.48±0.09% (Fig. 1.2), which may be due to the 10 min soaking in the citric acid solution used to initially soften the collagen of SDJ. Chang, Wang, Zhou, Xu, and Li (2010) reported a decrease in the thickness of fiber diameter and perimysial, or the sheath of connective tissue, during the marination of collagen-containing connective tissues with salts and/or weak organic acid solutions including citric acid. In this study, the DH rapidly increased from 3.96 to 20.11% during the span of 30 min to 3 h of incubation at 60°C. Similar results were reported in another study during the hydrolysis of tilapia skin, where the highest increment

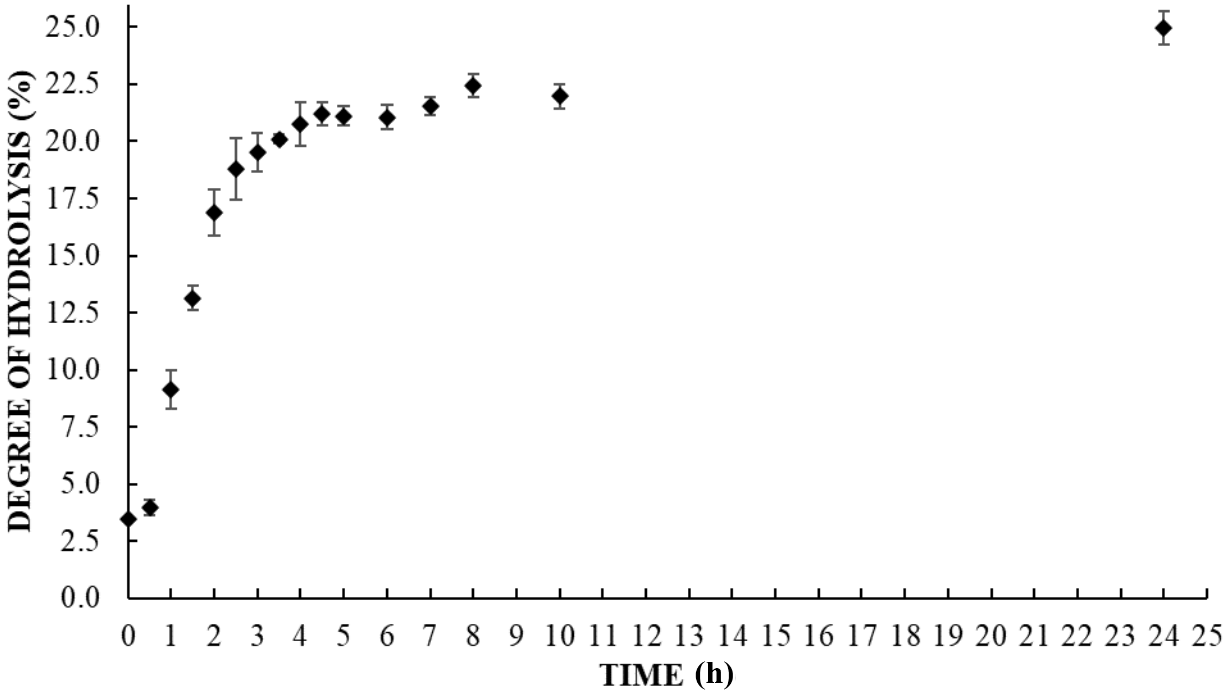

Fig. 1.2. – Monitoring the degree of hydrolysis of the salted, dried jellyfish (SDJ) over 24 h in a 60°C waterbath using the OPA method.

of DH was observed at the beginning of hydrolysis procedure and had the best hydrolysis at 60°C (Roslan, Kamal, Yunos, & Abdullah, 2015). It has been noted that the acid hydrolysis rate is high in the beginning due to rapid cleavage of peptide bonds and then begins to level out overtime (Senphan & Benjakul, 2014; Shahidi, Han, & Synowiecki, 1995). After 3 h, the DH plateaued at around 21% for the next 10 h. During the next 14 h, the DH only increased about 4% to 24.94±0.73%, which is where the plateau affect can be observed (Fig. 1.2). This plateau effect or slowing of hydrolysis for the jellyfish collagen is likely due to a decrease or total cleavage in the number of hydrolysis sites and/or inhibition of hydrolysis due to the presence of products (Senphan & Benjakul, 2014; Shahidi et al., 1995).

Similar DH research was performed using the jellyfish *Rhopilema esculentum* and received a 27.8% hydrolysis with trypsin and properase E (Zhuang et al., 2009). However, there have been no papers that monitored the DH of jellyfish using citric acid hydrolysis. The DH observed in this study of 21% after 4.5 h is very close to an expected range of 15-20% after 5 h of hydrolysis (Roslan et al., 2015). Giant catfish skin rapidly hydrolyzed to 43.51% in 2 h then plateaued at 45% over 12 h using enzymatic hydrolysis (Ketnawa, Benjakul, Martínez-Alvarez, & Rawdkuen, 2017); tilapia muscle had a DH of 25.16% using an enzymatic hydrolysis which was measured by the OPA method (Roslan et al., 2015); and bigeye snapper had a DH of 3.54 to 4.92% measured using a modified trinitrobenzenesulfonic acid (TNBS) assay, a method used to determine the number of free amino groups after hydrolyzation of a protein (Sukkwai, Kijroongrojana, & Benjakul, 2011). Preliminary studies conducted in our lab have suggested that at 4.5 h of an acid hydrolysis, an ideal jellyfish gel matrix is formed after refrigerated storage for 24 h (data not shown). Ahmed and Chun (2018) reported that the DH time highly influences the molecular weight of peptides and the terminal amino groups in the final product. Therefore, the

time of hydrolysis ultimately determines the specific size and number of peptides being formed, which leads to better or worse gel formation. In our study, 4.5 h of incubation created an ideal gelatin gel matrix. In the future, determining the peptides weight and number is important to understanding how the gelatin will behave in regards to cross-linking, antioxidant behavior, and the health benefits to the host when consumed. It has been mentioned that acid hydrolysis may be a cost-effective method to produce jellyfish or fish protein hydrolysates for use in the food/feed industry (Wisuthiphaet, Kongruang, & Chamcheun, 2015).

There have been 27 identified forms of collagen but Type I, II, and III are the most predominant forms (Schrieber & Gareis, 2007). A study using Ribbon jellyfish (*Chrysaora* sp., morphotype 1) found that the species predominantly had Type II collagen (Barzideh, Latiff, Gan, Benjakul, & Karim, 2014). Gelatin is formed when collagen is hydrolyzed with either an acid or alkali solution that partially breaks down the collagen fibers (Karim & Bhat, 2009). Depending on the type of solution used, Type A or B gelatin can be formed. In short, when treated with an acid solution, Type A gelatin is formed and Type B is formed when treated with an alkali solution. Differences in their physicochemical properties arise from this variation in treatment; for example, typically their viscosities and isoelectric points (pI) differ as Type A gelatins pI range from 7 to 9 while Type B gelatins pI range from 4.8 to 5.1 (Djagny, Wang, & Xu, 2001). Currently, no studies have reported the production of gelatin from Cannonball jellyfish; however, one study demonstrated a Type A gelatin extraction and tested the properties of the gelatin produced from the jellyfish *Lobonema smithii* (LS), while the second paper improved the Type A gelatin by optimizing the treatment and extraction conditions from LS (Lueyot et al., 2020; Rodsuwan, Thumthanaruk, Kerdchoechuen, & Laohakunjit, 2016). On the other hand, Type B gelatin was produced from the jellyfish *Rhopilema hispidum* and the physicochemical properties

were assessed (Cho et al., 2014). Both signify the possibility to produce either Type A or B gelatin from different jellyfish species.

Physicochemical properties of U-SDJ and H-SDJ

Moisture content and water activity (a_w)

Moisture content is the mass of water present in a known mass of a sample (Reeb, Milota, & Association, 1999). H-SDJ and U-SDJ had a moisture content (g/100g) of 4.82±0.32 and 1.08±0.25, respectively (Table 1.2). This may be due to U-SDJ having more minerals, especially Na from NaCl (Boudhrioua, Djendoubi, Bellagha, & Kechaou, 2009). Typical commercially produced bovine and porcine gelatins have a moisture content (g/100g) in the range of 9 to 12 (Rahman, Al-Saidi, & Guizani, 2008). Jellyfish gelatin powders produced from *Lobonema smithii* and *Rhopilema hispidum* via oven drying had moisture contents (g/100g) of 6.8 and 12.2, respectively (Cho et al., 2014; Lueyot et al., 2020). The lower moisture of H-SDJ and U-SDJ powders compared to the jellyfish gelatins produced via oven drying may be due to an extensive freeze-drying process or due to the excessive salt found in these samples.

It is known that a_w measures available free water in foods, which is responsible for specific biochemical reactions and is a crucial index in assessing the microbial stability, lipid oxidation, texture/mouthfeel, and enzymatic and non-enzymatic activities (Sablani, Kasapis, & Rahman, 2007; Solval, Sundararajan, Alfaro, & Sathivel, 2012). A variety of factors may influence moisture and the a_w of dried powders including drying time and conditions, storage conditions, number of solutes, chemical composition of product, and more depending on the product being used. H-SDJ and U-SDJ had a_w values of 0.089±0.02 and 0.092±0.00, respectively (Table 1.2). A typical range for the a_w values of dry powders is < 0.2. This low a_w indicates a very dry product and confirms the efficacy of the freeze-drying process. Having a a_w under 0.6 is

Table 1.2 – Physicochemical properties of the salted, dried jellyfish (SDJ), unhydrolyzed SDJ powders (U-SDJ) and hydrolyzed SDJ powders (H-SDJ).

Properties	**U-SDJ**	**H-SDJ**
Moisture (g/100 g)	1.08 ± 0.25	4.82 ± 0.32***
Water Activity (a_w)	0.092 ± 0.00	0.089 ± 0.02
Ash (g/100 g)	88.53 ± 0.14	56.61 ± 0.13***
Bloom (g)	<1.0	3.40 ± 0.30***
L*	77.44 ± 0.02	73.09 ± 0.05*
a*	0.52 ± 0.04	1.79 ± 0.03***
b*	4.14 ± 0.05	8.02 ± 0.02***
Hue	82.87 ± 0.54	77.43 ± 0.21***
Chroma	4.18 ± 0.05	8.21 ± 0.02***

*Values with * signify significant difference ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).
*Values are means ± SD of triplicate determinations.

typically considered to be microbially stable (Quek, Chok, & Swedlund, 2007).

Color

The color values of H-SDJ and U-SDJ powders are reported in L* (perceived lightness), a* (red/green), b* (blue/yellow) (Table 1.2). The calculated hue measures the property of a color based on the ratio of a* and b* while the chroma indicates the saturation of color intensity (Quek et al., 2007). The color, seen in Figures 1.3 and 1.4, shows H-SDJ compared to U-SDJ. From the picture it demonstrates that U-SDJ has a brighter white color compared to H-SDJ, which appears more as a light, pale-yellow powder. When comparing them based on the received values (L*, a*, b*) for H-SDJ and U-SDJ, the L* of 73.09±0.05 and 77.44±0.02, a* of 1.79±0.03 and 0.52±0.04, and b* of 8.02±0.02 and 4.14±0.05, respectively. Color values of U-SDJ were significantly (p<0.05) different than those of H-SDJ. This data confirms that the U-SDJ is lighter than the H-SDJ. A minimal red color in U-SDJ and more in H-SDJ (higher positive a*) and a slightly more yellowish tint (higher positive b*) in H-SDJ than U-SDJ, thus the perceived color correlates with the calculated L*, a*, b* values. The low chroma of 4.18±0.05 for U-SDJ compared to 8.21±0.02 for H-SDJ and a high hue of 82.87±0.54 for U-SDJ compared to 77.43±0.21 for H-SDJ both are significantly (p<0.05) different from one another. The visual appearance of U-SDJ is brighter white while H-SDJ shows a yellowish tint. A similar result was observed in the salted, dried jellyfish *Lobonema smithii*, where the initial product was light yellow and after hydrolysis the gelatin powder became darker in color into a light brown color (Lueyot et al., 2020). Again, this was observed in Type A gelatin from the jellyfish *Lobonema smithii* where the gelatin after hydrolysis was an orange/yellow color compared to bovine gelatin which appeared lighter in color (Rodsuwan et al., 2016). Lueyot et al. (2020) reported that the resulting darkening of the color is plausible due to the Maillard browning reaction, which could

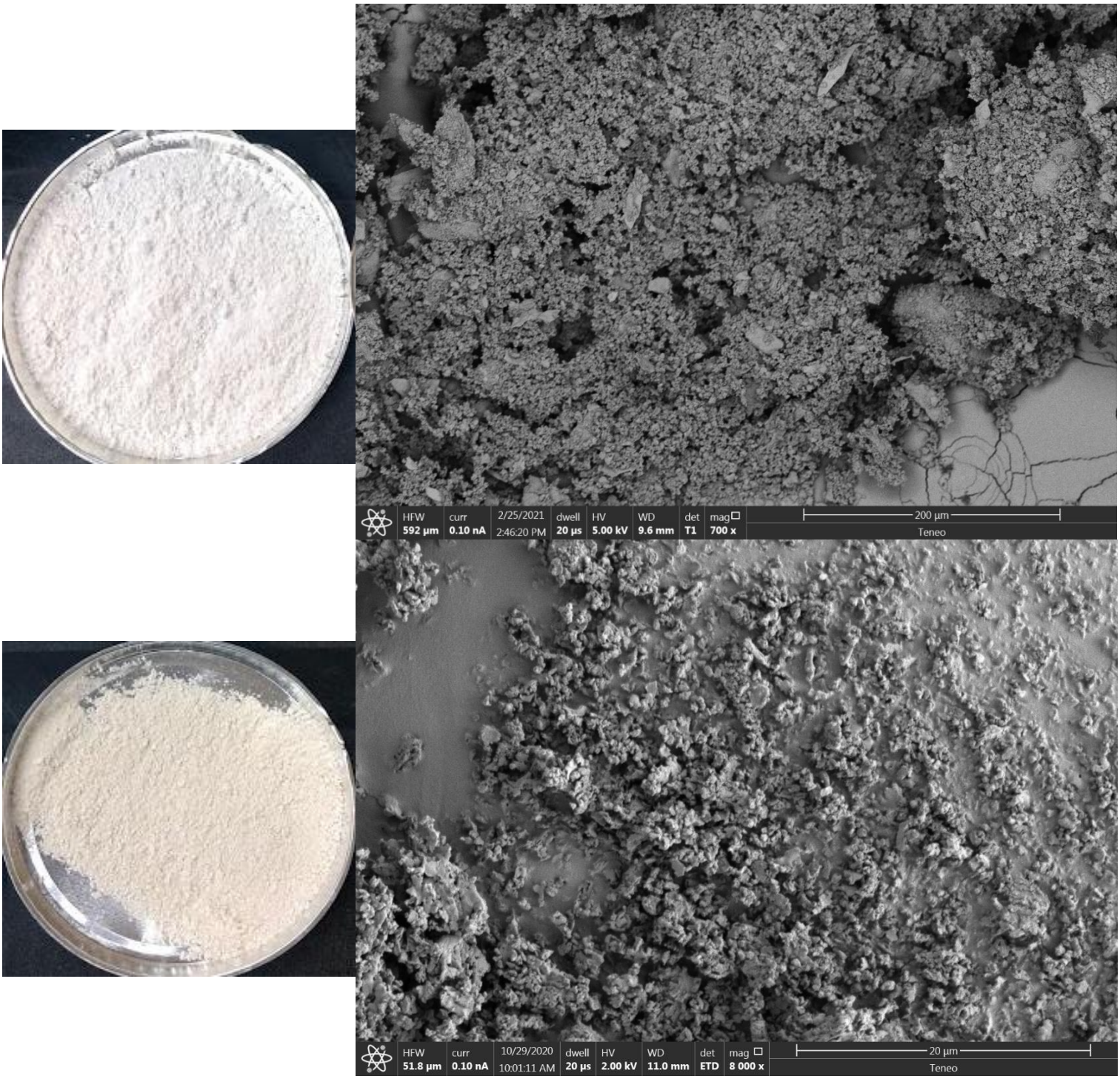

Fig. 1.3. (*top*) & 1.**4.** (*bottom) – top* – Powder (*left*) & Scanning electron microscopy (SEM) (*right*) images of unhydrolyzed salted, dried jellyfish (U-SDJ); *bottom* – Powder (*left*) & Scanning electron microscopy (SEM) (*right*) images of hydrolyzed salted, dried jellyfish (H-SDJ).

have developed between reducing sugars and amino acids as a result of the long time at increased temperatures during the hydrolysis process. When preparing gelatin, color does not affect the physicochemical properties but typically a more neutral color is preferred as it will have less of an impact when added into a product (Shyni et al., 2014).

Ash content and mineral analysis

According to XRF Scientific, ICP-MS is an analytical method that utilizes the ionization of a sample by hot plasma derived from argon gas. Once the ions are created, they are then passed into a mass spectrometer where the ions are separated by their mass-to-charge ratio which ultimately allows the user to determine the type and amount of minerals that are present within a sample, in this case the U-SDJ and H-SDJ jellyfish gelatin powders (What is ICP Spectroscopy, 2015). Foremost, the ash content (g/100g) of U-SDJ (88.53±0.14) was significantly (p<0.05) higher compared to that of H-SDJ (56.61±0.13) (Table 1.2). U-SDJ went through no previous washing and soaking steps to remove excess minerals like the H-SDJ, so it should contain a higher ash content, predominantly from sodium, illustrated in Table 1.3. A majority of the minerals present were in relatively small quantities besides sodium and aluminum (Table 1.3). The traditional salting process that is used to make SDJ employs approximately 1 kg of salt:alum mixture at a ratio of 1:10 per 8-10 kg of fresh jellyfish where it is then left to brine for ~20-40 days (Hsieh et al., 2001). Consequently, the ash content is high in both the U-SDJ and H-SDJ due to the initial high amounts of salt and alum that gets absorbed into the product, but this is higher in U-SDJ because no previously washing/soaking was performed. The Na and Al concentration (ppm, d.b.) in the U-SDJ were 372,541±10,686 and 970±62 while the H-SDJ powders had 227,283±1,069 and 4,260±18, respectively. Both concentrations are significantly different (p<0.05) from each other but in opposite ways as the U-SDJ contains more sodium

Table 1.3 – Mineral profile of unhydrolyzed salted, dried jellyfish (U-SDJ) and hydrolyzed-SDJ (H-SDJ) powder.

Element	Units	U-SDJ	H-SDJ
Ca	(g/100g, d.b.)	0.10 ± 0.00	0.08 ± 0.00**
K	(g/100g, d.b.)	0.20 ± 0.02	0.13 ± 0.01**
Mg	(g/100g, d.b.)	0.15 ± 0.01	0.11 ± 0.00***
P	(g/100g, d.b.)	0.02 ± 0.00	0.11 ± 0.00***
S	(g/100g, d.b.)	0.21 ± 0.01	0.31 ± 0.01***
Al	(ppm, d.b.)	970 ± 62	4260 ± 18***
B	(ppm, d.b.)	< 2.0	< 2.1
Cd	(ppm, d.b.)	< 0.8	< 0.8
Cr	(ppm, d.b.)	< 1.0	15.78 ± 0.32***
Cu	(ppm, d.b.)	< 1.5	16.81 ± 0.91***
Fe	(ppm, d.b.)	9.00 ± 1.91	126.74 ± 1.30***
Mn	(ppm, d.b.)	< 2.0	< 2.0
Mo	(ppm, d.b.)	< 1.0	< 1.0
Na	(ppm, d.b.)	372,541 ± 10,686	227,283 ± 1,069***
Ni	(ppm, d.b.)	< 1.0	13.68 ± 0.54***
Pb	(ppm, d.b.)	< 2.0	3.53 ± 1.37
Zn	(ppm, d.b.)	3.92 ± 0.50	14.58 ± 0.31***

*Values with * signify significant difference ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) b/t U-SDJ & H-SDJ gelatin.
*Values with < symbol were below detectable limits of the ICP-MS.

because less washing/soaking was performed compared to H-SDJ. However, the aluminum, and various other minerals, in the H-SDJ gelatin appeared to be higher than in U-SDJ. This may be explained due to the processing that occurs for the H-SDJ. As the preparation of H-SDJ requires washing/soaking, minerals (predominately sodium on the surface of the SDJ) are getting washed off while the aluminum from the salt:alum mixture may have already diffused beneath the surface. Because washing mostly removed the sodium, when tested for minerals after processing there is less sodium per known mass of sample but similar amounts of other minerals. Therefore, the concentrations of these minerals like Al and other minerals previously present in the jellyfish like S, Cr, Cu, Fe, Ni, and Zn appeared to significantly increase. Another plausible explanation for this phenomenon may be due to the minerals that were present in the tap water that got absorbed into the jellyfish when they were soaked overnight during the processing procedure.

The USDA considers SDJ a safe to eat food product and the nutritional information is available on the USDA's FoodData Central (USDA, 2009). However, some concerns have been surrounded around SDJ, specifically regarding its Na and Al concentrations. Diets that are high in Na are linked to hypertension (FDA, 2020). SDJ contains high amounts of Al from the addition of potassium aluminum sulfate, which reduces pH, prevents microbial spoilage, and acts as a hardening and firming agent (Hsieh et al., 2001). Moreover, it has been suggested that increased consumption of Al in the diet may play a role in the development of Alzheimer's and inflammatory bowel disease (Lerner, 2012; Pineton de Chambrun et al., 2014; Tomljenovic, 2011). A study performed in Hong Kong measured the amount of Al in 256 food products and found that ready-to-eat jellyfish products contained an average of 1200 mg of Al/kg. In addition with other Al-containing foods, individuals consumed on average 60% of the recommended maximum Al intake and 10% of that came from jellyfish; therefore, the study concluded that the

amount of Al in the diet is unlikely to cause adverse health effects on the general population (Wong, Chung, Kwong, Yin Ho, & Xiao, 2010). After washing/soaking the SDJ, the Na and Al levels are still present in high concentrations; therefore, finding alternative preservation methods and ways to further remove these minerals would be beneficial to consumers. Noteworthy is how one study demonstrated that sodium ions in increased concentrations led to interactions between ions and proteins, which caused large aggregates thus led to a disordered gel network and ultimately produced a weaker gel (Wang et al., 2018).

Particle size distribution

After standardizing the grinding process for the jellyfish gelatins, the particle size distribution data can be seen in Figures 1.5 and 1.6. The U-SDJ and H-SDJ exhibited a mean particle size D_{50} (µm) of 1.81±0.02 and 12.81±1.38, respectively. The particle size distribution graph for U-SDJ (Figure 1.5a) has 1 major peak and 2 minor peaks while the H-SDJ (Figure 1.6a) shows similar major and minor peaks with the addition of 2 major peaks at a larger particle size. The common small particle size peak may be due to the same minerals that are present in both samples, mainly Na and Al. As more minerals are present in the gelatin, the smaller the particle size. One possibility for larger particle sizes (>100 µm) may be due to an incipient agglomeration process, which is where the formation of irreversible link bridges, due to the hydrolysis of the proteins, may cause the production of the larger particle sizes observed (Tonon, Grosso, & Hubinger, 2011). Additionally, common peak sizes observed could be due to the powder grinder that was used. During the grinding process, it was observed that some powder caked up closer to the center of the blade causing more fine powder while powder towards the outside edges appeared more course. This may lead to the slight variation seen in the particle size distribution data. The D_{10} and D_{90} (µm), the average diameter of particles at 10% and 90% of the

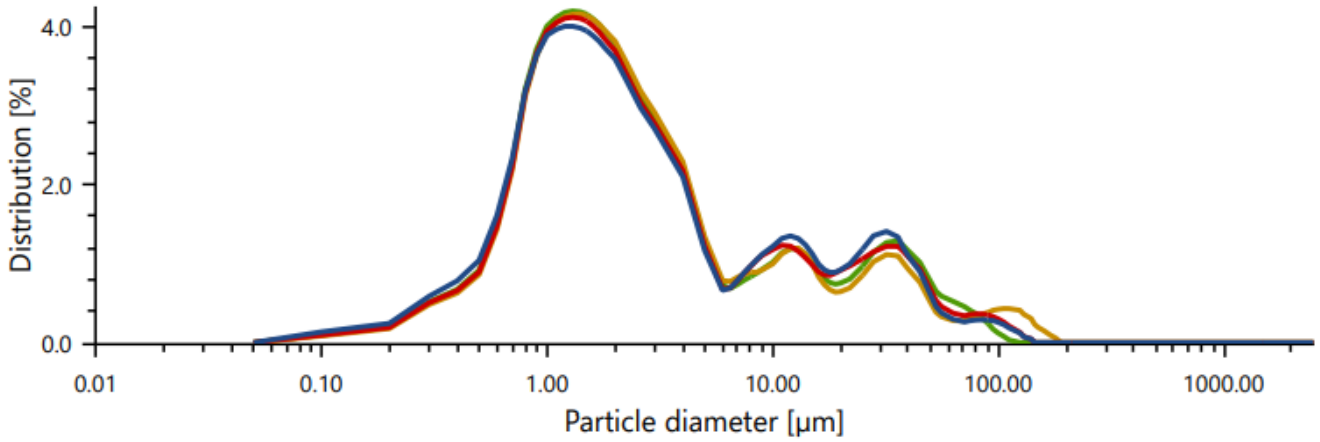

Fig. 1.5a. – Particle size distribution of unhydrolyzed salted, dried jellyfish powder (U-SDJ). *Different color lines indicate separate runs.

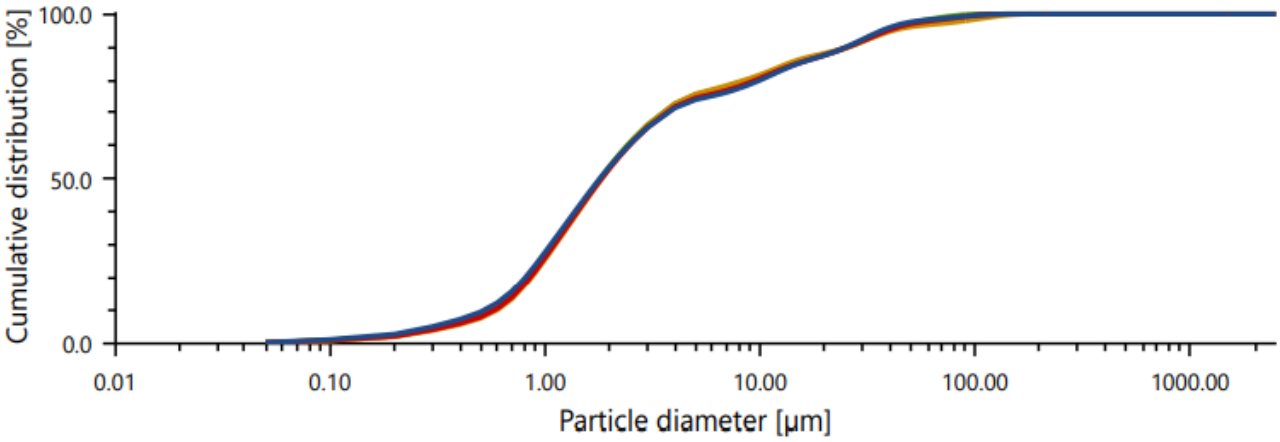

Fig. 1.5b. – Cumulative distribution (volume) - Undersize of unhydrolyzed salted, dried jellyfish powder (U-SDJ). *Different color lines indicate separate runs.

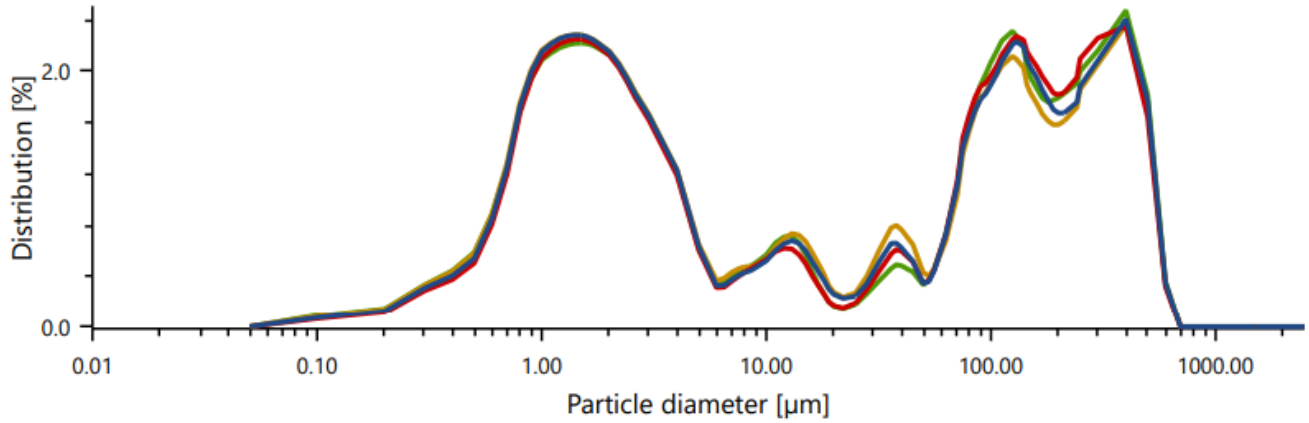

Fig. 1.6a. – Particle size distribution of hydrolyzed salted, dried jellyfish (H-SDJ) gelatin.

*Different color lines indicate separate runs.

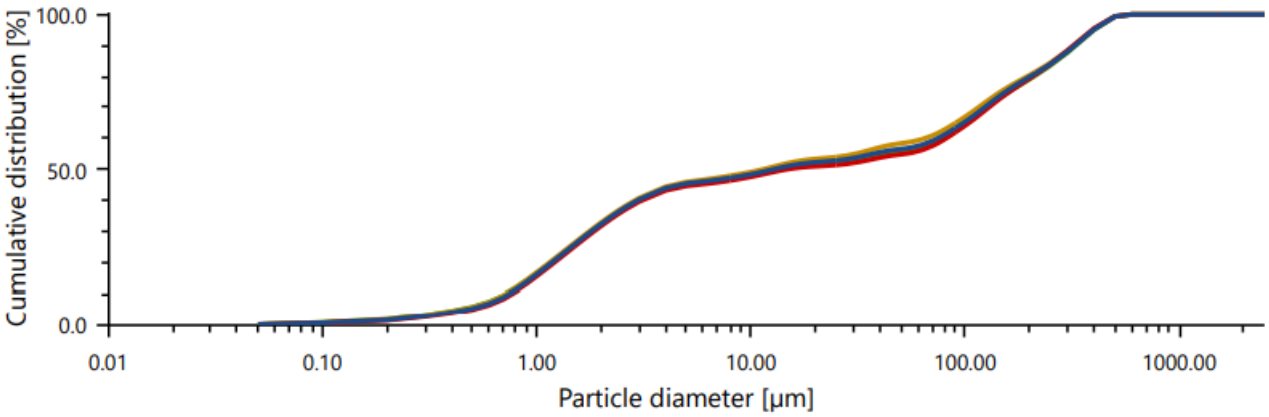

Fig. 1.6b. – Cumulative distribution (volume) - Undersize of hydrolyzed salted, dried jellyfish (H-SDJ) gelatin. *Different color lines indicate separate runs.

sample, for U-SDJ and H-SDJ was 0.57±0.03, 25.50±0.27 and 0.74±0.02, 324.12±3.56, respectively. These values in accordance with a span (μm) of 13.77±0.19 for U-SDJ and 25.47±2.69 for H-SDJ explains that U-SDJ has a much smaller particle size overall indicated by the much smaller D_{90} value and span value compared to H-SDJ, which can be further explain by Figures 1.5b and 1.6b. For U-SDJ, at a particle size of 10 μm or lower ~80% of the particles have been accounted while only ~50% were accounted for in H-SDJ, signifying that there are more larger sized particles in H-SDJ than U-SDJ. Again, the larger particles observed in H-SDJ is speculated to be due to either an agglomeration process or the freeing of protein molecules, which may be more difficult to grind, after removing significantly more minerals from during the processing steps.

Microstructure

The three-dimensional characterization of the freeze-dried, standardized ground U-SDJ and H-SDJ via SEM illustrates similar characteristics of particles that agglomerated into crumb-like groups with a porous structure (Fig. 1.3 and 1.4). The porous structure is likely to be due to ice crystals and/or air bubbles that occurred during the initial freezing process, evaporation of the water from sublimation, as well as mechanical stresses caused by inhomogeneous drying that can commonly occur during the freeze-drying process (Esfahani, Jafari, Jafarpour, & Dehnad, 2019). Powder aggregation may have occurred because after water is removed, droplets tend to come closer together due to water's surface tension properties (Ilyasoglu & El, 2014). These aggregating water molecules contain the protein and minerals thus causing the crumb-like group formations after most of the water has been removed. Additionally, it has been reported that gelatin gels with a lower concentration of Ca and/or Na ions had a more uniform, smoother surface, smaller pore size, and a more compact structure compared with gels that had a higher

concentration of these ions (Wang et al., 2018). In both SEM images for U-SDJ and H-SDJ, there appears to be flat-like shapes, which is hypothesized to be the collagen or protein molecules that are surrounded and covered by the minerals, mainly NaCl. Until now, SEM images of U-SDJ and H-SDJ jellyfish gelatin have not been performed.

Bloom Strength

The Bloom value for H-SDJ after following the Gelatin Manufacturers Institute of America, Inc. (GMIA) method for performing a Bloom test at 6.67% concentration for 17±1 h at 10°C incubation was reported to be 3.4±0.30 g (GMIA, 2019). Remarkably, H-SDJ successfully produced gelatin gels (Fig. 1.7). Meanwhile, no gelatin gels could be prepared with U-SDJ powders (e.g., no readings were obtained with the TA.XT Plus texture analyzer). Several studies have reported Bloom values of mammalian and fish gelatins. Eel (*Monopterus* sp.) skin gelatin at pH 5 and 8 had Bloom values of 213.2 and 214.7 g, respectively, while bovine gelatin had a Bloom value of 273.2 g (Nurul & Sarbon, 2015). Moreover, gelatin from Atlantic cod and salmon skin extracted at 56°C had Bloom values of roughly 70 and 101 g, respectively, and these Bloom values were lower than those reported for porcine gelatin (220 g) (Arnesen & Gildberg, 2007). Gelatin from a species of jellyfish (*Lobonema smithii*) received Bloom values between 8.79 g to 323.74 g while another paper reported the Bloom value for this species of jellyfish from 18 g to 118 g, thus demonstrating that Bloom is highly dependent on the extraction conditions used (Lueyot et al., 2020; Rodsuwan et al., 2016). The Bloom value of gelatins from the jellyfish species *Rhopilema hispidum* (31.2 kPa) was lower compared to those of bovine (107.9 kPa) and porcine (147.4 kPa) (Cho et al., 2014). Compared to other gelatins from mammalian and marine species, it is evident that the gelatin gel produced from H-SDJ was a weak gel. Nevertheless, a gel network did develop and was detectable by the TA.XT Plus texture analyzer. This provides

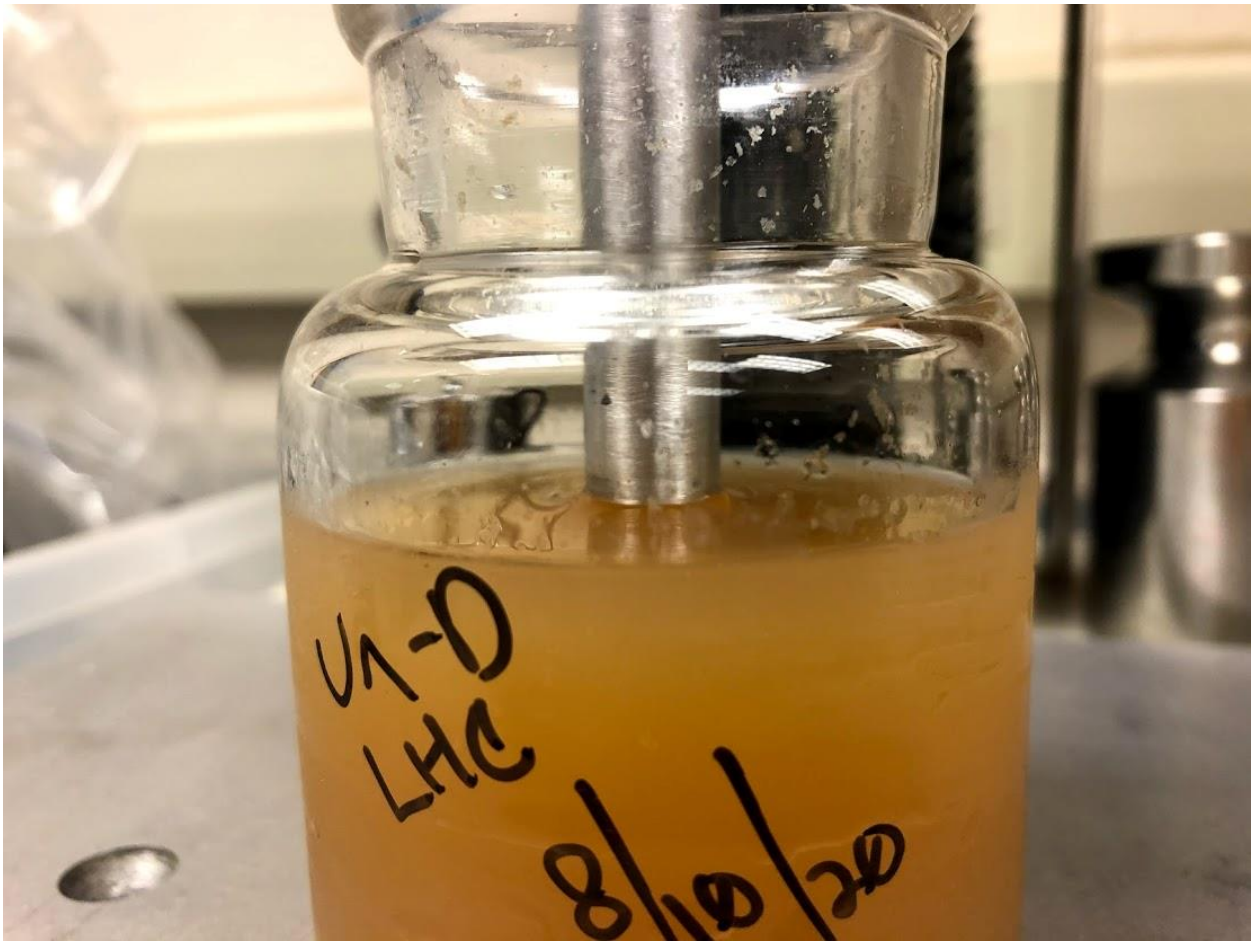

Fig. 1.7. – 6.67% hydrolyzed salted, dried jellyfish (H-SDJ) gelatin during the Bloom test.

potential for improvement as no optimization or modification of the H-SDJ gelatin was performed.

The predominant amino acids in the jellyfish *Rhopilema hispidum* are glycine, proline, alanine and hydroxyproline, and the total amount of these amino acids is an important factor in the thermal stability of a gelatin (Cho et al., 2014). Typically, jellyfish gelatin contains lower amounts of the imino acids (proline & hydroxyproline) than mammalian gelatins, which are important in hydrogen bonding (Ledward, 1986). The amount of hydrogen bonding is believed to be to the hydroxyproline level, which is lower in jellyfish gelatin than in mammalian gelatin. This hydroxyproline level is key to stabilizing the triple-stranded collagen helix by hydrogen bonding, which comes from hydroxyprolines hydroxyl group (Balti et al., 2011; Cho et al., 2004; Lueyot et al., 2020). In addition, it has been reported that low levels of alanine can cause poor gelation while lysine is used to stabilize the gelatin structure by cross-linking. Consequently, low levels may affect gelation, and both types of amino acids are reported to be low within the jellyfish species *Stomolophus meleagris*, *Lobonema smithii* and *Rhopilema hispidum* (Cho et al., 2014; Gómez-Guillén et al., 2002; Nagai et al., 1999; Rodsuwan et al., 2016). It has been suggested that the strength and other physicochemical properties of a gelatin differ due to the amino acid profile originating from the collagen; for example, it has been reported that marine species, like jellyfish, are typically lower in imino acids, proline and hydroxyproline, which contribute to the weaker gel matrixes that are observed when producing gels (Karim & Bhat, 2009). This phenomenon is hypothesized to be another reason why weak Bloom was observed for H-SDJ.

It has been suggested that gelatin gels from jellyfish need a higher concentration of solids to begin forming a noticeable gelatin network than their mammalian counterparts (Cho et al.,

2014). This confirms the information mentioned above because with less crucial amino acids to form more stable gelatin networks, it may require a higher concentration of solids to form a stronger gel network. Overall, the amino acid composition of a raw material will determine the amino acid profile of the resultant gelatins and thus play a role in the gelatin's functional properties (Rodsuwan et al., 2016). In addition, it has been reported that increasing NaCl concentration and maturation temperature reduces the strength and stiffness of gelatin networks (Chatterjee & Bohidar, 2006; Ipsen, 1997). The sodium ions (Na^+) can inhibit gelatin formation by shielding or screening protein molecules, which leads to a decrease in the capability for molecules to aggregate or gel (Cheng, Lim, Chow, Chong, & Chang, 2008). Therefore, finding a way to decrease the high levels of sodium ions and other mineral ions within H-SDJ may improve the gel formation. These results suggest that jellyfish gelatin may have different functional properties than traditional gelatins that may be desirable for various types of products either in the food, cosmetic, or biotechnological industries.

Conclusion

This study illustrates (for the first time) the feasibility of utilizing Georgia-caught salted, dried jellyfish (SDJ) as a source of collagen rich material that can be used to produce a novel marine gelatin. An acid hydrolysis and freeze-drying procedure was developed to hydrolyze collagen and produce a powder from SDJ. Remarkedly, the hydrolyzed-SDJ (H-SDJ) produced a weak gel with a strong potential for improvement. The study demonstrated the feasibility of producing novel gelatin powders from SDJ that can potentially be used in food applications as thickening, stabilizing, emulsifying, and/or microencapsulating agents.

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CHAPTER 2

ENGINEERING NOVEL GELATIN POWDERS FROM SALTED AND DRIED CANNONBALL JELLYFISH (*STOMOLOPHUS MELEAGRIS*): THE EFFECT OF MINERAL CONTENT, PH, SOLID CONCENTRATION, AND MATURATION TEMPERATURE ON THE BLOOM STRENGTH OF GELATIN GELS[2]

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Abstract

Cannonball jellyfish is a good source of collagen. Gelatin is a hydrocolloid derived from the partial hydrolysis of collagen. The aim of this study was to develop gelatin powders from salted, dried jellyfish (SDJ) and determine the effect of mineral removal, pH adjustment, solid concentration, and maturation temperature on the Bloom strength of resultant gelatin gels. SDJ was hydrolyzed using citric acid solution at 60°C for 4.5 h, demineralized via dialysis, freeze-dried, and pulverized to produce dialyzed jellyfish gelatin powders (D-SDJ). Hydrolyzed (non-dialyzed) jellyfish powders were used as the control (H-SDJ). Demineralization of H-SDJ removed ~97% of the minerals, predominately Na, Ca, Mg, and K. D-SDJ showed larger mean particle sizes, lower bulk density, smoother microstructures, and a higher crude protein content than H-SDJ. The isoelectric point of H-SDJ and D-SDJ was close to 4.4. Gelatin gels prepared with D-SDJ showed higher Bloom strengths than those produced with H-SDJ. Highest Bloom values were observed in gelatin gels produced with 10% (w/v) D-SDJ, at pH 4.4 and/or 6.5 and maturated at 4 and/or 7°C. This study demonstrates the effectiveness of demineralization, pH adjustment, maturation temperature, and solid concentration on the Bloom strength of jellyfish gelatin gels, creating a novel marine gelatin powder that could be used in several food applications.

Keywords: jellyfish, collagen, gelatin, demineralization, Bloom, dialysis, isoelectric point

Introduction

After a feasibility study that demonstrated that hydrolyzed salted, dried jellyfish powders (H-SDJ) produced a weak gelatin gel, it was observed that H-SDJ had a high mineral content and low pH. Therefore, it was hypothesized that removing excess minerals and modifying the pH of the H-SDJ could result in stronger gelatin gels.

Gelatin (hydrolyzed collagen) is a hydrocolloid used in several food applications including confections (chewiness, texture, and foam stabilization), low-fat spreads (creaminess, fat reduction, and mouthfeel), dairy (stabilization and texturization), baked goods (emulsification, gelling, and stabilization), meats (water-binding) as well as lowering caloric density (Karim & Bhat, 2009). Interestingly, gelatin is also used as a low-calorie thickener in gluten-free products. Commercial gelatins are normally obtained from mammalian collagen, mainly from bovine and porcine sources (Leone, Lecci, Durante, Meli, & Piraino, 2015). Gelatins obtained from marine sources have gained the attention of the global food industry in recent years due to their unique functionalities including lower gelling and melting temperatures compared to bovine and porcine gelatins which may allow for a quick release of encapsulated aromas and flavors and these marine gelatins can be excellent encapsulating agents for vitamins, bioactives, colors, and probiotic bacteria as well (Borza et al., 2010; Karim & Bhat, 2009; Li, Chen, Cha, Park, & Liu, 2009). However, poor rheological and mechanical properties, variable quality, off-flavors, production costs and low yields are often associated with marine gelatins.

According to Karim and Bhat (2009), the properties of gelatin gels such as viscosity, gel strength, gelling and melting temperature are affected by the molecular weight and distribution of peptides, solid concentration of the gelatin solution, gel maturation time and temperature, pH, and salt content. Recent studies on a Type A gelatin produced from the jellyfish (*Lobonema*

smithii) reported that pH, maturation temperature, time of pretreatment, and the extraction process initially performed on the raw material affects the yield and strength of the resultant gelatins' gels (Lueyot et al., 2020; Rodsuwan, Thumthanaruk, Kerdchoechuen, & Laohakunjit, 2016).

In a previous study, it was found that H-SDJ contains a high amount of minerals, especially Na and Al. According to Chatterjee and Bohidar (2006), a high concentration of NaCl reduces the strength and stiffness of gelatin gel networks. Moreover, sodium ions (Na^+) can inhibit gelatin formation by shielding or screening proteins, which lead to a decrease in the capability for the protein molecules to aggregate or gel (Cheng, Lim, Chow, Chong, & Chang, 2008). Hence, our team hypothesized that removing excess salts in H-SDJ using a dialysis process may improve the Bloom strength of the resultant gelatin gels. Dialysis is a size-based separation method that allows for selective-diffusion of molecules, mostly minerals in this case, to travel from a concentrated solution through a semipermeable membrane into a dialysis buffer, like deionized water (Evans, Romero, & Westoby, 2009). Researchers have demonstrated the removal of excess salts from a protein solution and an aloe polysaccharide solution using dialysis (Phillips & Signs, 2004; Tan, Li, Xu, & Xing, 2012). Currently, no studies have demonstrated how mineral removal may affect the Bloom strength of jellyfish gelatins.

Modifying the pH of gelatin gels may affect its Bloom strength (Etxabide, Urdanpilleta, Gómez-Arriaran, De La Caba, & Guerrero, 2017). According to Li, Li, Li, Yang, and Jin (2020), the stability of marine collagens can be understood by determining their isoelectric points (pI) through the determination of zeta-potential values. Collagen molecules begin to form aggregates when they are suspended in a solution close to their pI (zeta potential = 0). It has been noted that collagen extracted from different organisms tend to have different pIs due to a varying amino

acid composition. For example, collagens obtained from organisms that have more acidic amino acid residues like glutamic acid tend to have lower pI (Li et al., 2020). An interesting study on the effect of pH adjustment on functional, rheological, and structural properties of eel skin gelatin showed that eel skin treated at a higher pH (~8) exhibited greater emulsifying, foaming, fat binding, gel strength, gelling and melting temperature, and viscoelasticity properties (Nurul & Sarbon, 2015).

Osorio, Bilbao, Bustos, and Alvarez (2007) reported that mammalian gelatin gels with three Bloom strengths (180, 220, and 240 g) were utilized and an increase in gel strength was observed with an increase in solid concentration (5, 7, and 10% w/v). Interestingly, the melting (T_m) and gelling (T_g) temperatures also increased with an increase in solids concentration and pH (from 3 to 6) for all gelatin gels, while maturation temperatures of the gelatin gels did not affect their T_g and T_m. Every gelatin demonstrated higher storage modulus (G′) (more elasticity) values than loss modulus (G″) (less viscosity) for temperatures lower than T_m. The study also reported a model that correlates T_m and T_g with pH and solid concentration at a fixed gel strength (Osorio et al., 2007). This information is extremely useful in finding new and innovative ways to make suboptimal gelatins into gelatins with more desirable properties for use in products ranging from foods to cosmetics, pharmaceuticals, or even in medical applications.

To date, there are no scientific studies that have reported on the effect of pH adjustment on the physicochemical properties of gelatin obtained from cannonball jellyfish. Scientific studies conducted in this area may contribute to the utilization and optimization of marine gelatins. Preliminary studies in our lab have successfully demonstrated the removal of minerals from SDJ by dialysis. Hence, the objective of this study was to produce gelatin powders from

SDJ and determine the effect of mineral removal, pH adjustment, solid concentration, and maturation temperature on the Bloom strength of resultant gelatin gels.

Materials/Methods

Materials

Commercial salted, dried cannonball jellyfish (*Stomolophus meleagris*) (SDJ) were purchased from Golden Island International LLC (Darien, GA, USA). Dialysis tubing was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). Sodium hydroxide anhydrous pellets was purchased from MilliporeSigma (Burlington, MA, USA). Food-grade citric acid was from Milliard (New Jersey, USA).

Production of dialyzed gelatin powders from SDJ (D-SDJ)

The process for turning SDJ into dialyzed SDJ powders (D-SDJ) was created based on preliminary studies and added upon in this research (Fig. 2.1). Briefly, 1 kg of SDJ was rinsed and soaked in 8 L of tap water overnight for rehydration and removal of excess minerals. Rehydrated SDJ was rinsed with clean tap water, chopped, and soaked in a 3 L citric acid solution (1.5% w/v) for 10 min. Then, SDJ was drained of excess citric acid solution, homogenized in a commercial grade blender (Model BL610, NINJA, SharkNinja Operating LLC, Needham, MA, USA) at medium and high power for 8 and 4 min, respectively. Afterwards, the mixture was further processed with an ultra-shearing homogenizer (Homogenizer 850, Fisherbrand, Fisher Scientific UK Ltd, Loughborough, UK) at 8000 rpm for 6 min then at 10000 rpm for 8 min, until a visibly thin liquid was observed. Then, the liquified product was incubated at 60°C for 4.5 h in a water bath (Model 2872, Precision, Thermo Electron Corporation, Waltham, MA, USA) to allow the hydrolyzation of the jellyfish collagen. Once the hydrolysis process had been finished, a dialysis procedure was performed to remove

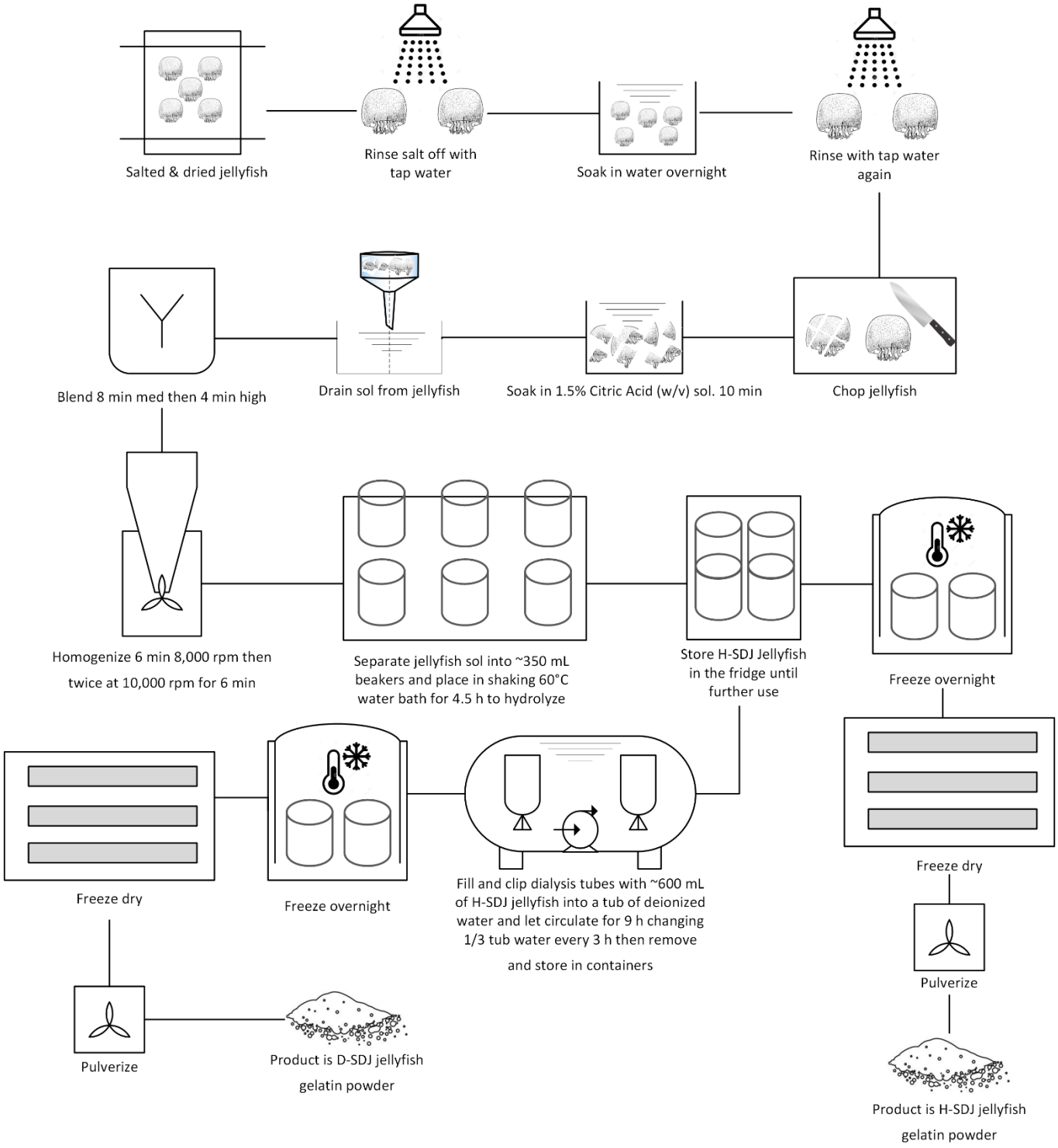

Fig. 2.1. – Process diagram to turn salted, dried jellyfish (SDJ) into hydrolyzed jellyfish powders (H-SDJ) and/or hydrolyzed and dialyzed jellyfish powders (D-SDJ).

excess minerals. The dialysis was performed by placing 600 mL of liquid hydrolyzed jellyfish into regenerated cellulose dialysis tubing (cut 25-30 mm in length) (MWCO: 6-8 kD, Spectra/Por® 1 Dialysis Membrane, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) at room temperature. Then, dialysis tubes were sealed with plastic clips and placed into a 65 L bath of deionized water (DIW), which was used as the dialysis buffer. The water was continuously agitated/stirred to speed up dialysis. The water was completely replaced after 18 h of circulation then continued to circulate for an additional 6 h while half the water was changed every 1.5 h until completion (total dialysis time = 24 h). The time frames selected were based on preliminary studies conducted in our lab (data not shown). Afterwards, the hydrolyzed and dialyzed jellyfish was frozen at -4°C for 12 h then freeze-dried at -55°C for 2 days and gradual increasing of the chamber temperature to 20°C for 3 days was performed using a pilot-scale lyophilizer (Virtis, the Virtis Company, Gardiner, NY, USA). Then, freeze-dried samples were pulverized using an electric grain grinder mill (Model SUS304, SLSY & MOONCOOL, Shanghai, China) to obtain the hydrolyzed and dialyzed SDJ powders (D-SDJ). Concurrently, un-dialyzed SDJ powders (H-SDJ) were prepared using the procedure described above (except the dialysis process) as the control. The powders were stored in a desiccator jar at room temperature until needed for analysis.

Physicochemical properties of D-SDJ

Moisture and water activity (a_w)

Moisture content of the powders was determined by the AOAC Official Method 934.01 (AOAC, 2020) using an Isotemp® Vacuum Oven Model 281A (Thermo Fisher Scientific, Waltham, MA, USA). Water activity (a_w) was determined using an Aqualab water activity meter (Model Series 3 TE, Decagon Devices, Inc., Pullman, WA, USA).

Ash and mineral analysis

The ash content of H-SDJ and D-SDJ was calculated by following the general AOAC Official Method for ash analysis, performed in triplicate (Marshall, 2010). In short, samples were weighed, and the program followed was set at 550°C for 12-18 h in a muffle furnace (Model F-A170, Thermolyne, Dubuque, IA, USA). After ashing, the samples were then taken out, weighed, and the ash content was calculated. Then, the mineral profile of both H-SDJ and D-SDJ powders was determined by an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Model FHS16, Ametek Inc., Berwyn, PA, USA) method. The minerals P, K, Ca, Mg, Mn, Fe, Al, B, Cu, Zn, Na, Pb, Cd, Ni, Cr and Mo were quantified using a microwave-acid (HNO_3) digestion, ICP method using CEM Mars5 microwave digestion system (Model 61E ICP, Thermo Jarrell-Ash, Franklin, MA, USA). The mineral analysis was performed at the University of Georgia (UGA) - Soil, Plant, and Water Laboratory in Athens, GA.

Color

The color of the powders was measured using a Lab Scan XE Colorimeter (Hunter Associates Laboratory, Inc., Resbon, VA, USA) which was reported in CIE L.A.B color scales (L*, a*, b*). To determine the color, petri dishes (100mm x 15mm) were filled with the freeze-dried powders until their bottom was completely covered. Chroma and hue were quantified using Eqs. (1) and (2).

$$Chroma = [(a^*)^2 + (b^*)^2]^{\frac{1}{2}} \quad (1)$$

$$Hue = tan^{-1}(\frac{b^*}{a^*}) \quad (2)$$

Particle Size Distribution

Particle size analysis was conducted according to a method described in chapter 1, using a Particle Size Analyzer (Model PSA 1190, Anton Paar, Austria) equipped with laser diffraction.

The freeze-dried powders were fed into the machines hoper and transported via Venturi/free fall to the analytical area. In this area, the powders were illuminated using three separate lasers from low to high angles, each laser simultaneously diffracted light which was read and analyzed by the equipment. In this study, a 10 s run time with dispersion parameters of 40% vibrator duty cycle, 40 Hz vibrator frequency, and 1200 mBar of air pressure was utilized. The whole light scatter pattern was collected and analyzed to calculate the particle size distribution in accordance with the Fraunhofer reconstruction mode which quantifies the angular distribution of backscattered light. Particle size distribution data was reported as D_{10}, D_{50}, and D_{90} which is the average diameter of the particles at 10%, 50%, and 90% of the sample being tested, respectively.

Scanning electron microscopy (SEM)

SEM images of the powders was collected using the scanning electron microscope (1450 EP, Carl Zeiss MicroImaging, Thornwood, NY, USA) located at the Georgia Electron Microscopy facility (Athens, GA). Powdered samples were first sputtered-coated with gold then images were collected using an acceleration potential of 2 kV, which provided the greatest resolution of the sample morphologies. This process was previously used in our laboratory and described by Jiang, Dev Kumar, Chen, Mishra, and Mis Solval (2020).

Bulk density

The bulk density was determined using the method previous described by Yihong, Yisheng, Geoff, Lirong, and William (2009) with slight modifications. Briefly, a 100 mL graduated cylinder was tared on an analytical balance then powder was filled to the 10 mL mark. The cylinder was taken off the balance and tapped 100 times. If the powder fell below the 10 mL mark then more powder was filled to reach the 10 mL mark and tapped 100 more times. This process was repeated until the tapped powder read 10 mL. At this point, the graduated cylinder

with powder at the 10 mL mark was weighed. The mass of the powder (grams) was divided by the volume (10 cm^3) to receive the bulk density (g/cm^3), each performed in triplicate.

Crude protein analysis

Crude protein analysis was determined by following the AOAC Official Method 993.13 for nitrogen (total) by dry combustion (AOAC, 2019). In short, the Dumas method was followed, which quantified the total nitrogen by a dry combustion method using an automated nitrogen analyzer (Rapid N Exceed, Elementar, Langenselbold, Germany), described according to Jung et al. (2003). A conversion factor of 5.8 was utilized to determine crude protein content (Binsi, Shamasundar, Dileep, Badii, & Howell, 2009; Khong et al., 2016).

Isoelectric point (pI) determination

pI of H-SDJ and D-SDJ was determined by following the method of Li et al. (2020) with slight modifications. In short, H-SDJ and D-SDJ powders were weighed and mixed with 2 M acetic acid (99.7+% purity, ACROS Organics, Fair Lawn, NJ, USA) to give a final concentration of 0.1 mg of powder per mL of acetic acid solution. After mixing, the solution was allowed to hydrate for at least 15 min then was incubated at 40°C in a water bath (Model 2872, Precision, Thermo Electron Corporation, Waltham, MA, USA) for 15 min, swirling periodically. Then, the pH of the solution was adjusted from ~2.4 (initial) up to 6.0 using 1 M NaOH solution. Afterwards, the liquid mixture was injected into an Omega Cuvette (Cuvette number 225288, Anton Paar, Austria) and placed into the zeta potential analyzer (Model Litesizer 500, Anton Paar, Austria). The parameters were a target temperature of 20°C, equilibration time of 1 min, Smoluchowski equation, Henry factor of 1.50 and adjusting the solvent to acetic acid (refractive index of 1.3717, viscosity of 0.0011550 Pa•s, and relative permittivity of 6.20 ε_r). Zeta potential (mV) readings were recorded at a given pH value.

Bloom strength of gelatin gels produced with different solid concentrations of H-SDJ and D-SDJ at different pH values and maturation temperatures

Bloom strength (g) of gelatin gels produced with H-SDJ and D-SDJ powders was determined by following the official method of the Gelatin Manufacturers Institute of America, Inc. (GMIA) (GMIA, 2019) with slight modifications. Gelatin gels were prepared in Bloom jars (Height = 59+1mm H. ∅ (diameter) = 85mm) (Brookfield Engineering, Middleboro, MA, USA) with three solid concentrations (5, 6.67, and 10% (w/w)) formulated with 5.63, 7.50, and 11.25 g of H-SDJ or D-SDJ and 106.9, 105.0, and 101.3 g of DIW, respectively. The samples were allowed to completely swell for ~1.5 h at room temperature. Then, the pH of the sample was either kept at ~2.4 or adjusted to 4.4 or 6.5 using a 1 M NaOH solution. These pH values were selected based on the results obtained for pI values of the H-SDJ and D-SDJ powders. Next, dissolution of powders was achieved by incubating the samples in a 65°C water bath for 15 min, swirling periodically. Afterwards, the samples were allowed to temper at room temperature for ~20 min. Finally, samples were incubated at three temperatures 4, 7, and/or 10°C for 17±1 h to allow maturation and formation of gelatin gels. After maturation, a texture analyzer (TA.XT Plus, Stable Micro Systems Ltd, Godalming, UK) was used to determine the Bloom strength (g) of the gelatins at a 4 mm penetration depth. The parameters used were a 12.7 mm diameter probe, which depressed the surface of the gel by 4 mm at a speed of 1 mm/sec. The peak force in grams was recorded and is referred to as the Bloom strength of a gelatin (GMIA, 2019).

Statistical analysis

All experiments and analyses were carried out in triplicate replication. The mean and standard deviations (SD) were calculated then statistical tests were run to determine if significant differences arose in the collected data. For Bloom strength results, a two-way analysis of

variance (ANOVA) (two independent variables), and post-hoc Tukey's studentized range tests ($\alpha = 0.05$) were employed; while for the rest of the data a one-way ANOVA and post-hoc Tukey's studentized range tests ($\alpha = 0.05$) were conducted to determine the statistical significance of observed differences among the means. This was conducted using RStudio statistical software version 1.2.5033 (RStudio, Inc. Boston, MA, USA).

Results/Discussion

Physicochemical properties of H-SDJ and D-SDJ

Moisture content and a_w

The moisture content and a_w of H-SDJ and D-SDJ was 4.03±0.13 g/100 g, 0.079±0.01 and 4.63±0.33 g/100 g, 0.060±0.01, respectively (Table 2.1). Although the a_w of D-SDJ was similar to that of H-SDJ; H-SDJ had a significantly ($p<0.05$) lower moisture content compared to D-SDJ. Because of their low moisture and a_w values, both H-SDJ and D-SDJ are considered to be dry and a shelf-stable product. Currently, no studies have demonstrated how the removal of minerals of powdered foods may affect their moisture content and a_w. The lower moisture content values for the H-SDJ compared to the D-SDJ may suggest that the amount of water in the sample for a given mass was lower in H-SDJ. As salt concentration increases, research has shown that moisture content decreases (Boudhrioua, Djendoubi, Bellagha, & Kechaou, 2009). Thus, H-SDJ having significantly more minerals is likely driving out more moisture in the powder than in the D-SDJ. Water activity is a parameter used to determine the shelf stability of foods, in regards to microbial growth, by measuring the amount of free water available in a sample (Solval, Sundararajan, Alfaro, & Sathivel, 2012). Typically, dried foods (a_w values < 0.6) are considered shelf stable where microbial growth is limited, assuming no moisture absorption will occur during storage (Fellows, 2009).

Table 2.1 – Characterization of hydrolyzed salted, dried jellyfish (H-SDJ) and dialyzed-SDJ (D-SDJ) powders.

		H-SDJ	**D-SDJ**
	Water activity (a_w)	0.079 ± 0.01	0.060 ± 0.01
	Ash (g/100 g, dry basis)	56.17 ± 0.13	1.44 ± 0.06 ***
	Moisture (g/100 g, wet basis)	4.03 ± 0.13	4.63 ± 0.33 *
	Bulk Density (g/cm^3)	0.29 ± 0.00	0.037 ± 0.00 ***
	Crude Protein (%)	31.77 ± 0.04	85.35 ± 1.28 ***
Color	L*	70.91 ± 0.03	60.95 ± 0.01 **
	a*	1.31 ± 0.04	5.73 ± 0.04 ***
	b*	8.78 ± 0.03	21.71 ± 0.04 ***
	Hue	81.49 ± 0.29	75.21 ± 0.10 ***
	Chroma	8.88 ± 0.03	22.45 ± 0.04 ***
Particle size analysis	D10 (µm)	0.70 ± 0.03	5.80 ± 0.12 ***
	D50 (µm)	6.46 ± 1.13	54.89 ± 0.54 ***
	D90 (µm)	314.90 ± 3.61	251.60 ± 8.53 ***
	Mean (µm)	99.42 ± 1.22	97.48 ± 2.60
	Span	49.62 ± 8.75	4.48 ± 0.12 ***

†Values are means ± standard deviation (SD) of triplicate determinations.
††Rows with * signify significant difference ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) between H-SDJ & D-SDJ. H-SDJ = Gelatin powders produced with hydrolyzed salted, dried jellyfish; D-SDJ = Gelatin powders produced with hydrolyzed and dialyzed salted, dried jellyfish.

Color

Color was determined using the L* (lightness) a* (red/green), b*(blue/yellow) color scale. The color values of D-SDJ were significantly (p<0.05) different than those of H-SDJ (Table 2.1). The color results indicated that D-SDJ was darker, slightly redder and yellower than H-SDJ, which confirm the findings obtained from the images of the powders presented in Figure 2.2. It was observed that after dialysis, samples became darker. This may be due to the removal of salts, which show a whitish color, and/or the exposure of entrapped polyphenols within the jellyfish tissues, which was previously illustrated in three different species of jellyfish described by Leone, Lecci, Milisenda, and Piraino (2019). Also, potential Maillard browning reactions may have taken place as reducing sugars and amine groups from the amino acids react and ultimately can cause the browning (Lueyot et al., 2020). Commonly, commercial gelatins have a color that ranges from pale yellow to a darker amber (Alfaro, Biluca, Marquetti, Tonial, & de Souza, 2014).

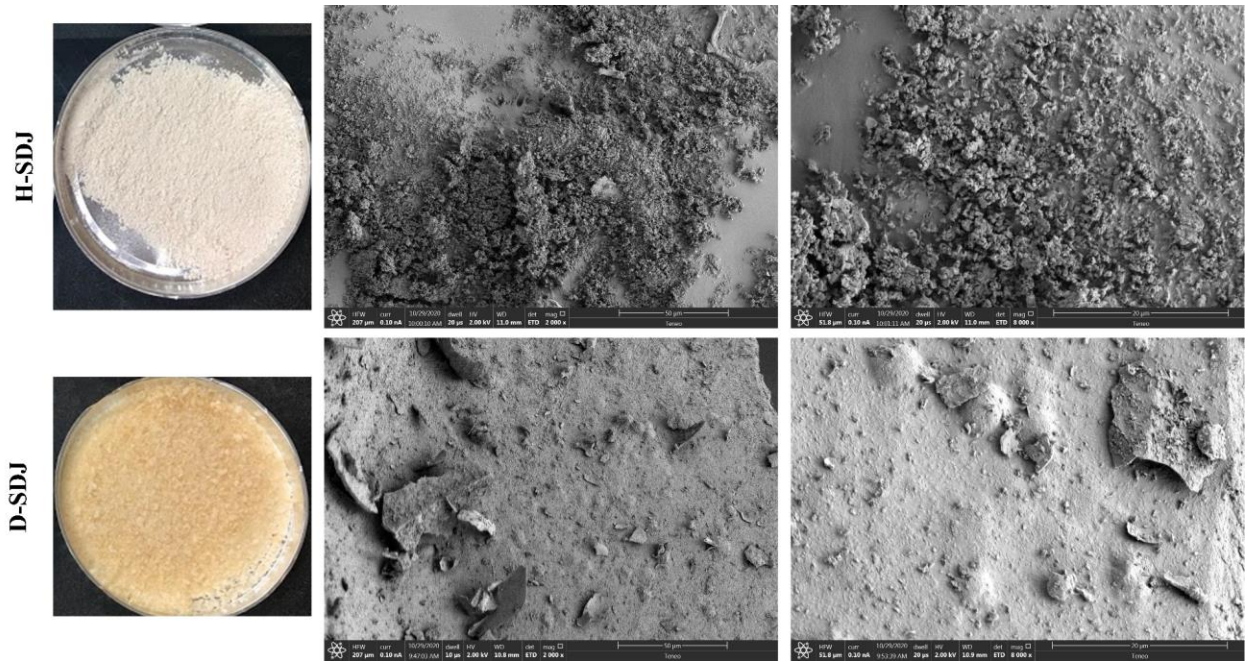

Fig. 2.2. – Powder (*left*) & Scanning electron microscopy (SEM) (*right*) images of hydrolyzed salted, dried jellyfish (H-SDJ) and dialyzed salted, dried jellyfish (D-SDJ).

Ash content and mineral profile

The ash content of D-SDJ was significantly (p<0.05) lower than that of H-SDJ (Table 2.1). Remarkably, D-SDJ had ~97% less ash than H-SDJ. These results confirm the effectiveness of the dialysis process to remove excess minerals. According to Soria, Brokl, Sanz, and Martínez-Castro (2012), dialysis is defined as the diffusion of solutes and ultrafiltration of fluids that pass through a semi-permeable membrane into a dialysis buffer. Preliminary studies conducted in our lab determined that 24 h of dialysis was sufficient to remove a majority of the minerals present in H-SDJ. Figure 2.1 shows the process to turn SDJ into D-SDJ.

Table 2.2 lists the mineral profile of the H-SDJ and D-SDJ with the percent change in minerals. After dialysis, the reduction of minerals ranged from the lowest 46.2±12.6% in Cr up to the highest of 98.6±0.3% for Na. Receiving a ~99% reduction in the Na content from ~239,205 to ~3,311 (ppm, dry basis (d.b.)) demonstrates that the dialysis process was extremely effective. Contrary, numerous minerals reported an increase in concentration (e.g., P, S, Cu) but most notably Al increased by 21.2±6.3% to ~5,465±277 (ppm, d.b.). In preliminary studies, some minerals reported an increase in concentration from unhydrolyzed-SDJ (U-SDJ) to H-SDJ and this phenomenon may have been observed because with much less sodium per known mass of sample, the concentrations of these minerals, most importantly Al, appeared to significantly increase. To our knowledge, there is no research that has looked into how Al binds to collagen, especially after heating during hydrolyzation, but it is predicted that the Al may bind more tightly to the collagen proteins similar to how Al binds to proteins in the body like albumin and transferrin (Cheng, Wang, Xi, Cao, & Jiang, 2018). Thus, removing Al by dialysis was not very effective and may be why Al appeared to increase from U-SDJ to H-SDJ and again after the creation of D-SDJ. This study demonstrates that dialysis was very effective at removing Na

Table 2.2 – Mineral profile for hydrolyzed salted, dried jellyfish (H-SDJ) and dialyzed-SDJ (D-SDJ) and the percent change in minerals from H-SDJ to D-SDJ.

Mineral	Units	H-SDJ	D-SDJ	% change from H- to D-SDJ
Ca calcium	g/100g d.b.	0.09 ± 0.00	<0.01***	93.5 ± 0.4
K potassium	g/100g d.b.	0.09 ± 0.02	0.02 ± 0.00**	84.7 ± 5.8
Mg magnesium	g/100g d.b.	0.13 ± 0.00	<0.01***	92.0 ± 0.0
P phosphorus	g/100g d.b.	0.11 ± 0.00	0.14 ± 0.01**	22.0 ± 5.3
S sulfur	g/100g d.b.	0.38 ± 0.01	0.53 ± 0.02***	42.6 ± 2.4
Al aluminum	ppm, d.b.	4,150 ± 26	5,465 ± 277**	21.2 ± 6.3
B boron	ppm, d.b.	<2.67	<5.7	CBD
Cd cadmium	ppm, d.b.	<1.06	<2.28	CBD
Cr chromium	ppm, d.b.	17.47 ± 0.38	9.40 ± 2.12**	46.2 ± 12.6
Cu copper	ppm, d.b.	17.26 ± 0.78	22.86 ± 1.52**	32.6 ± 10.3
Fe iron	ppm, d.b.	196 ± 18	93 ± 2.5***	52.2 ± 3.7
Mn manganese	ppm, d.b.	<2.65	<5.7	CBD
Mo molybdenum	ppm, d.b.	<1.32	<2.85	CBD
Na sodium	ppm, d.b.	239,205 ± 5967	3,311 ± 655***	98.6 ± 0.3
Ni nickel	ppm, d.b.	14.13 ± 1.91	<2.33*	CBD
Pb lead	ppm, d.b.	<3.39	13.95 ± 6.84	CBD
Zn zinc	ppm, d.b.	40.29 ± 7.93	14.33 ± 2.04***	63.0 ± 12.1

†Values are means ± standard deviation (SD) of triplicate determinations.
††Rows with * signify significant difference ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) between H-SDJ & D-SDJ.
†††Values with "<" symbol were below detectable limits during the ICP-MS. ppm = parts per million. d.b. = dry basis. CBD = could not be determined. H-SDJ = Gelatin powders produced with hydrolyzed salted, dried jellyfish; D-SDJ = Gelatin powders produced with hydrolyzed and dialyzed salted, dried jellyfish.

and other minerals like Ca, K, Mg, etc. while not ideal for removing certain minerals like Al, P, S, and Cu. Although no research has reported the demineralization of jellyfish gelatin by dialysis, an interesting study demonstrated the successful demineralization of sea brim gelatins using ethylenediaminetetraacetic acid (EDTA) for 12 h, which reduced the mineral content of gelatins to 0.57±0.10 g/100 g from 59.8±0.3 g/100 g (Akagündüz et al., 2014). In another study, demineralization of gelatins obtained from lizardfish (*Saurida* spp.) using a combination of NaCl and NaOH solutions reduced the ash content of gelatins while improving the gel strength (Wardhani, Rahmawati, Arifin, & Cahyono, 2017). Other studies have reported the successful demineralization of collagen and gelatins derived from grass carp fish scales, camel bone, and spotted golden goatfish scales using HCl (AL-Kahtani et al., 2017; Chuaychan, Benjakul, & Nuthong, 2016; Zhang, Xu, & Wang, 2011). To the best of our knowledge, no reports have been conducted on the demineralization of jellyfish gelatins using a dialysis procedure.

Particle size distribution

The particle size distribution of D-SDJ is given in Table 2.1. The mean particle size (D_{50}, µm) of D-SDJ was 54.89±0.54, which is significantly ($p<0.05$) larger than that of the H-SDJ powder (6.46±1.13). Currently, there are limited reports on how excess minerals in gelatin powders may reduce their particle size. One possible explanation for a larger particle size observed in D-SDJ may be related to its lower mineral content. As more minerals are released, this left a lighter/fluffier, less dense gelatin powder compared to the H-SDJ (Fig. 2.3). A significantly ($p<0.05$) lower bulk density in D-SDJ than H-SDJ illustrates that demineralization created a lighter product (Table 2.1). It was hypothesized that when the standardized grinding procedure is performed, the lower-density D-SDJ fills the chamber in a cloud-like form which

Fig. 2.3. – *Left* – hydrolyzed, salted, dried jellyfish (H-SDJ) 7.5 g. *Right* – dialyzed-SDJ (D-SDJ) 7.5 g.

does not grind the powder as fine while the more mineral dense H-SDJ is heavier thus being able to grind into a finer powder. Moreover, D-SDJ contains more proteinaceous material, which may be more difficult to break down during the grinding process. Particle size data is an important parameter in food powders because it can give crucial information on behavior of food powders; for example, determining if a sample is free flowing or not, the capability to form stable emulsions/suspensions, its texture/mouthfeel in a product (gritty, chalky, sandy characteristics if too large) as well as potentially affecting the rheological properties of a product (Van der Meeren, Dewettinck, & Saveyn, 2004).

Microstructure

The H-SDJ and D-SDJ microstructure was analyzed via SEM (Fig. 2.2). SEM imaging shows what appears to be chunks of protein surrounded by minerals in H-SDJ, this effect is not as prevalent in D-SDJ thus being a reason for its fluffier and lighter particles. This difference may have caused less fine grinding and a larger particle size to occur. The D-SDJ powders appeared to be either relatively large and flat-like structures with a small pore size (which suggests proteinaceous material) or extremely small dust-like particles (suggesting minerals). In comparison, the H-SDJ showed crumb-like, agglomerated groups with a porous structure. Wang et al. (2018) reported that gel powders containing less Na and Ca ions show smoother surfaces and smaller pore sizes. Moreover, it has been reported that a small pore size in gelatin powders may lead to the absorption of higher quantities of water, which serves to texturize, stabilize, gelatinize, or give an emulsifying effect to the gelatins (Abdelhedi et al., 2019). After demineralization of spotted golden goatfish scales using an HCl treatment, the scales appeared to became less rigid and became more porous which suggests that minerals were removed (Chuaychan et al., 2016). Preliminary studies hypothesized that the agglomerated clumps were

believed to be NaCl, which may in turn affect the strength of the gelatin due to the proteinaceous material being completely covered/surrounded. In the D-SDJ SEM images, it can be observed that a majority of these clumps have been removed after dialysis and what is left is the flat-like proteinaceous material that used to be surrounded by these clumps of presumably minerals. With the removal of these minerals, it was hypothesized that freeing more proteinaceous material would allow for improvement in the Bloom strength of the resultant gelatin gels.

Bulk density and crude protein content

Bulk density is a measure of sample mass divided by its volume (Cheng et al., 2008). The H-SDJ and D-SDJ bulk density (g/cm^3) was 0.29±0.00 and 0.037±0.00, respectively (Table 2.1). According to the USDA (2021), loose, well-aggregated porous materials rich in organic matter, like proteins, tend to have a lower bulk density. The D-SDJ was significantly ($p<0.05$) less dense than the H-SDJ (Fig. 2.3). The less dense (lighter/fluffier) nature of the D-SDJ correlates with the mineral removal as ~97% of the minerals were removed. Using the SEM images (Fig. 2.2), it is hypothesized that with more minerals there is less space for air to be trapped within the product while removing minerals produced more open space thus allowing more air and producing the significantly smaller bulk density in D-SDJ. Brizzi, Funiciello, Corbi, Di Giuseppe, and Mojoli (2016) mentioned that gelatin density increases with increasing salt concentration. Similar results were observed where fish gelatin alone observed a bulk density of 0.77 g/cm^3 while the addition of pectin significantly increased the bulk density (Cheng et al., 2008). Another report demonstrated that fish gels had a significantly lower bulk density (0.127-0.142 g/cm^3) than starch gels (0.411-0.523 g/cm^3) (Wang, Zhang, & Mujumdar, 2013). However, no studies have reported how the bulk density of jellyfish gelatin is affected by demineralization.

Additionally, the crude protein content of H-SDJ and D-SDJ was ~32 and ~85%, respectively (Table 2.1). As the minerals were removed, discussed above, this allowed for the crude protein to become more concentrated, which led to the significantly (p<0.05) larger amount of protein observed in the D-SDJ. Based on preliminary studies, this trend was continued from the original SDJ as the crude protein (g/100 g, d.b.) for the oral arms was ~8 while the umbrellas were ~6. This demonstrates that after the washing, soaking and demineralization steps, this caused removal of predominately minerals while concentrating the proteins that were present in the jellyfish. Research found that desalted ready-to-eat cannonball jellyfish had a protein content of ~4-5% (Hsieh, Leong, & Rudloe, 2001). Another study reported the crude protein (g/ 100 g, dry mass) of three species of jellyfish *A. hardenbergi, R. hispidum,* and *R. esculentum* ranged from 21-38 for the umbrella and 33-53 for the oral arms (Khong et al., 2016). This demonstrates that the H-SDJ being produced has a protein content within the range of three different species of jellyfish. To our knowledge, no research has demonstrated how demineralization affects crude protein content.

Isoelectric point of H-SDJ and D-SDJ

The pI is defined as the pH at which the surface charge of a protein is zero (Barzideh, Latiff, Gan, Benjakul, & Karim, 2014). Measuring pI of proteinaceous material is normally accomplished by zeta potential determinations. Furthermore, collagen proteins with zeta potential values closer to zero (pI) form aggregates due to an increase in hydrophobic interactions between the collagen molecules in the solution (Ahmad, Benjakul, & Nalinanon, 2010; Li et al., 2020). The zeta potential values of H-SDJ and D-SDJ at different pH conditions is shown in Figure 2.4. It can be observed that as pH increased from ~2.4 to 6.0, the zeta potential values (mV) of the powders decreased. For both the H-SDJ and D-SDJ, they were

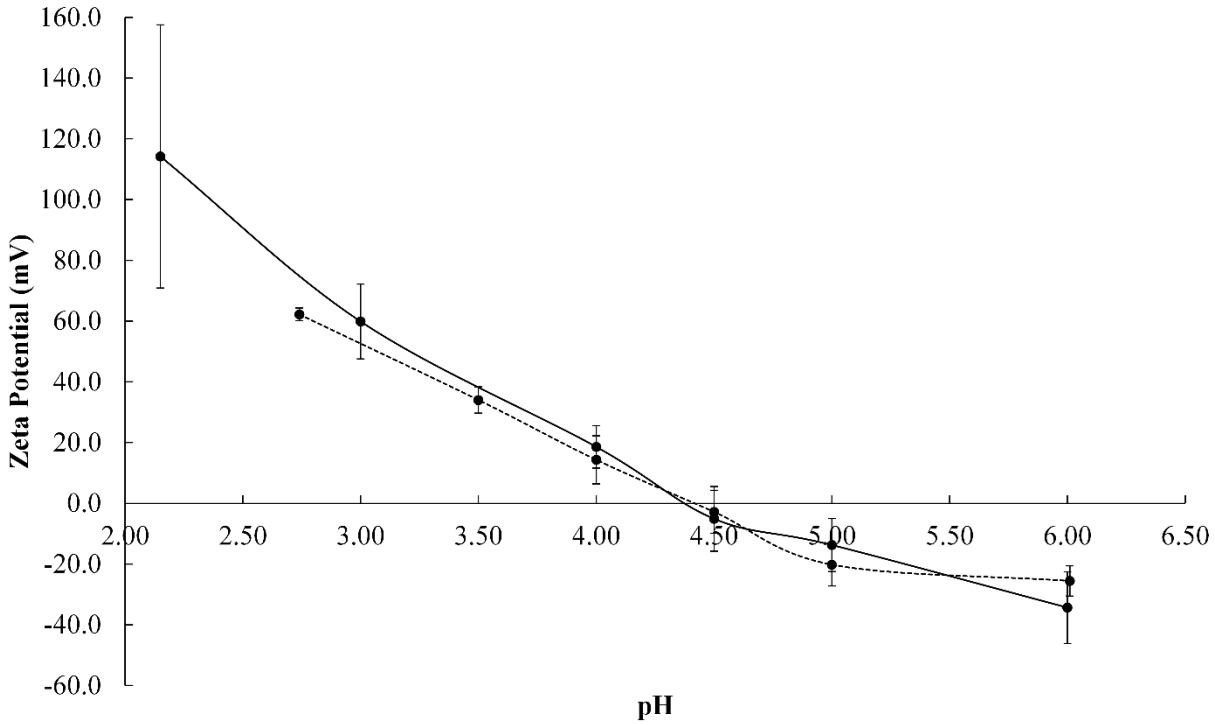

Fig. 2.4. – Zeta potential (mV) values of hydrolyzed salted, dried jellyfish (H-SDJ) (—) and dialyzed (D-SDJ) (---) at different pH values.

essentially the same where they were positively charged from their initial pHs until ~4.4 then were negatively charged thereafter. Therefore, it was determined that the pI for H-SDJ and D-SDJ was approximately 4.4. It has been reported that most collagens have an pI between 6 to 9 and this variation in pI is related to the amino acid composition of the collagen (Li et al., 2020). Barzideh et al. (2014) reported an pI for the Ribbon jellyfish (*Chrysaora* sp.) close to 6.64. Furthermore, Li et al. (2020) reported that lower pIs of collagen molecules may be due to a higher concentration of acidic amino acids, specifically glutamic acid. Interestingly, Khong et al. (2018) reported the pI values of collagen from jellyfish *Acromitus hardenbergi* to be 4.92 for the bell and 5.40 for the oral arms. Meanwhile, the pI of collagen from sea cucumber (*Acaudina molpadioides*) was 4.25 (Li et al., 2020). It has been reported that cold-water fish gelatin extracted from skins, appeared to be more stable at its pI ~4.5 than at pH 3.0 (Cheng et al., 2008). Therefore, our team hypothesized that the pH of the resultant gelatin gels may influence their Bloom strength.

Bloom strength of gelatin gels produced with H-SDJ and D-SDJ

The resistance or Bloom strength is a measure of a gel's compressibility, firmness, consistency, and hardness at a given temperature and it is determined by the weight in grams to depress a specific depth under normal conditions (Kempka, Souza, Ulson de Souza, Prestes, & Ogliari, 2014). Factors that been known to affect Bloom strength of gels are solid concentration, mineral content (specifically NaCl), maturation temperature, and pH (Chatterjee & Bohidar, 2006; Choi & Regenstein, 2000; Kamel & Deman, 1977). Gelatin gels were prepared at three solid concentrations (5, 6.67, 10% (w/v)) with H-SDJ and/or D-SDJ, three pHs (2.4, 4.4, 6.5), and maturated at three different temperatures (4, 7, 10°C) to determine how varying different

parameters effects the gelatin strength of the novel jellyfish gelatins. The original Bloom value of H-SDJ tested at 10°C maturation temperature, pH = 2.4, 6.67% solids was 3.4 g.

Effect of demineralization

The effect of removing minerals from the H-SDJ to produce D-SDJ by a dialysis method had remarkedly improved the Bloom strength of the jellyfish gelatin gels (Fig. 2.5, 2.6, and 2.7). Regardless of the parameters being tested (solid concentration, maturation temperature, and pH), the Bloom strength (g) of the gelatin gels produced with D-SDJ were significantly ($p<0.05$) stronger than that of gelatin gels produced with H-SDJ.

It is believed that the higher Bloom strengths observed in gelatin gels produced with D-SDJ was due to the significant reduction of minerals, especially sodium (Na), during the dialysis process. More than 96% of the Na present in H-SDJ was removed by dialysis (Table 2.2). Previous studies have shown that the removal of Na may impact the functionality of the resultant gelatin gels. For example, the addition of NaCl in concentrations of 0.5 to 2% was found to reduce the strength and stiffness of gels produced from protein isolates, which is speculated to be due to delayed structure development and this was not seen with the addition of $CaCl_2$ signifying that the sodium ions caused the weaker gels (Ipsen, 1997). Similar findings were reported when NaCl was added to gelatin gels produced with dextran dialdehyde T-70, where the salts caused a decrease in ionic interactions within the gelatin matrix (Schacht et al., 1993). Another study reported that increasing salt concentrations soften the resultant gelatin gels similar to raising its temperature as the salt acts to inhibit/interfere with the gels ability to form electrostatic interactions, likely hydrogen bonding, thus causing softening of the gelatin to occur (Chatterjee & Bohidar, 2006). Because H-SDJ contains high quantities of minerals, the results obtained in this study suggest that the excess minerals may interfere in the formation strong gel networks.

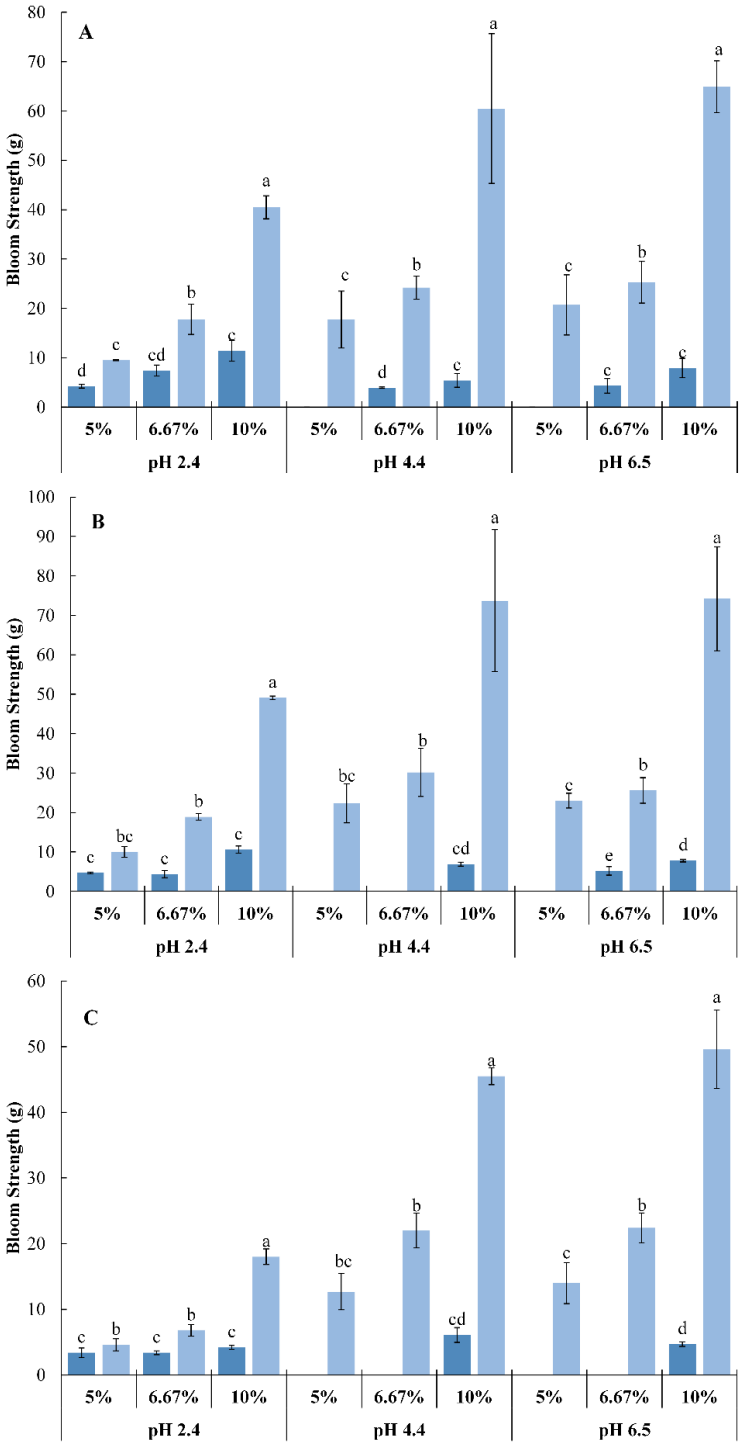

Fig. 2.5. – Effect of solid concentration (%) on Bloom strength (g) of jellyfish gelatin gels (■ = H-DSJ; ■ = D-SDJ) maturated at different temperatures (A = 4°C; B = 7°C; C = 10°C). H-SDJ = Gelatin gels produced with hydrolyzed salted, dried jellyfish; D-SDJ = Gelatin gels produced with hydrolyzed and dialyzed salted, dried jellyfish. [abcd]Means treatments with different letters at the same pH and maturation temperature (°C) are significantly different (p<0.05).

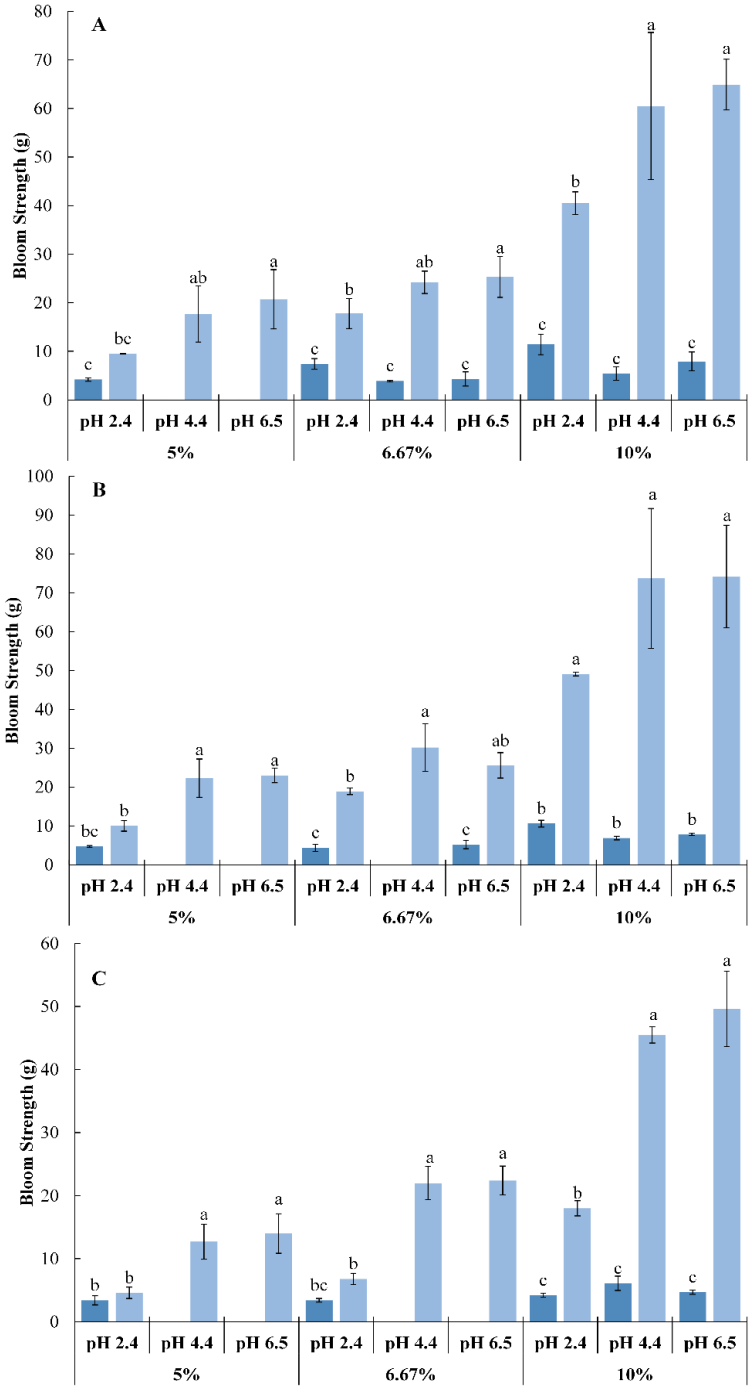

Fig. 2.6. – Effect of pH on Bloom strength (g) of jellyfish gelatin gels (■ = H-DSJ; ■ = D-SDJ) maturated at different temperatures (A = 4°C; B = 7°C; C = 10°C). H-SDJ = Gelatin gels produced with hydrolyzed salted, dried jellyfish; D-SDJ = Gelatin gels produced with hydrolyzed and dialyzed salted, dried jellyfish. [abcd]Means treatments with different letters at the same solid concentration (%) and maturation temperature (°C) are significantly different (p<0.05).

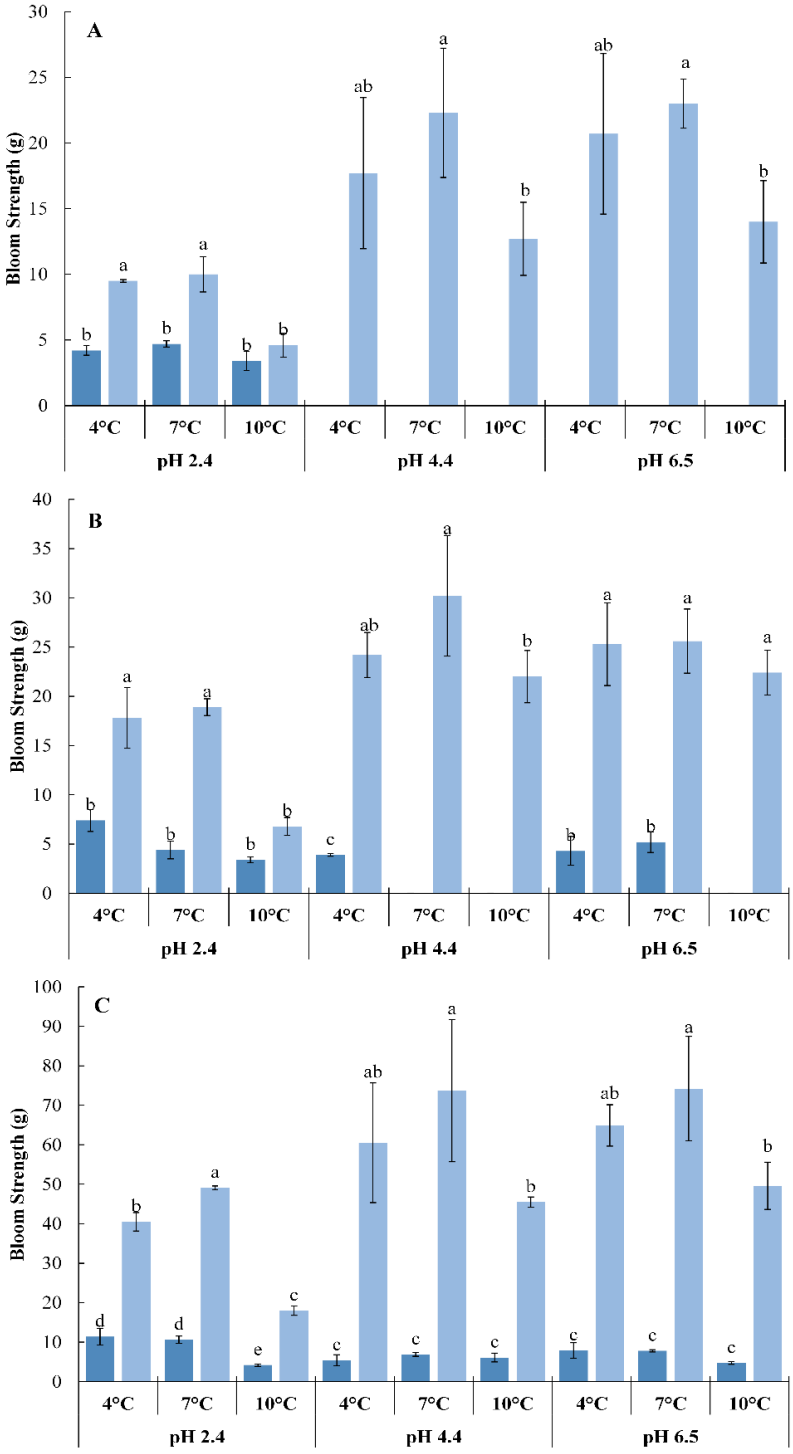

Fig. 2.7. – Effect of maturation temperature (°C) on Bloom strength (g) of jellyfish gelatin gels (= H-DSJ; = D-SDJ) prepared at different solid concentrations (A = 5%; B = 6.67%; C = 10%). H-SDJ = Gelatin gels produced with hydrolyzed salted, dried jellyfish; D-SDJ = Gelatin gels produced with hydrolyzed and dialyzed salted, dried jellyfish. [abcd]Means treatments with different letters at the same pH and solid concentration (%) are significantly different (p<0.05).

Increasing salt concentrations were shown to decrease Bloom strength of multiple types of gelatin gels (Choi & Regenstein, 2000). This has been further backed up in a study that found that salt weakens a gels structure, specifically the difference between rigidity (G′) and viscosity (G″) proportionally decreases with increasing salt concentrations (Brizzi et al., 2016). Brizzi et al. (2016) deemed that highly concentrated salted gelatins have quasi-viscoelastic behavior, a theory used to model viscoelasticity of soft tissues (Kohandel, Sivaloganathan, & Tenti, 2008).

Contrary that all sodium removal is good for gel strength, research has also shown that small amounts of minerals may improve the functionality of gelatin gels. For example, gelatin gels derived from lizardfish (*Saurida* Spp.) prepared with 0.8% NaCl and 0.5% NaOH showed higher gel strengths over various evaluated conditions using less NaCl (Wardhani et al., 2017). Another study reported that the addition of salt at 1.5% had a shielding or screening effect on protein and pectin molecules which prevented it from coalescing (Cheng et al., 2008). On the other hand, looking at the second most predominant mineral in H-SDJ and D-SDJ (Al), researchers found that the addition of aluminum potassium sulfate acted to increase the mechanical strength of a gelatin with up to ~10% NaCl concentration (Siimon, Mõisavald, Siimon, & Järvekülg, 2015). Given that Na and Al are still present in higher proportions in D-SDJ (compared to other minerals), further research will need to be conducted to determine the effect that these minerals have at different concentrations on the physicochemical properties of the resultant jellyfish gelatin gels.

Effect of solid concentration

Typically, higher solid concentrations produce stronger gelatin gels and this pattern was observed in gelatin gels produced with H-SDJ and D-SDJ at 4°C pH 2.4 (Figure 2.5 A). Choi and Regenstein (2000) demonstrated that stronger fish and mammalian gelatin gels are produced

with higher solid concentrations. At incubation temperatures of 4, 7 and 10°C, similar trends were observed for gelatin gels produced with both H-SDJ and D-SDJ (Figure 2.5 A, B and C). In general gelatins gels produced with 10% solids concentration showed significantly (p<0.05) higher Bloom strengths than those produced with 5 and 6.67% solids. Meanwhile, similar gel strengths were achieved with gelatin gels produced with 5 and 6.67% solids concentrations. At 10°C maturation temperature, increasing the solids concentration of gelatin gels prepared with D-SDJ from 6.67 to 10% increased their Bloom strength by 264, 206, and 221% when they were prepared at a pH of 2.4, 4.4 and 6.5, respectively (Figure 2.5 C). Similar results were observed when testing the Bloom strength of gelatin gels produced from jellyfish (*Rhopilema hispidum*), where low gel strengths were observed at solids concentrations lower than 5% then Bloom strength drastically improved when the gels were prepared at 5 and 6.67% solids (Cho, Ahn, Koo, & Kim, 2014). Because of differences in the amino acid profile of collagen obtained from porcine, bovine, the jellyfish (*Rhopilema hispidum*) and cannonball jellyfish, specifically in hydroxyproline, proline and glycine, the resultant gelatin gels produced at different solids concentrations will show different Bloom strengths (Cho et al., 2014; Gómez-Guillén et al., 2002). In short, the strength of gelatin gels depends on solids concentration whereas a gel with less solids tends to act more like a liquid while at higher solids may behave more as semi-solid material (Kamel & Deman, 1977). Additionally, higher concentrations have been associated with a faster gelation process due to second order kinetics (Djabourov, Nishinari, & Ross-Murphy, 2013).

Effect of pH

Adjustments to the pH of the gelatin gels produced with H-SDJ and D-SDJ were performed to understand pH's effect on the Bloom strength (Fig. 2.6). Gelatin gels were prepared

with H-SDJ and D-SDJ at three pH conditions including pH 2.4 (original), 4.4 (pI) and 6.5. The results revealed that gelatin gels with significantly (p<0.05) higher Bloom strengths were produced at pH values of 4.4 and 6.5 regardless of the solid concentration and maturation temperatures (Fig. 2.6 A, B and C). These findings may suggest that stronger gelatin gels are produced at pH values close or higher than the pI of the jellyfish collagen. The effect of pH on Bloom strength was more evident with gels prepared with 10% solids (Fig. 2.6). Furthermore, at 10°C maturation temperature, increasing the pH of gelatin gels produced with D-SDJ from 2.4 to 4.4 increased their Bloom strength by 276, 323, and 252% when they were prepared with 5, 6.67, and 10% solids, respectively (Figure 2.6 C).

In short, if using a higher maturation temperature and a stronger gelatin is desired then pH adjustments to a more neutral pH, like 6.5, may positively affect the strength of the gelatin if using a solids content of 6.67% or higher. Researchers demonstrated that gel strengths decreased significantly below a pH of 4 and above a pH of 8 while maximum gel strength occurred between pH 4 and 8 (Choi & Regenstein, 2000). Similar results were seen that as pH was decreased (below 4), weaker gels were observed due to increased degradation of the proteins (Papadopoulou, Rizos, & Aggeli, 2016). Research found that increasing the pH towards the isoelectric point has been shown to form a more compact and stiffer gelatin network (Gudmundsson & Hafsteinsson, 1997).

Effect of maturation temperature

Effect of maturation temperatures on the Bloom strength of gelatin gels produced with H-SDJ and D-SDJ at varying percent solids and pHs is shown in Figure 2.7. The three maturation temperatures that were utilized were 4, 7, and 10°C. In general, gelatin gels maturated at 4 and 7°C had significantly (p<0.05) higher Bloom values than those maturated at 10°C. It is believed

that these results may be due to 10°C being closer to the gelling and melting temperatures of the gelatin gels. The novel jellyfish gelatins presented in this study were developed using an acidic hydrolysis treatment which likely formed a Type A gelatin. Research has shown that lower maturation temperatures enhance Type A gelatin gel formation while destabilizing occurs at higher temperatures (Chatterjee & Bohidar, 2006). Furthermore, at 6.67% solids concentration, increasing the maturation temperature of gelatin gels produced with D-SDJ from 7 to 10 °C, reduced their Bloom strength by 64, 28, and 12% when they were prepared at pH of 2.4, 4,4 and 6.5, respectively (Fig. 2.7 B). This may suggest that increasing the pH of gelatin gels can improve their stability at higher maturation temperatures closer to their melting points. In short, if using a higher maturation temperature, like 10°C, with a solids content from 5 to 10% then using a pH near the gelatins pI can improve the gelatin as if lower maturation temperatures were being used. Choi and Regenstein (2000) reported similar trends to what was observed in this research at pH 2.4 for 5 and 6.67% solids where Bloom decreased at a higher maturation temperature. However, their research did not take into consideration how pH and concentration in addition to maturation temperature may affect the Bloom strength of the resultant gelatin gels.

Conclusion

The production of a novel gelatin powder from salted, dried jellyfish was successfully achieved through acid hydrolysis, demineralization by dialysis, and freeze-drying. The dialysis process was able to remove ~97% of the minerals present in hydrolyzed salted, dried jellyfish. The hydrolyzed and dialyzed gelatin powders were both dry and shelf stable with low moisture content and water activity (<0.2) values. Demineralization significantly increased the Bloom strength of the jellyfish gelatin gels regardless of the pH, solid concentration, and maturation temperature utilized. Moreover, demineralized jellyfish gelatin gels produced with 10% solids

concentration, at pH 4.4 and 6.5 and maturated at temperatures of 4°C had higher Bloom strengths (>60-65 g). This was a tremendous improvement compared to gelatins gels produced with 10% solids concentration, at pH 4.4 and 6.5 and maturated at temperatures of 4°C using hydrolyzed, un-dialyzed jellyfish gelatin powders (Bloom strengths <8 g). Stronger gelatin gels were produced when maturated at 4 and 7°C, with 10% solids and pH values of 4.4 and 6.5. Also, it was demonstrated that a pH near the gelatins isoelectric point produced no significant differences in the gel strength from 4 to 10°C at 5 to 10% solids while at a low pH differences arose. The study successfully demonstrated that novel gelatin powders with low mineral content can be produced from salted, dry jellyfish which can potentially be used as a functional ingredient in numerous food applications.

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CHAPTER 3

EFFECT OF SODIUM ALGINATE AND POMEGRANATE POLYPHENOLS ON BLOOM STRENGTH AND THEIR EFFECT ON MICROENCAPSULATION OF *LACTOBACILLUS RHAMNOSUS* GG WITH NOVEL GELATINS DERIVED FROM SALTED AND DRIED CANNONBALL JELLYFISH PREPARED AT DIFFERENT PH'S: THE OPTIMIZATION OF JELLYFISH GELATIN & PROBIOTIC INSERTION[3]

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Abstract

The production of alternative gelatins from marine sources remains low as they are considered less desirable. The process to produce a gelatin from hydrolyzed and dialyzed salted, dried jellyfish (D-SDJ) has shown promising results. This study focused on optimizing the novel D-SDJ using the addition of pomegranate polyphenols (PPs) and sodium alginate (Na-Alg) at concentrations of 360 μg/g of gelatin and 0.4% (w/v), respectively. Evaluating the effect of PPs and Na-Alg addition on the Bloom strength and cell viability using *Lactobacillus rhamnosus* GG (LRGG) was assessed. Gel strength was significantly increased at 10% solids after being crosslinked with PPs and the addition was strongest at pH 4.4 & 6.5 especially at 4°C maturation temperature which improved gel strength from 60.5 to 120.6 g and 64.9 to 113.5 g a 49.8% and 42.8% increase in Bloom strength over the non-crosslinked D-SDJ (control), respectively. Both Na-Alg and PPs were stronger than the control at native pH (2.4) at temperatures 4 and 7°C. After probiotic microencapsulation using freeze drying (FD) and spray drying (SD), data found that SD with the addition of PPs and Na-Alg at neutral pH (6.5) significantly improved the survival of LRGG before and after SD by ~205 and ~410% compared to pH 2.4, respectively, and during the 28-day shelf-life study by ~1 log CFU/g solids compared to the non-crosslinked D-SDJ. Low pH (2.4) reported minimal LRGG survival while the potential discovery of an antimicrobial peptide from cannonball jellyfish could serve as a potential antimicrobial food additive. The observations in this study suggests that optimization of D-SDJ with the addition of PPs and/or Na-Alg can significantly improve gel strength in accordance with pH, concentration, and maturation temperature adjustments as well as increase cell viability for encapsulated LRGG.

Keywords**:** LRGG, microencapsulation, antioxidants, crosslinking, sodium alginate, gelatin, jellyfish, Bloom, spray drying

Introduction

The probiotic market is one of the fastest growing sectors in the food industry expected to be worth 57.2 billion USD in 2022 with a 7.8% compound annual growth rate (Greenberg, 2019). Probiotics are live microorganisms, which when administered in adequate quantities confer health benefits to the host (Reid, 2016). Therefore, the use of microencapsulated probiotic cultures has gained more attention because they are relatively easy to handle, transport, store, and have the capability to be used in various food applications. However, microencapsulation often exposes probiotic cells to harsh environmental conditions that reduces cell viability; thus, novel strategies (e.g., complex coacervation, spray drying, electrospraying) have been investigated to increase the survivability of probiotics during microencapsulation (Eratte, Dowling, Barrow, & Adhikari, 2018).

According to Florou-Paneri, Christaki, and Bonos (2013), *Lactobacillus rhamnosus* GG (LRGG) is a Gram-positive, non-spore forming, rod shaped, homofermentative lactic acid bacteria with probiotic properties. LRGG has the capability to survive and proliferate in gastric acid pH, bile-containing medium conditions as well as the capability to adhere to enterocytes (Capurso, 2019; Gorbach, 1990). Furthermore, LRGG has shown positive effects in treating various diseases like irritable bowel syndrome, atopic dermatitis, and diarrhea (Blaabjerg, Artzi, & Aabenhus, 2017; Reid, 2016). It has been reported that children treated with LRGG at a dosage of 5×10^9 (colony forming units (CFU)/day for 14 d was shown to be successful against *C. difficile* while 1×10^{10} CFU/day was successful after 10 d in children (<12 kg) with antibiotic-associated diarrhea (Biller, Katz, Flores, Buie, & Gorbach, 1995; Blaabjerg et al., 2017).

Microencapsulation is a process where thin films or polymer coats are applied to small solid particles, droplets of liquids or even gases (Jackson & Lee, 1991). This process has been used for several decades to improve the stability of ‘active’ or sensitive materials (e.g., flavors,

aromas, antioxidants, fragrances, lipids, etc.) by surrounding the 'active' materials within a protective matrix that is inert to what it is encapsulating (Ré, 1998). According to Jackson and Lee (1991), microencapsulation requires the use of a wall material or microencapsulating agent (e.g., gums, lipids, hydrocolloids, carbohydrates, and even proteins like gelatin) and the selection of the microencapsulating agent depends on properties of the core material and how that microencapsulated product will be utilized, stored, and further processed. Generally, spray drying microencapsulation involves three steps (1) droplet production (atomization); (2) droplet-to-particle conversion by drying (solvent vaporization); and (3) sample collection (separating sample from drying air) (Bah, Bilal, & Wang, 2020). The food industry uses microencapsulation for six major reasons: to reduce the core materials reactivity with environment factors, to decrease the core materials transfer rate with the environment, to promote easier handling, to control the release of the core material, to mask a taste, and to dilute the core material (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). A challenge of microencapsulating probiotics is to find a compatible microencapsulating agent that can protect the probiotic cells against environmental stressors (e.g., storage temperature, oxygen exposure, relative humidity, light, moisture content), which can affect the viability of the bacterial cells during processing and storage (Tripathi & Giri, 2014). It has been shown that using oxygen impermeable packaging material for the storage of probiotics can largely impact the survival of the bacteria (Lopes, Bueno, Aguiar Jr., & Finkler, 2017).

Marine gelatins have unique functional properties that may be of great interest to the food industry; for example, they show lower gelling and melting temperatures compared to bovine and porcine gelatins which may allow for a quick release of encapsulated materials (Karim & Bhat, 2009). Karim and Bhat (2009) also reported that the gelling and melting temperatures of

gelatins can be modified by crosslinking with hydrocolloids, polymers, plant phenolics, genipin, and enzymes like transglutaminase. In addition, crosslinked gelatins using natural plant PPs have shown increased mechanical strength and thermal stability but contains fewer amino groups and holds less water (Strauss & Gibson, 2004). Zhao and Sun (2017) reported successful crosslinking of bovine gelatin with PPs from *Fructus chebulae*, or Helile, and found that crosslinking at 360 µg PPs/g of gelatin resulted in the highest thermal stability and the strongest mechanical properties in the gelatin hydrogels. The study also reported that polyphenolic crosslinking of gelatin was more cost-effective than genipin crosslinking, the most common natural crosslinking agent. Additionally, it has been reported that polyphenol-containing extracts from six cultivars of Georgia-grown pomegranate peels had ~6% hydrolyzable tannins (majority ellagitannins, specifically punicalagin isomers and punicalin) and less than 1% catechin followed by quercetin and epicatechin (Pande & Akoh, 2009).

Other strategies have been used to improve the mechanical strengths and physicochemical properties of gelatins; for example, Etxabide, Urdanpilleta, Gómez-Arriaran, De La Caba, and Guerrero (2017) demonstrated that gelatin films processed at a pH of 5.4 had higher decoloration and lower solubility which signified a greater extension of crosslinking compared to pH 2.0. Meanwhile, Sow, Toh, Wong, and Yang (2019) reported that the addition of sodium-alginate (Na-Alg) into fish gelatin at a concentration of 0.4% (w/v) significantly improved the gel strength of the resultant gelatin gels. Microencapsulation of *Lactobacillus plantarum* ST16Pa at various pHs (3, 4, 5, 6, 7, 9, 11, 13) showed the lowest growth curves at low pH (3, 4) while the addition of Na-Alg, regardless of the concentration used (2, 3 or 4%), protected the bacteria from acidic conditions during the microencapsulation process (Todorov, LeBlanc, & Franco, 2012). In another study, it was reported that free *Lactobacillus casei* NCDC-

298 cells were drastically reduced at pH 1.5 while those microencapsulated with Na-Alg at 2, 3, or 4% were significantly higher than the free cells (Mandal, Puniya, & Singh, 2006).

Freeze drying is the preferred method to microencapsulate probiotic cultures compared to spray drying (SD) (Moayyedi et al., 2018). However, SD is a continuous and a cost-effective alternative to microencapsulate probiotic cultures. It has been reported that microencapsulation by SD can improve the cell viability of LRGG over a period of 6-months using a double-encapsulation technique (Sohail, Turner, Coombes, & Bhandari, 2013). Preliminary studies in our lab have successfully microencapsulated LRGG with fish gelatin by mixed-flow spray-drying (MFSD) at 140°C inlet and 65-75°C outlet air temperatures. The resultant probiotic powders had higher biofilm forming capabilities over probiotic powders produced by concurrent spray-drying, freeze drying, and the non-encapsulated cells (Jiang, Dev Kumar, Chen, Mishra, & Mis Solval, 2020).

The crosslinked gelatins with PPs and/or Na-Alg as a microencapsulating agent for probiotics can improve cell viability by creating a more compact/denser environment; thus, reducing oxygen and light exposure (Johnston, 2013; Zhao & Sun, 2017). Currently, limited studies have been conducted on the effects of Bloom strength and the survival of microencapsulation LRGG *via* spray drying using crosslinked gelatins from cannonball jellyfish. Moreover, the effect of pH on the survival of probiotic cells after microencapsulation with crosslinked jellyfish gelatins is not fully understood. The feasibility of microencapsulating the probiotic LRGG with novel gelatins derived from cannonball jellyfish and prepared at different pHs with Na-Alg and/or PPs was evaluated in this study. Therefore, the objective of this study was to use jellyfish gelatin crosslinked with pomegranate peel PPs and/or Na-Alg to improve the

gelatins Bloom strength and to reduce the effect of environmental stressors on the microencapsulated LRGG, thus improving cell viability during processing and storage.

Materials/Methods

Materials

Hydrolyzed and dialyzed gelatin powders from salted and dried jellyfish (D-SDJ) was previously prepared based on preliminary studies. Fresh pomegranates were received from the Georgia Pomegranate Association (Alma, GA, USA). Na-Alg was purchased from Cape Crystal Brands (Summit, NJ, USA). LRGG (ATCC 53103) was obtained in a capsulated powdered form from Culturelle Probiotics (Cromwell, CT, USA) and was grown on de Man, Rogosa and Sharpe (MRS) agar from Thermo Fisher Scientific (Waltham, MA, USA). Butterfield's phosphate buffer (BPB) from Hardy Diagnostics (Santa Maria, CA, USA) was used to serially dilute cultures for enumeration purposes.

Isolation of polyphenols from pomegranate peels

Polyphenol extraction was followed according to a method previously described by Srivastava et al. (2010) with slight modifications. Fresh pomegranate peels were air dried and powdered using a kitchen blender (Model BL610, Ninja, Shark Ninja Operating LLC, Needham, MA, USA). Then, dry pomegranate peels powders were weighed out and placed into an Erlenmeyer flask with a mixture of ethanol-water (70% ETOH) at a ratio of 1:10 of extracted solute to solvent. The mixture was homogenized using an ultra-shearing processor (Homogenizer 850, Fisher Brand, Fisher Scientific UK Ltd, Loughborough, UK) for 6 min at 8000 rpm then for 4 min at 10000 rpm and then placed into a preheated shaking water bath (50 ± 1°C) for 45 min. Next, the liquid was placed into test tubes and centrifuged (Model J2-21M, Beckman Instruments Inc., Palo Alto, CA, USA) for 20 min at 4,000 x *g*. The supernatant was then filtered

through Whatman No.1 filter paper (Whatman International Ltd., Maidstone, England) using a Büchner funnel. The extraction was repeated again as described above using 70% ETOH with the filtered solids. The supernatants, containing the polyphenolic compounds, were pooled and transferred to a round-bottom flask. The ETOH was removed using a Rotavapor (Büchi RE 111, Flawil, Switzerland) (connected to a vacuum pump system) set at 60-65°C as reported by Srivastava et al. (2010). Lastly, the concentrated polyphenol extract was first frozen overnight for 12 h at -4°C then was freeze-dried at -55°C for 2 days and -4°C for 3 days in order produce a polyphenol powder, which was stored in amber glass vials at -4°C until further use.

Crosslinking D-SDJ with PPs

The crosslinking of D-SDJ with PPs (D-SDJ+PP) was carried out by following the official method of the Gelatin Manufacturers Institute of American Inc. (GMIA) with slight modifications (GMIA, 2019; Zhao & Sun, 2017). Briefly, 7.5 g of D-SDJ was hydrated in 70 mL of deionized water (DIW) for 2 h and then placed into a 65°C waterbath for 15 min, swirling periodically, until D-SDJ was fully dissolved. Then, 360 µg of PPs per gram of D-SDJ was dissolved in 20 mL of DIW and added to the D-SDJ solution. Gelatin solutions containing 105 mL of DIW and 7.5 g D-SDJ were prepared (6.67% solids, w/v) in a Bloom jar. Gelatin solutions with 5 and 10% solids (w/v) were also prepared. Next, the pH of the gelatin solutions was adjusted with a 1 M NaOH solution; three pHs were evaluated, 2.4 (original), 4.4 (isoelectric point (pI) of D-SDJ), and pH 6.5. Afterwards, the gelatin solutions were placed into a 40°C waterbath for 30 min to allow for crosslinking to occur. The solution surface was then skimmed off of any foam/bubbles on the surface, lid wiped, and allowed to temper at room temperature for 15 min. Next, the gelatin solutions were placed at 4, 7, and/or 10°C for 17±1 h then tested for Bloom strength by following the Bloom strength method described below.

Preparation of D-SDJ with Na-Alg

The gelatins gels were prepared at either 5, 6.67, or 10% solids (w/v) using D-SDJ. The procedure was followed according to the official method of the Gelatin Manufacturers Institute of American Inc. (GMIA) with slight modifications (GMIA, 2019; Sow et al., 2019). For 6.67% concentration (w/v), 7.5 g dry wt. D-SDJ + 0.45 g Na-Alg + 104.5 mL of DIW was used. Half the DIW was used to hydrate the D-SDJ and allowed to completely swell for 2 h at room temperature. During hydration, Na-Alg was hydrated by pouring half of the remaining DIW into the Na-Alg beaker and heating the Na-Alg-water mixture in a 65°C waterbath for 5 min then it was stirred until fully dissolved. After D-SDJ hydration, the solution was placed into a 65°C waterbath for 15 min, swirling periodically. Once complete, the D-SDJ was put on a stir plate and the Na-Alg-water mixture was added to the D-SDJ mixture in a Bloom jar. As previously described, the pH of the gelatin solutions was adjusted with a 1 M NaOH solution; three pHs were evaluated, 2.4 (original), 4.4 (pI of D-SDJ), and 6.5. The solution surface was then skimmed off of any foam/bubbles on the surface, lid wiped, and allowed to temper at room temperature for 15 min. Next, the gelatin solutions were placed at 4, 7, and/or 10°C for 17±1 h. After maturation, the Bloom strength of the resultant gelatin gels was measured according to the Bloom strength method described below.

Bloom strength determination

After creation of the gelatin gels, a TA.XT Plus texture analyzer (Stable Micro Systems Ltd, Godalming, UK) was used to determine the Bloom strength (g) of the gelatins at 4 mm penetration depth following the official method of the Gelatin Manufacturers Institute of American Inc. (GMIA, 2019). In short, three samples were tested which were D-SDJ+PP, D-SDJ+Na-Alg, and D-SDJ alone (control). After maturation at one of the three temperatures, a

sample was then placed on the TA unit where a 12.7 mm in diameter probe depressed the surface of the gel by 4 mm at a speed of 1 mm/s. The peak force in grams was recorded and this peak forced is referred to as the Bloom strength of the gelatin.

Microencapsulation of LRGG

Preparation of LRGG cultures

LRGG was cultured using a previously described protocol (Jiang et al., 2020). In short, one probiotic capsule containing LRGG was dissolved in 9 mL of sterile BPB. Then, 100 µL of the solution was spread plated on MRS agar plates (100mm x 15mm) (prepared according to the instructions described on the MRS agar packaging) using a sterile disposable cell spreader (HS8151, Heathrow Scientific, Vernon Hills, IL, USA) then placed into a zip-lock bag and incubated for 24 h at 37°C. After 24 h, 1 mL of sterile BPB was added to the plate, cells were harvested using a cell spreader then another 1 mL BPB was added and again the cells were collected using a pipette and pipetted into microcentrifuge tubes (one plate provided one LRGG pellet). The resulting slurry was centrifuged at 13,000 x *g* for 1 min using a microcentrifuge (MiniSpin plus, Eppendorf, Hamburg, Germany) then the supernatant was poured out. This was performed to obtain a cell pellet. A washing step, repeated twice was done, where 1 mL of BPB was added to the pellet then thoroughly vortexed to dissolve the pellet, centrifuged again, and the supernatant was poured out to receive the washed LRGG pellet. The pellets were stored for no more than 1 week under refrigeration, which minimized cell loss in the pellets based on preliminary research (Jiang et al., 2020; Klu, Williams, Phillips, & Chen, 2012).

Preparation of LRGG suspensions

Stable suspensions were prepared by homogenizing 100 g/L of the dried, modified jellyfish gelatin powders in deionized water. Then, suspensions were autoclaved at 121°C for 15

min and allowed to cool down to 37°C. Afterwards, LRGG pellets were added into the sterile suspensions while being constantly stirred to produce LRGG suspensions (~9 to 10 log CFU/g solids) which were kept in refrigeration (~4°C) for 12 h before drying (Jiang et al., 2020).

Drying of LRGG suspensions

LRGG suspensions were spray dried in a pilot-scale spray dryer (equipped with a two-fluid nozzle) (Anhydro, PSD 52, Denmark) under mixed-flow conditions at the University of Georgia (UGA) Food Product Innovation and Commercialization Center (Food PIC) in Griffin, GA. The inlet temperature was set at 140°C and outlet was kept between 60 to 75°C. The feed flow rate was set to 13.5 mL/min. This process produced nine microencapsulated LRGG powders (LRGG with D-SDJ, LRGG with D-SDJ+PP, LRGG with D-SDJ+Na-Alg) each microencapsulating agent was evaluated at three pHs (2.4, 4.4, 6.5). Concurrently, suspensions containing LRGG and D-SDJ were also freeze-dried using a pilot-scale freeze dryer (Virtis, The Virtis Company, Gardiner, NY, USA) and used as the control. After the drying procedure, LRGG powders were immediately enumerated, 1 gram of powder allotments were weighed into 15 mL test tubes, placed into a vacuum sealable bag with desiccant, vacuum sealed and stored at 4°C to begin the shelf-life study.

Enumeration of the probiotics

The viability or cell counts were measured following the method previously described by Jiang et al. (2020). In short, 100 µL of the probiotic suspension or 1 gram of dried powders was serial diluted into 99 mL of sterile BPB. The dried powders were allowed to hydrate in the BPB before dilution in order to ensure homogenous samples. Then, 100 µL were pipetted and spread plated on MRS agar. The samples were performed in triplicate and the plates were then placed into a zip-lock bag and incubated for approximately 40 h at 37°C in aerobic conditions. After,

individual colonies were counted and were expressed in log (CFU) per gram of dried solids (log CFU/g) (Jiang et al., 2020).

Shelf-life study

Cell counts in LRGG suspensions and powders were determined before and after the drying procedure, respectively. The shelf-life study was conducted by storing the LRGG powders into 1 gram allotments into 15-mL test tubes which were then further stored in plastic vacuum sealable pouches with desiccant to reduce moisture within the packaging as much as possible. The samples were placed at ~4°C for the shelf-life study. Cell counts of LRGG were determined every 7 d for 28 d by following the procedure described in the enumeration of the probiotics section.

Statistical Analysis

The data collected from this study was determined in triplicate for the plate counts then means and standard deviations were created from the data and analyzed using an analysis of variance (ANOVA) test with an alpha (α) value of 0.05 in order to determine if statistical differences arose between the various LRGG jellyfish gelatin powders that were produced. For the Bloom strength results, a two-way Analysis of Variance (ANOVA) (two independent variables), and post-hoc Tukey's studentized range tests ($\alpha = 0.05$) were employed; while for the rest of the data a one-way ANOVA and post-hoc Tukey's studentized range tests ($\alpha = 0.05$) were conducted to determine the statistical significance of observed differences among the means. This was conducted using RStudio statistical software version 1.2.5033 (RStudio, Inc. Boston, MA, USA).

Results/Discussion

Bloom strength of gelatins gels produced with D-SDJ, D-SDJ+PP, and D-SDJ+Na-Alg

Preliminary studies in our lab have demonstrated the feasibility of producing gelatin gels from hydrolyzed salted and dried jellyfish (H-SDJ). Additionally, a demineralization process using H-SDJ to produce hydrolyzed and dialyzed SDJ (D-SDJ) was effective at removing the minerals and improving the Bloom strength of the resultant gelatin gels. In this study, a further improvement D-SDJ with the addition of PPs isolated from pomegranate peels and Na-Alg was evaluated. It has been reported that marine gelatins can be preferred over mammalian gelatins as they typically have a lower melting point, which results in faster dissolution in the mouth without the chewy mouth-feel (Karim & Bhat, 2009). In short, further optimization of the D-SDJ will provide more application usages for this marine gelatin.

Effect of solid concentration

The effect of the gelatin powders concentration (5, 6.67, and/or 10%, w/v) at three pHs (2.4, 4.4, and/or 6.5) and three maturation temperatures (4, 7, and/or 10°C) on the Bloom strength of the resultant gelatin gels was first evaluated (Fig. 3.1). There was a slight improvement in the Bloom strength of gelatin gels prepared with 6.67% (w/v) gelatin powders over those prepared with 5% gelatin powders. Surprisingly, gelatin gels prepared with D-SDJ+PP at 10% solid concentration showed significantly (p<0.05) higher Bloom strengths than the rest of the gelatins at all evaluated pHs and all three maturation temperatures. This large increase in gel strength at 10% concentration, which was not observed at the other concentrations is believed to be due to the presence of more noncovalent bonds in gelatins formed with 10% gelatin powders. It is believed that the addition of PPs to D-SDJ provided more opportunity for the exposed functional groups that were not bonded in D-SDJ alone to bond with the added PPs thus allowing more crosslinking, like hydrogen bonding, to occur. This ultimately produced the significantly stronger gelatin gels at 10% concentrations compared to the D-SDJ+Na-Alg and

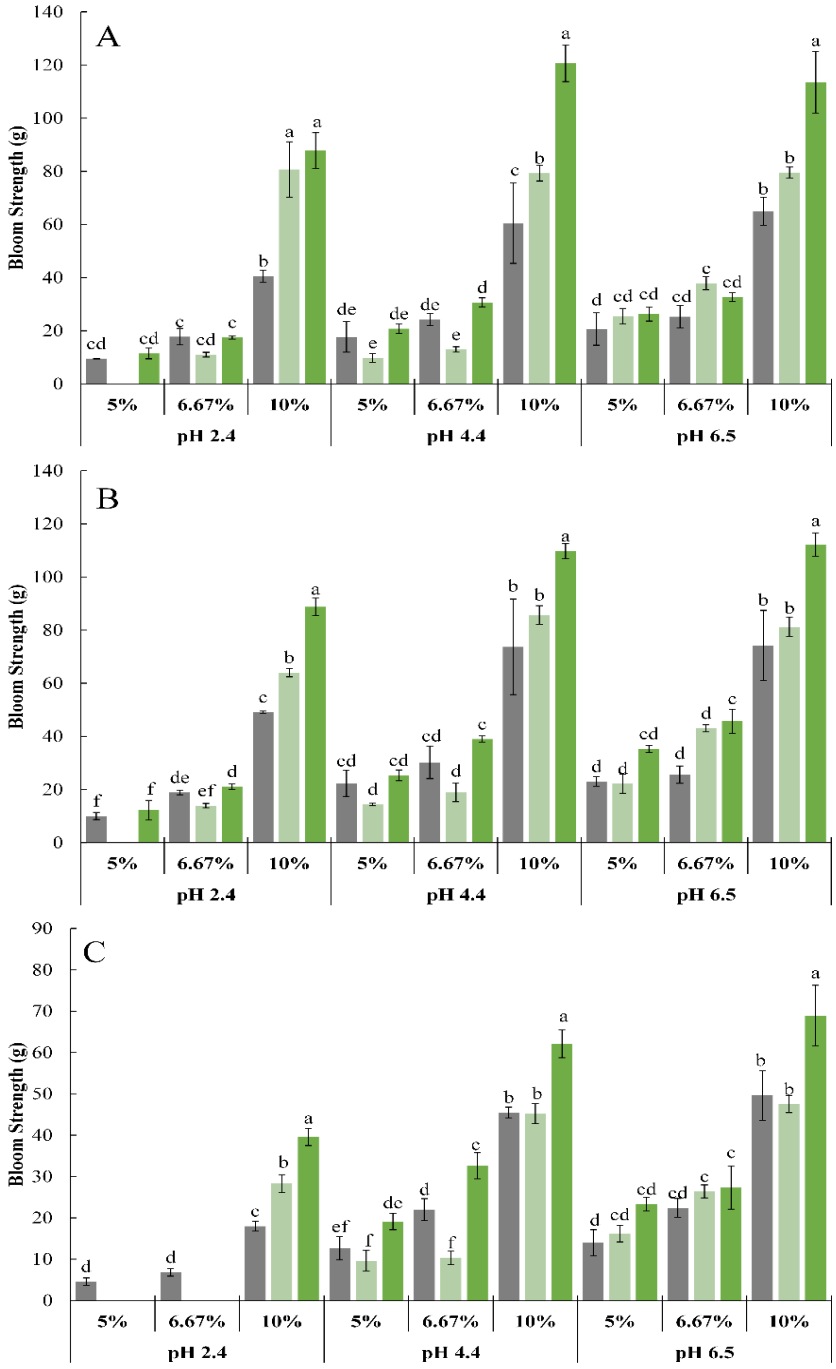

Fig. 3.1. – Effect of solid concentration (%) on Bloom strength (g) of jellyfish gelatin gels (■ = D-SDJ; ■ = D-SDJ+Na-Alg; ■ = D-SDJ+PP) maturated at different temperatures (A = 4°C; B = 7°C; C = 10°C). D-SDJ = Gelatin gels produced with hydrolyzed and dialyzed salted, dried jellyfish (D-SDJ); D-SDJ+Na-Alg = Jellyfish gelatin gels produced with added sodium alginate; D-SDJ+PP = Jellyfish gelatins gels produced with added polyphenols. [abcd]Means treatments with different letters at the same pH and maturation temperature (°C) are significantly different (p<0.05).

control (D-SDJ). Similar results were observed where tea polyphenols were added to a gelatin+Na-Alg network and the researchers hypothesized that the lower molecular weight PPs inserted themselves into the network, which resulted in more covalent crosslinking (Dou, Li, Zhang, Chu, & Hou, 2018). This may not have been observed at the lower concentrations due to limited functional groups present for the PPs to insert themselves into. Previous research mentioned that the increased gel strength with the addition of PPs is likely due to the formation of more hydrogen bonds; however, adding too high of PPs concentrations will decrease the Bloom strength of the gelatin gels as PPs can also be used to precipitate out gelatin/collagen components (Zhao & Sun, 2017).

Having a lower concentration of D-SDJ+Na-Alg and D-SDJ+PP (<6.67% solids) resulted in no difference in the gel strength compared to the control (Fig. 3.1 A-C). At 5 and 6.67% solid concentration, the concentration of gelatin with PPs was not sufficient to form crosslinks and produce stronger networks like seen at 10% solids. In the case of D-SDJ+Na-Alg, researchers have reported that without the presence of free Ca^{2+}, then alginate may be confined to the gelatin matrix and interfere with the gel network formation while with Ca^{2+} it forms a bi-continuous network thus improving gel strength (Sow et al., 2019). This phenomenon may be due to the creation of *Egg-box junctions*, which is where charged polysaccharides, in this case the added alginate, establishes bridges between two parallel polymer molecules using divalent cations, like Ca^{2+}, present in the D-SDJ, thus leading to more rigid junctions (Damodaran & Parkin, 2017). Moreover, researchers have reported that Na-Alg improved gel modulus when added to a gelatin at its native pH (Goudoulas & Germann, 2017). In this study, as the concentration of Na-Alg increased to 10% solids at pH 2.4, the native pH, it produced significantly stronger gels compared to the control which may be due to Na-Alg preferring the native pH as well as having

more Ca^{2+} at the higher solid concentration. However, as pH was increased (4.4 and 6.5) the control significantly improved and there was a comparable gel strength between D-SDJ+Na-Alg and D-SDJ. Preliminary studies demonstrated that the Bloom strength of the gelatin gels produced with D-SDJ can be improved by increasing the pH from 2.4 to 4.4 or 6.5. Importantly, at a low pH (2.4) significantly stronger gels using D-SDJ can be produced without having to do an adjustment to the pH with the addition of Na-Alg (0.4%, w/v) and/or PPs (360 µg/g gelatin) at 10% concentration using a maturation temperature from 4 to 10°C. Practical application where a low pH is necessary and an increase in gel strength or stiffness may be desired like in jellies/jams that are kept in refrigeration.

Effect of pH

When comparing the effect of pH, significantly ($p<0.05$) weaker gels were observed at pH 2.4 compared to pH 4.4 and 6.5 but not typically between 4.4 and 6.5 (Fig. 3.2 A-C). This is likely due to the fact that pH increases will not impact the Bloom strength past a given pH (presumably the pI of gelatins). An optimum pH range for fish gelatin was found to be between 4 to 8 while lowering the pH below 4 and raising the pH greater than 8 decreased gel strength (Choi & Regenstein, 2000). Additionally, with a higher pH (above 2.4), producing stronger gels may be explained due to being closer to the pI where stronger gels have been associated with increased pH. It has been mentioned that proteins generally carry a net charge which causes electrostatic repulsion among the protein molecules thus potentially hindering gelatin formation (Damodaran & Parkin, 2017). Additionally, this was shown to occur in gelatin from cod skins as the pH approached their pI, the gelatins became more neutrally charged and formed more compact and stiffer gels (Gudmundsson & Hafsteinsson, 1997). As the pH is increased towards the pI or above the pI this electrostatic repulsion is not nearly as strong and, in this data, appears to allow

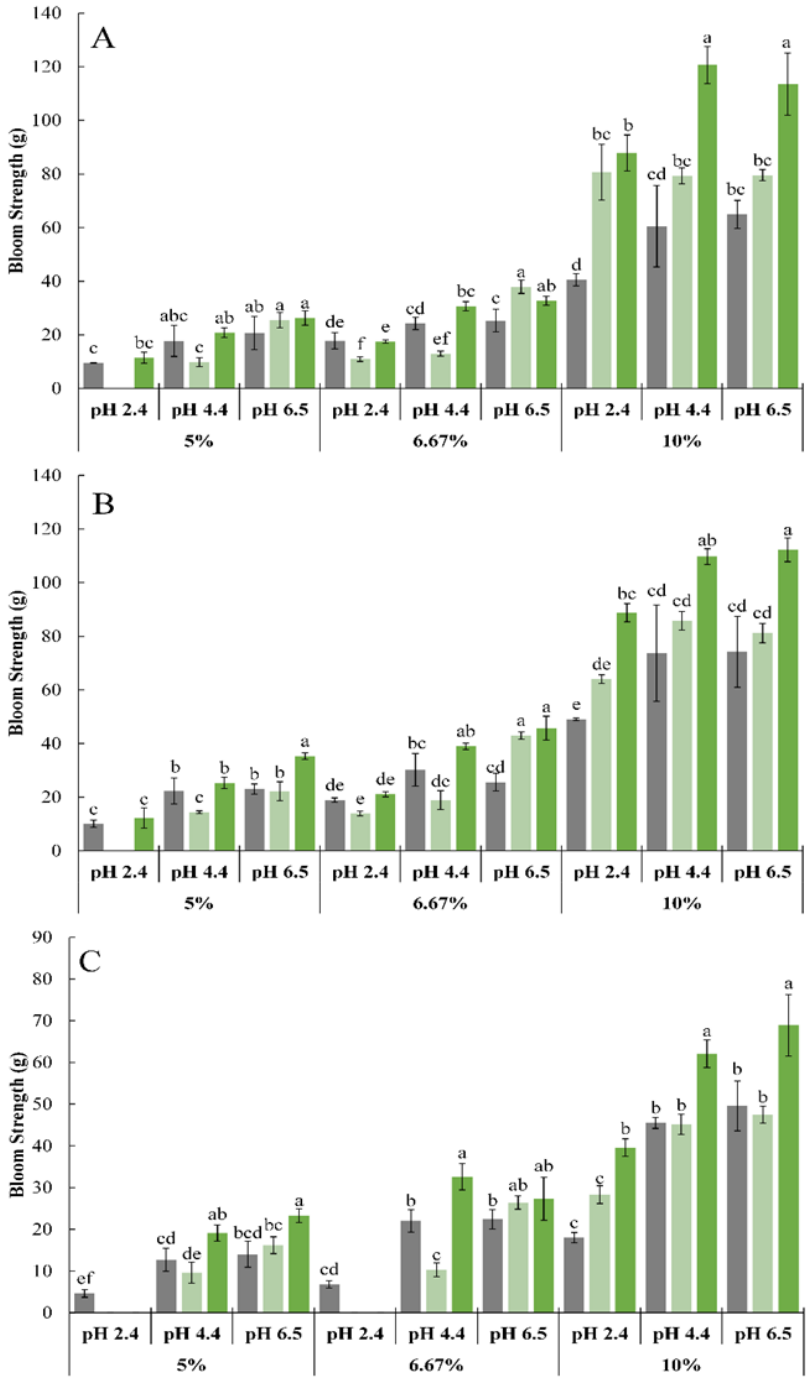

Fig. 3.2. – Effect of pH on Bloom strength (g) of jellyfish gelatin gels (■ = D-SDJ; ■ = D-SDJ+Na-Alg; ■ = D-SDJ+PP) maturated at different temperatures (A = 4°C; B = 7°C; C = 10°C). D-SDJ = Gelatin gels produced with hydrolyzed and dialyzed salted, dried jellyfish (D-SDJ); D-SDJ+Na-Alg = Jellyfish gelatin gels produced with added sodium alginate; D-SDJ+PP = Jellyfish gelatins gels produced with added polyphenols. [abcd]Means treatments with different letters at the same solid concentration (%) and maturation temperature (°C) are significantly different (p<0.05).

for increased gel strength for the gelatins at pH 4.4 and 6.5 compared to pH 2.4 for most of the three treatments (D-SDJ, D-SDJ+Na-Alg and D-SDJ+PP). This may be due to experiencing more electrostatic repulsion at the lower pH than at the higher pH's. Bloom strength of gelatin gels is then controlled to some extent by the pH of that gelatin (Gudmundsson & Hafsteinsson, 1997).

The data shows that D-SDJ+Na-Alg was the most affected by low pH of 2.4 and with increasing pH the gel strength was significantly stronger at pH 6.5 with 5 and 6.67% concentration at each of three temperatures compared with the other two lower pH's and the control; while at 10% concentration there was generally no significant differences in the gel strength between D-SDJ+Na-Alg and the control and at pH 6.5 from the other pHs (Fig. 3.2). Sow et al. (2019) mentioned that electrostatic interactions occurred in fish gelatin gels in the pH range of 6.41 to 6.67. This increase in gel strength at pH 6.5 may be attributed to those electrostatic interactions that occurred at this pH in the D-SDJ+Na-Alg complex thus resulting in the significantly stronger gels. Therefore, it is predicted that the D-SDJ+Na-Alg complex has a synergists effect where there is an optimum pH and concentration as well as some *Egg-box junctions* that are occurring, each of which is necessary to create significantly stronger gels than the control; however, if those optimum levels are not meet, weaker gels are likely to be produced.

Increasing the pH of the gelatin gels prepared with D-SDJ+PP significantly ($p<0.05$) improved their gel strengths at 10% solids concentration. An important trend to mention is at low concentration, 5% solids, at ideal conditions (low maturation temperature, neutral pH) for the most part each of the treatments produced comparable gels (Fig. 3.2 A). As the conditions worsened for a gel, increasing maturation temperature, significant differences began to arise

where PPs produced significantly stronger gels while the once comparable treatments were weakened at these non-ideal conditions (Fig. 3.2 B & C). This data demonstrates that the addition of PPs to D-SDJ at pHs 4.4 and 6.5 can produce significantly stronger gelatin gels when maturated at 10°C compared to the other treatments (Fig. 3.2 C).

Effect of maturation temperature

Higher maturation temperatures resulted in weaker gelatin gels (Fig. 3.3). Moreover, maturation temperatures of 4, 7, and 10°C at 10% solids (pH 2.4) demonstrates significantly (p<0.05) stronger gelatin gels for D-SDJ+PP and D-SDJ+Na-Alg than D-SDJ. Meanwhile at pH's 4.4 and 6.5, D-SDJ+PP was significantly (p<0.05) stronger at 4 and 7°C than the other treatments (Fig. 3.3 C). Surprisingly, the strength of gelatin gels prepared with D-SDJ+PP at 5% solids was comparable to that of D-SDJ at 4, 7, and 10°C for most of the pHs (Fig. 3.3 A). With more solids, more crosslinks/covalent bonds were able to form at 6.67% solids (Fig. 3.3 B) and again at 10% concentration where the significantly strongest gelatin gels were formed for D-SDJ+PP at 4 and 7°C (pH 4.4 and 6.5). Interestingly, gelatin gels prepared with D-SDJ+PP and D-SDJ+Na-Alg were significantly (P<0.5) stronger than those prepared with D-SDJ alone at 4 and 7°C maturation temperatures (pH 2.4) (Fig. 3.3 C). This demonstrates that lower temperatures (4 and 7°C) and higher concentrations (10% solids) may provide an ideal condition for an increase in the number of covalent bonds given the significantly stronger gels formed at these conditions for D-SDJ+PP and D-SDJ+Na-Alg. Similar results have been observed for porcine and fish gelatin as a decrease in maturation temperature resulted in increased Bloom strengths (Choi & Regenstein, 2000).

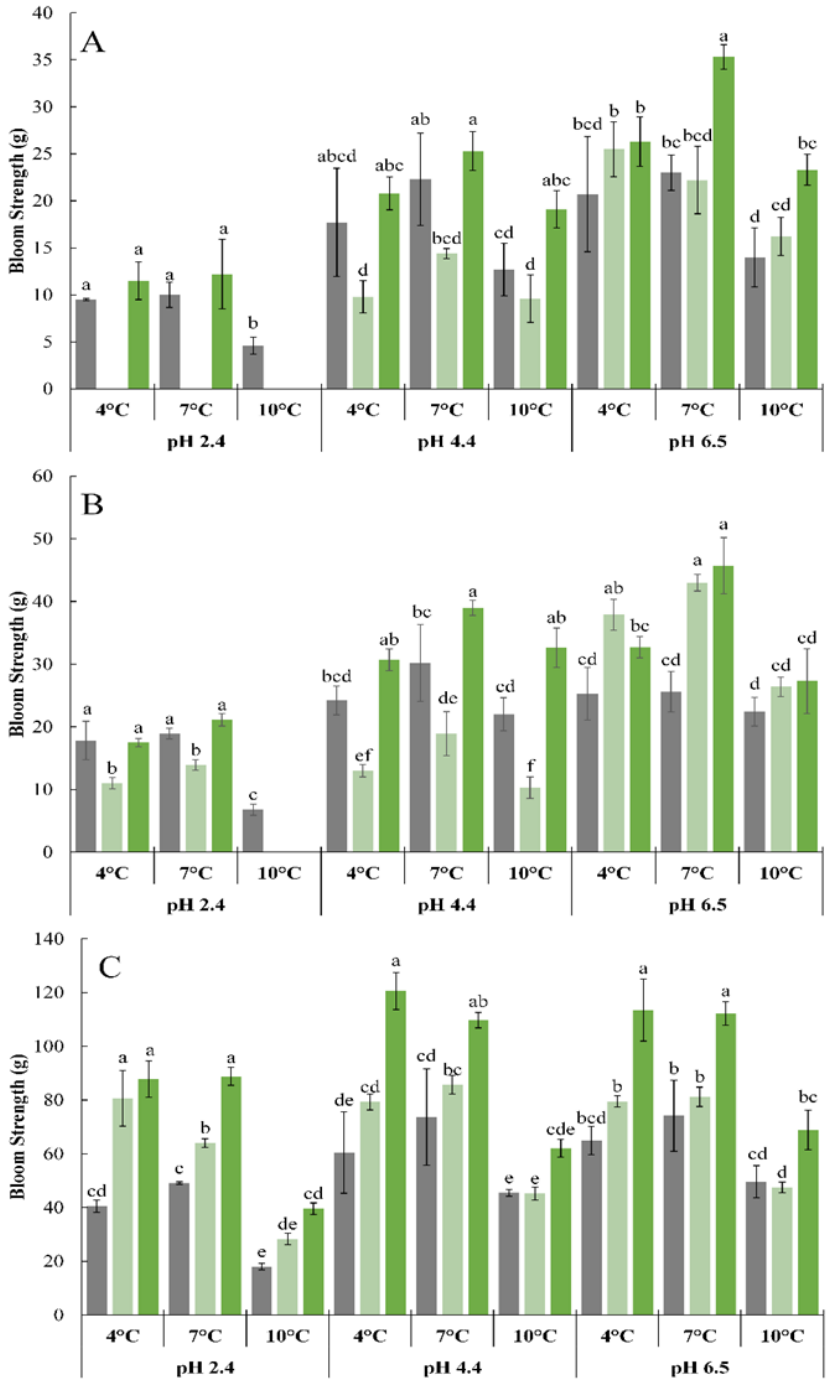

Fig. 3.3. – Effect of maturation temperature (°C) on Bloom strength (g) of jellyfish gelatin gels (= D-SDJ; = D-SDJ+Na-Alg; = D-SDJ+PP) produced at different solid concentrations (A = 5%; B = 6.67%; C = 10%). D-SDJ = Gelatin gels produced with hydrolyzed and dialyzed salted, dried jellyfish (D-SDJ); D-SDJ+Na-Alg = Jellyfish gelatin gels produced with added sodium alginate; D-SDJ+PP = Jellyfish gelatins gels produced with added polyphenols. [abcd]Means treatments with different letters at the same pH and solid concentration (%) are significantly different (p<0.05).

At 10°C maturation temperature, the weakest gels were formed which may suggest that this maturation temperature is closer to the gelatin's melting temperature thus causing a weaker gel strength. For Type A gelatins, lower maturation temperatures enhance gelatin formation while destabilization occurs at higher temperatures (Chatterjee & Bohidar, 2006). The melting point of a gelatin gel is the temperature at which the gelatin network will begin to melt while the gel point is where the solution begins to gel (Wardhani, Rahmawati, Arifin, & Cahyono, 2017). Gelling and melting temperatures of marine gelatins have been reported to be 11-12°C and 13.8°C for cod, 15-18°C and 23-27°C for catfish, and 6.6°C and 19.1°C for the jellyfish *Lobonema smithii*, respectively (Karim & Bhat, 2009; Lueyot et al., 2020). The melting point of the D-SDJ is hypothesized to begin slightly above 10°C as gelation is occurring but as maturation temperature is further decreased, significantly stronger gel networks were formed at 4 and 7°C, in most cases, compared to 10°C for each of three treatments. In short, when looking at temperature and concentration, D-SDJ+PP improved gel strength the most with an increasing concentration (10% solids) and lower temperatures (4 and 7°C) while D-SDJ+Na-Alg improved gel strength at a lower pH (2.4) and a higher solid concentration (10% solids) compared to the control. Future studies will use rheological techniques to determine the actual gelling and melting temperatures of the D-SDJ treatments. The temperature dependence of a gel state, unique for each gelatin, offers numerous opportunities in food manufacturing (Damodaran & Parkin, 2017).

Microencapsulation of LRGG with D-SDJ, D-SDJ+PP and D-SDJ+Na-Alg

Refrigeration storage before drying

Preliminary studies demonstrated that after 12 h storage at 4°C, LRGG suspensions prepared with D-SDJ had a ~3 log CFU/g solids reduction in viable cells of LRGG at pH 2.4

while no significant reduction was observed at pH 4.4 and 6.5 (data not shown), which may be due to an antimicrobial peptide within the D-SDJ gelatin. Additionally, LRGG suspensions prepared with D-SDJ+Na-Alg and D-SDJ+PP experienced a ~1 and ~0.29 log CFU/g solids reduction in viable cells of LRGG at pH 2.4, respectively. It has been reported that a 4.18 log CFU/g solids reduction in viable cells was seen in free, nonencapsulated cells of *Lactobacillus casei* NCDC-298 after 3 h at pH 1.5 compared to pH 6.5 (Mandal et al., 2006). This shows that low pH of probiotic suspensions has a negative effect on the cell survival of the probiotics. Additional tests were performed (data not shown) to determine whether (1) the pH of suspensions was harming the LRGG cells, (2) Na-Alg and/or PPs at pH 2.4 were protecting LRGG cells during 12 h storage at 4°C, and/or (3) if D-SDJ has an antimicrobial affect.

Therefore, three solutions of pH 2.4, 4.4 and 6.5 were prepared with deionized water and pH was adjusted with citric acid and/or 1 M NaOH. Then, the samples were inoculated with ~10 log CFU/g solids of LRGG and stored for 12 h at 4°C. The results revealed a ~1 log CFU/g solids reduction of LRGG cells at pH 2.4 and no significant reduction of cells at pHs 4.4 and 6.5. This suggests that D-SDJ may have some peptides with antibacterial properties that are more active at lower pH's. One study has reported antimicrobial compounds in Cannonball jellyfish (CBJ) which were identified using a bioassay-direct fractionation which found that the antimicrobial activity was present in two fractions, a low-molecular-weight and neutral terpenoid containing fraction (Betz, 1988). However, since the initial finding from this study, no further researched has been conducted on CBJ antimicrobials since. Other studies have reported antimicrobial properties in a variety of species of jellyfish including *Aurelia aurita, Cyanea capillata*, and *Nemopilema nomurai* to name a few (Moon et al., 2011; Ovchinnikova et al., 2006; Zhou et al., 2018). This provides evidence that antimicrobial peptides in jellyfish do exist;

however, further research needs to be conducted to test for the presence of antimicrobial properties in the D-SDJ gelatin.

Cell survival (%) before and after drying

When comparing the cell survival (%) before and after either freeze drying (FD) or spray drying (SD) differences arose at pH 2.4 (Figure 3.4 A). There was minimal cell survival in each of the tested groups because, as mentioned previously, low pH of suspensions was not an ideal environment for the survival of LRGG. One sample, however, did receive slight survival (D-SDJ+Na-Alg) and this persisted to have viable cells for the 28-day shelf-life storage. These results suggest that adding 0.4% (w/v) Na-Alg to D-SDJ at low pH may improve the cell survival of LRGG during microencapsulation. It has been reported Na-Alg is generally used at a concentration from 0.75 to 2% (w/v) while concentrations below this range tend to exhibit a decreased viscosity and less ability to crosslink and concentrations above this range are too viscous, both have been seen to cause difficulties during encapsulation (Lopes et al., 2017). Given this information, using a higher Na-Alg concentration may led to an increase in the cell survival of the LRGG.

As pH of encapsulants was increased to 4.4 and 6.5 differences arose in cell survival of LRGG as well. Surprisingly, probiotic powders prepared with D-SDJ+Na-Alg showed significantly ($p<0.05$) higher cell survival after drying at pH 4.4 and 6.5, an increase in survival of ~3.2 and ~3.6 log CFU/g solids, compared to pH 2.4, respectively (Fig. 3.4). These results demonstrated that the cell survival of D-SDJ+Na-Alg powders (dried *via* spray drying) was even higher than that of probiotic powders prepared via freeze drying (D-SDJ-FD). This could indicate that FD is not inactivating heat sensitive peptides, which may result in cell loss.

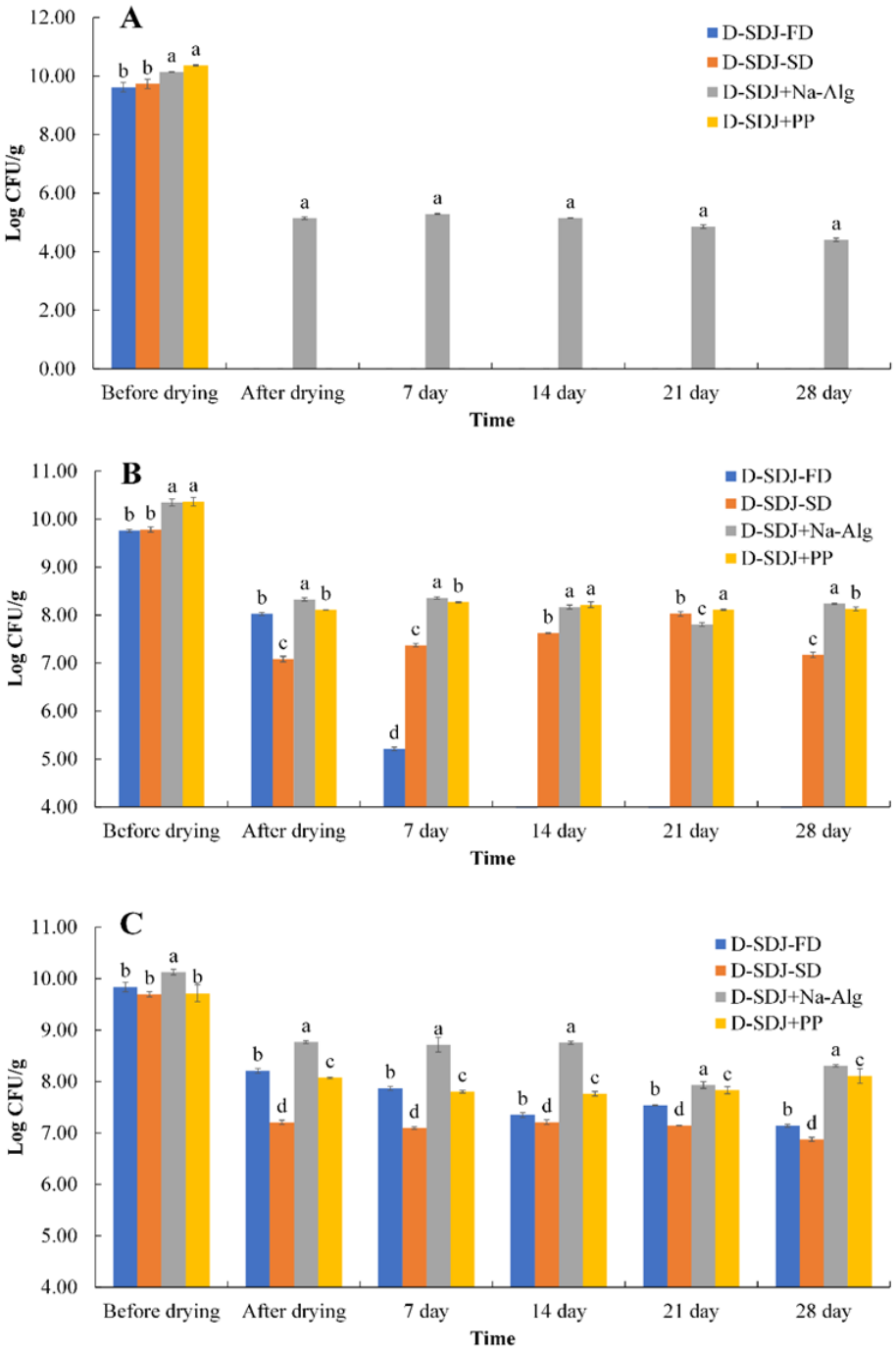

Fig 3.4. – Cell counts (Log CFU/g solids) of microencapsulated *Lactobacillus rhamnosus* GG during shelf storage using hydrolyzed and dialyzed salted, dried jellyfish (D-SDJ) *via* freeze drying (D-SDJ-FD), D-SDJ *via* spray drying (D-SDJ-SD), D-SDJ with sodium alginate *via* spray drying (D-SDJ+Na-Alg) and D-SDJ with pomegranate peel polyphenols *via* spray drying (D-SDJ+PP) at pH A) 2.4, B) 4.4 and C) 6.5. [abcd]Means treatments with different letters at the same time and pH are significantly different ($p<0.05$).

Additionally, this suggests that Na-Alg may be adding additional protection to LRGG cells during the microencapsulation process. Research has demonstrated that alginate crosslinked microspheres formed electrostatic interactions between other biopolymers in solution during microencapsulation which created a thicker encapsulating layer thus leading to a more ideal barrier to protect probiotic bacteria against stressors like pepsin and acid (Annan, Borza, & Hansen, 2008). Li, Chen, Cha, Park, and Liu (2009) demonstrated that Na-Alg when added to gelatin then soaked in sodium citrate increased the survival encapsulated *L. casei* ATCC 393 due to a more beneficial structure and higher pH which led to greater stability compared to the other samples. Given this, the Na-Alg allows for more electrostatic interactions to occur with the D-SDJ gelatin during the microencapsulation process which produces a more ideal structure and also works synergistically with a more neutral pH to better protect and improve the viability for the LRGG cells.

When looking at Figure 3.5, the D-SDJ alone at pH 4.4 had minimal survival while Na-Alg and PPs had a ~75% and ~36% increase in survival of LRGG, respectively. At pH 6.5, similar trends were observed while Na-Alg and PPs samples compared to D-SDJ alone demonstrated a ~410% and a ~205% increase in survival of LRGG. When comparing the same samples at different pH's before and after SD, all samples outperformed pH 2.4 while pH 6.5 resulted in significantly increased cell survival over pH 4.4 only with the addition of Na-Alg and PPs (Fig. 3.5). There was a ~347% increase in cell survival from pH 4.4 to 6.5 for Na-Alg and PPs had a ~181% increase in cell survival from pH 4.4 to 6.5. Producing a less harsh environment for the probiotics by increasing pH while producing a more ideal encapsulant by adding Na-Alg and/or PPs acted to further protect the cells from the high temperatures during SD compared to D-SDJ alone.

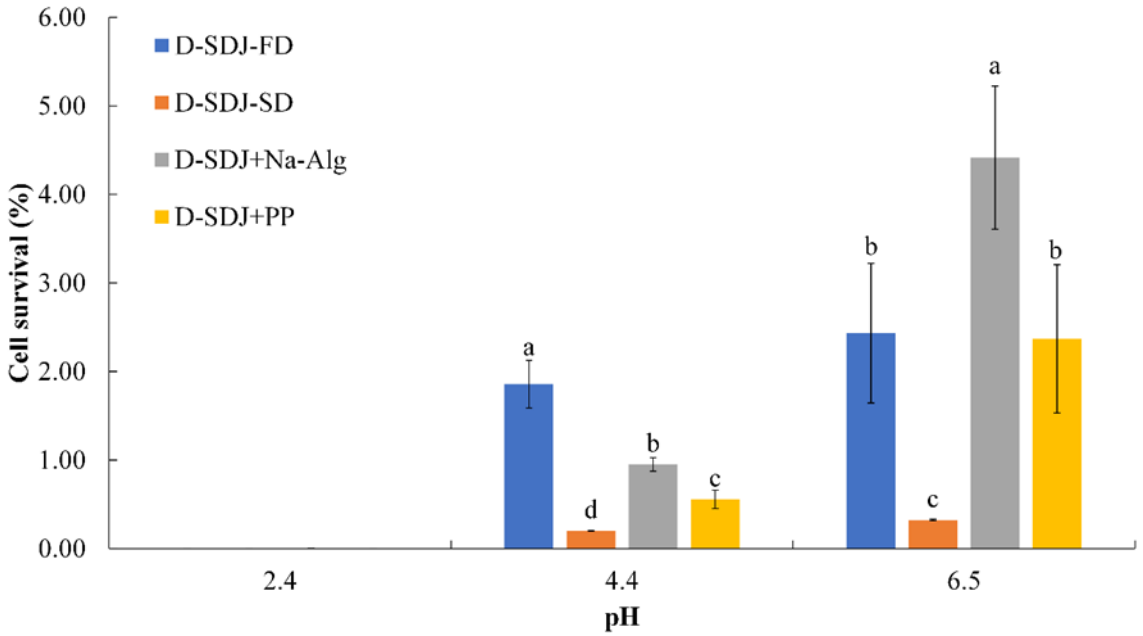

Fig. 3.5. – A) Cell survival (%) of LRGG after microencapsulation with D-SDJ *via* freeze drying (D-SDJ-FD), D-SDJ *via* spray drying (D-SDJ-SD), D-SDJ with Na+Alg *via* spray drying (D-SDJ+Na-Alg), and with D-SDJ with PPs *via* spray drying (D-SDJ+PP) at three different pH values. [abcd]Means treatments with different letters at the same pH are significantly different (p<0.05).

The addition of Na-Alg significantly improved LRGG survival over PPs and D-SDJ alone for the SD samples signifying that with Na-Alg using a more neutral pH (4.4 & 6.5) provided the most ideal environment for the survival of LRGG (Fig. 3.4 and 3.5). This data provides evidence that the potential creation of *Egg-box junctions* and crosslinking between Na-Alg and PPs, mentioned in Bloom strength section, provided a stronger gel network. This increased gel network may also produce a denser and more compact network compared to the control gelatin. Even though this is not a drastic change in cell survival, the difference was significant. Researchers found that crosslinking with natural polyphenols created a denser structure in gelatins (Zhao et al., 2016; Zhao & Sun, 2017). While the addition of Na-Alg was hypothesized to form physically stronger microspheres due to their observed resistance to degradation in simulated gastro-intestinal juices (Annan et al., 2008). Importantly, as pH increased from 2.4 up to 6.5, the length of survival of LRGG for the FD samples went from day 0 at pH 2.4 up until day 7 at pH 4.4 then at pH 6.5 the LRGG persisted for 28 d. This further signifies that a more neutral pH provides a more ideal environment for the probiotic bacteria. Mandal et al. (2006) demonstrated that pH 6.5 provided an increase in survival of *L. casei* NCDC-298 compared to pH 1.5. The low survival of FD LRGG is likely due to a low pH but also can be due to environmental stressors during the FD process. Li et al. (2009) mentioned that FD, like SD, evokes numerous environmental stressors that can cause major cell loss like formation of ice crystals, high osmolarity, macromolecular denaturation and the removal of water which can affect hydrophilic macromolecules in the probiotic bacteria.

Shelf life of microencapsulated LRGG

The shelf-life conditions for storing the probiotic powders at 4°C in a vacuum packaged material provided a relatively good means to monitor the shelf life of the probiotic gelatins as

viability persisted in most samples after 28 d. After the shelf-life study, it was found that Na-Alg was the only sample at pH 2.4 to still have viable cells, which was hypothesized to be due to D-SDJ+Na-Alg forming a more protective encapsulating layer over the other samples tested. At pH 4.4 and 6.5, Na-Alg and PPs had approximately a ~1 log increase in the survival of viable cells compared to the D-SDJ alone (control) after the 28 d (Fig. 3.4 B & C). This may be due to the Na-Alg and PPs forming increased electrostatic interactions with the D-SDJ gelatin in conjunction with an ideal pH to produce a more favorable environment for the LRGG cells to survive. Similar results were seen when *Lactobacillus casei* ATCC 393 was encapsulated with alginate + gelatin then soaked with sodium citrate which showed an improvement in the number of viable cells over 1 week (Li et al., 2009). This was also observed when genipin, a common natural crosslinking agent, crosslinked chitosan was added to an alginate-Hylon starch which significantly increased the survival of numerous types of probiotic bacteria strains, including *L. rhamnosus* ATCC 7469, compared to the non-crosslinked form (Zanjani, Ehsani, Tarzi, & Sharifan, 2018). Also, the cell counts of LRGG microencapsulated in D-SDJ+Na-Alg, D-SDJ+PP, D-SDJ-SD, and D-SDJ-FD remained relatively stable during the 28-d shelf-life storage at pH 6.5. It is was observed that the D-SDJ can extend the shelf-life of LRGG cells with minimal loss over 28 d, especially for Na-Alg and PPs, but further optimizing of the wall material will need to be considered in order to have less cell loss during the drying process.

To our knowledge, there is no previous research that has demonstrated improved survival of LRGG using Na-Alg and natural polyphenols from pomegranates to crosslink a novel D-SDJ. Cell viability for both SD encapsulated Na-Alg and PPs was ~8 log CFU/g solids at pH 4.4 and 6.5 while non-crosslinked cells for D-SDJ-SD was ~7 log CFU/g solids for pH 4.4 and 6.5 and ~7 log CFU/g solids for D-SDJ-FD at pH 6.5 while no survival persisted at pH 4.4. Overall, it

was observed that as the gel strength of a gelatin was increased due to an increase in pH and with the addition of Na-Alg and PPs, there was also an increase in survival rates for microencapsulated LRGG. The creation of more *Egg-box junctions* and/or electrostatic interactions may produce a more compact/dense gel network which not only improved the gel strength for D-SDJ but improved the survival of LRGG. More research will need to be conducted to further protect the LRGG cells from the harse conditions during the drying process, which caused a majority of the cell loss.

Conclusion

The effect of two additional modifications (sodium alginate (Na-Alg) & pomegranate polyphenols (PPs)) on dialyzed salted, dried jellyfish (D-SDJ) gel strength and its effect on viability of *Lactobacillus rhamnosus* GG (LRGG) was investigated in this research. The data demonstrated that D-SDJ is significantly improved at 10% solids when adding PPs at a concentration of 360 µg of PPs/ g gelatin and 0.4% Na-Alg (w/v). The increase in gel strength is likely due to increased electrostatic interactions, likely hydrogen bonding, in the gelatin matrix that were able to be created from crosslinking with Na-Alg and PPs. This created a more dense/compact and stiffer gel matrix that was optimized at 10% concentration with a lower maturation temperature (4 or 7°C) and at a higher, more neutral, pH (4.4 or 6.5). Also, the addition of Na-Alg and PPs in D-SDJ gelatin can be used to receive a ~8 log CFU/g solids of LRGG after 28 d of encapsulation which was significantly improved over the spray drying and freeze drying samples for D-SDJ alone at pH 4.4 and 6.5. It was observed that as gel strength was increased for D-SDJ that the survival of microencapsulated LRGG also increased. Therefore, having a higher pH, concentration, lower maturation temperature, and the addition of Na-Alg and PPs to D-SDJ increased gel strength while also increasing microencapsulated LRGG

survival. The information in this study can find application in modifying gelatins, especially marine gelatins, for different uses in the food industry as well as for improving the survival of microencapsulated LRGG using a gelatin complex as the wall material.

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ENDING STATEMENT/FUTURE RESEARCH

The research conducted illustrates (for the first time) the feasibility of utilizing Georgia-caught salted, dried jellyfish (SDJ) as a source of collagen-rich material that can be used to produce a novel marine gelatin. An acid hydrolysis and freeze-drying procedure was developed to hydrolyze collagen and produce a powder from SDJ. Remarkedly, the hydrolyzed-SDJ (H-SDJ) produced a weak gel with a strong potential for improvement. The utilization of a dialysis process was able to remove ~97% of the minerals present in hydrolyzed salted, dried jellyfish to produce hydrolyzed and dialyzed salted, dried jellyfish (D-SDJ). The demineralization process significantly increased the Bloom strength of the jellyfish gelatin gels regardless of the pH, solid concentration, and maturation temperature utilized. Moreover, demineralized jellyfish gelatin gels produced with 10% solids concentration, at pH 4.4 and 6.5 and maturated at temperatures of 4°C had higher recorded Bloom strengths (>60-65 g). This was a tremendous improvement compared to gelatins gels produced with 10% solids concentration, at pH 4.4 and 6.5 and maturated at a temperature of 4°C using hydrolyzed, un-dialyzed jellyfish gelatin powders (Bloom strengths <8 g). Further modification was performed to the D-SDJ by crosslinking with either pomegranate polyphenols (PPs) or sodium alginate (Na-Alg). The study demonstrated that the addition of PPs and Na-Alg at concentrations of 360 μg PPs/g of gelatin and 0.4% (w/v) significantly increased gel strength using 10% solids, pH 4.4 & 6.5 at 4°C maturation temperature which improved gel strength from 60.5 to 120.6 g and 64.9 to 113.5 g a 49.8 and 42.8% increase in Bloom over the non-crosslinked D-SDJ (control), respectively. Additionally, Na-Alg and PPs in D-SDJ gelatin can be used to receive a ~8 log CFU/g solids of LRGG after 28

d of microencapsulation which was significantly improved over the spray drying and freeze drying samples for D-SDJ alone at pH 4.4 and 6.5.

Future studies will be conducted on the cannonball jellyfish based on the results and observations collected during this project. Exploring into fresh jellyfish, not the salted, dried form, will be assessed as well as providing real-world applications for the novel gelatins being created. Reducing odor, color, and masking flavor will be crucial for producing a more useable product in the U.S. market. The gelatins have great potential for use in foods, cosmetics and even in pharmaceutical or medical applications. Potential antimicrobial peptides will be further explored with potential for producing antimicrobial edible films for use in the seafood industry. The future of how cannonball jellyfish will be utilized in the U.S. market is still unknown but one thing is for sure, that jellyfish production and consumption will soon begin to makes it mark in the U.S.