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Development and characterization of a novel, edible bigel system with the potential to protect probiotics during *in vitro* digestion

by

Mark A. Bollom

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science & Technology

Program of Study Committee:
Nuria Acevedo, Co-major Professor
Stephanie Clark, Co-major Professor
Rodrigo Tarté

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2020

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NOMENCLATURE

SL	Soy Lecithin
SA	Stearic Acid
WPC80	Whey Protein Concentrate 80
OE	Oleogel Emulsion
HY	Hydrogel
PL	Phospholipid
SAXS	Small Angle X-ray Scattering
CSLM	Confocal Scanning Laser Microscopy
GC	Gas Chromatography
SSF	Simulated Salivary Fluid
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
O/W	Oil-in-Water
W/O	Water-in-Oil
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
XRD	X-ray Diffraction
MFGM	Milk Fat Globule Membrane
TAG	Triacylglycerol
DAG	Diacylglycerol
MAG	Monoacylglycerol
pI	Isoelectric Point

USDA	United States Department of Agriculture
FFA	Free Fatty Acid
FAME	Fatty Acid Methyl Ester
MRS	De Man, Rogosa, and Sharpe
GI	Gastrointestinal

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ABSTRACT

Bigels are semi-solid biphasic systems. They are composed of an organic phase, called an organogel (or oleogel, if edible), and aqueous phase, called a hydrogel. They have been used to deliver drugs, but their application in the food industry is still relatively new. The two broad objectives of this work include: first, to develop and characterize the structure of an edible bigel, and second, to assess the ability of a bigel to protect probiotics from harsh digestive tract conditions.

For the first broad objective, three main methodologies were used: small angle X-ray scattering, rheology (amplitude sweeps, frequency sweeps, and temperature ramps), and fluorescence microscopy. The developed bigel was made from an oleogel emulsion containing soybean oil, soy lecithin, stearic acid, and water and hydrogel containing whey protein concentrate 80 and water. Two water usage levels within the oleogel emulsion and two protein usage levels within the hydrogel were explored. Moreover, five ratios of oleogel emulsion:hydrogel were examined. The gels were stable for a minimum of five months. Small angle X-ray scattering revealed that the oleogel emulsion retained its basic structural units, a reverse micelle from soy lecithin and bilayer from stearic acid, at every level of hydrogel addition. Rheology affirmed the solid-like behavior of the bigels and showed that a bigel could have improved mechanical properties over a monogel (oleogel emulsion or hydrogel on their own) at certain water usage levels, protein usage levels, and ratio of oleogel emulsion:hydrogel. Rheology furthermore revealed that a bigel has a higher critical strain than a pure oleogel emulsion, which is a major advantage of using a bigel over a pure oleogel emulsion. Fluorescence microscopy showed the continuity and interaction of phases.

For the second broad objective, a standardized *in vitro* digestion system was used, and the viability of *Lactobacillus acidophilus* and *Bifidobacterium lactis* were assessed at various time points throughout digestion. A specific objective of the second phase was to understand the effect, if any, of phospholipids on probiotic survival during digestion. Two gels with similar macro properties, but different in that one had phospholipids (soy lecithin acted as the phospholipid source) and one did not, were used to understand this effect. Gas chromatography affirmed enzyme activity during digestion, and the control, with no gelators, underwent the greatest lipolysis. Additionally, no probiotics in the control survived gelation, but those entrapped within a bigel did survive – affirming the suitability of a bigel to protect probiotics during digestion. Phospholipids did not have a significant effect on probiotic viability, likely because they are broken down by digestive enzymes.

This research has laid the groundwork for bigel implementation into foods. Additional work may need to be done to optimize bigel structure for a particular application, but this work has shown how bigels are assembled, how their phases interact, their ability to protect probiotics during *in vitro* digestion, and the inability of phospholipids to extend probiotic viability during *in vitro* digestion.

CHAPTER 1. GENERAL INTRODUCTION

What is a gel?

Gels are viscoelastic substances that manifest themselves in the food industry through applications like jams and jellies, desserts, and yogurt. Most gelling agents currently used in the food industry are either a polysaccharide or protein (Banerjee & Bhattacharya, 2012). The definition of a gel has been debated for some time, and some generally accepted definitions are briefly described herein. One definition, by Flory (1953), states that a gel:

1. Has solid-like rheological properties
2. Contains two or more components
    a. One of the components is present in substantial quantity as a liquid
    b. At least one of the non-liquid components is heavily cross-linked to form a tangled, interconnected network structuring the liquid.

Another gel definition, offered by Almdal, Dyre, Hvidt, and Kramer (1993), characterizes a gel using the following physical and rheological attributes:

1. Material with soft, solid, or solid-like characteristics
2. Has two or more components
3. Has a storage modulus, G', with a “pronounced plateau” extending for at least a few seconds and a loss modulus, G", that “is considerably smaller than the G' in the plateau region.”

Yet another definition, also from a rheological perspective, is offered by Ross-Murphy (1995) where a gel:

1. Has G'>G"

2. The relationship between G' and G" remain constant over a range of frequencies.

Ross-Murphy goes on to describe the rheological properties necessary for something to be called a “true gel,” “weak gel,” or “fluid gel.” Table 1.1 outlines the terminology used to describe various types of gels.

Table 1.1. Classification of gel types. From Einhorn-Stoll and Drusch (2015).

Gel terminology	Oscillation behavior	Frequency dependence	Gel structure	Example
True Gel	G'>G"	No	Continuous network	Gelatin, pectin
Weak Gel	G' and G" crossover	Yes	Highly viscous liquid	Xanthan gum, λ-carrageenan
Fluid Gel	G'>G"	Yes	Small particles in fluid matrix	Stirred yogurt

Gelation can occur via many processes that modify environmental conditions, such as elevated temperature, ionic strength, pH, and enzymatic activity (Aguilera & Baffico, 1997; Bryant & McClements, 1998; Lauber, Krause, Klostermeyer, & Henle, 2003; Lucey & Singh, 1997). Different physical characteristics and appearances of the gel result from these many processing options.

Gelators commonly used in the food industry are whey, gelatin, starch, soy protein, and alginate (Ahmed, Ptaszek, & Basu, 2017). These gelators immobilize water to make what is called a hydrogel; however, an emerging class of gelators are arriving, called organogelators, that can immobilize an organic phase to make what is called an organogel. When edible, an organogel is called an oleogel (Marangoni & Garti, 2011). This thesis focuses on edible applications; thus, the term oleogel shall be used henceforth.

Gels are of particular interest in encapsulation of sensitive or bioactive ingredients (Einhorn-Stoll & Drusch, 2015). For a gel to be used for this application, however, they must be designed so that the gel is strong enough to protect the ingredient from harsh environmental factors (pH, chemical alterations) but "weak" enough to allow the release of the ingredient at the desired endpoint. Gels have been developed to encapsulate ingredients like probiotics (Singh, Medronho, Miguel, & Esquena, 2018; Zhang et al., 2018), caffeine (Gunasekaran, Ko, & Xiao, 2007; Gunasekaran, Xiao, & Eleya, 2006), or β-carotene (O'Sullivan, Davidovich-Pinhas, Wright, Barbut, & Marangoni, 2017), for example. This review now digresses to discuss the two major classes of gels: oleogels and hydrogels.

Oleogels

An oleogel is a gel where the liquid phase is oil (Rogers, 2009). Oleogelators, which help form the gels, are often low molecular weight, whereas traditional hydrogelators are polymeric and thus high molecular weight (Co & Marangoni, 2012). Selecting an appropriate oleogelator is challenging because there must be a balance between the gelator being soluble enough that it does not precipitate out, but not so soluble that it forms a solution. Examples of oleogelators that have been developed include: various waxes like sunflower, carnauba, candelilla, bee, berry, or rice bran (Dassanayake, Kodali, Ueno, & Sato, 2009; Patel, Babaahmadi, Lesaffer, & Dewettinck, 2015); soy lecithin and stearic acid (Gaudino, Ghazani, Clark, Marangoni, & Acevedo, 2019); or even fatty acids, like 12-hydroxystearic acid or ricinelaidic acid (Rogers, Wright, & Marangoni, 2009; Wright & Marangoni, 2006).

There is a strong impetus to develop alternative hardstock fat structuring techniques (or fat mimetics) as traditional methods have led to *trans* fat consumption concerns (Rogers, 2009). High intake of saturated fatty acids and *trans* fats are reported to be deleterious to human health

because they increase the LDL:HDL ratio and increase the risk for heart attack (Aro, Jauhiainen, Partanen, Salminen, & Mutanen, 1997; Ascherio, Hennekens, Buring, Master, Stampfer, & Willett, 1994). As a result, the American Heart Association (2015) has recommended limiting consumption of saturated and *trans* fats by limiting foods such as red meat, fried foods, and baked goods.

Oleogel applications in food are beginning to emerge, with studies done in ice cream (Zulim Botega, Marangoni, Smith, & Goff, 2013a, 2013b), breakfast sausages (Barbut, Wood, & Marangoni, 2016), frankfurter-type sausages (Kouzounis, Lazaridou, & Katsanidis, 2017; Wolfer, Acevedo, Prusa, Sebranek, & Tarté, 2018), composite cream-filled chocolate confections (Hughes, Marangoni, Wright, Rogers, & Rush, 2009), cream cheese (Bemer, Limbaugh, Cramer, Harper, & Maleky, 2016), and chocolate paste (Patel et al., 2014). An oleogel's final physical and mechanical characteristics are strongly dependent on how it was prepared, with cooling rate (Morales-Rueda, Dibildox-Alvarado, Charo-Alonso, Weiss, & Toro-Vazquez, 2009; Ojijo, Neeman, Eger, & Shimoni, 2004; Toro-Vazquez, Morales-Rueda, Dibildox-Alvarado, Charó-Alonso, Alonzo-Macias, & González-Chávez, 2007), storage time/ conditions (Ojijo, Kesselman, Shuster, Eichler, Eger, Neeman, & Shimoni, 2004; Toro-Vazquez et al., 2007), shear (Ojijo, Neeman, et al., 2004), gelator concentration (Hwang, Fhaner, Winkler-Moser, & Liu, 2018), and the presence of crystal habit modifier(s) (Morales-Rueda et al., 2009) all playing important roles.

Another advantage of oleogels is that they have the potential to help delay oxidation in liquid oil compared to unstructured oils. Hwang et al. (2018) showed that an oleogel structured with waxes slowed fish oil oxidation compared to non-structured oil. Da Pieve, Calligaris, Panozzo, Arrighetti, and Nicoli (2011) structured cod liver oil with monoglycerides and found that structuring conferred minimal protection against the formation of primary oxidation

products but did help protect against the formation of secondary oxidation products due to the network structure.

The oleogel composition utilized in this work is, in fact, an oleogel emulsion because a significant amount of water is added. Previous work by Gaudino et al. (2019) developed the oleogel emulsion comprised of soy lecithin, stearic acid, canola oil, and water. Soy lecithin and stearic acid serve as the oleogelators, with water being an essential partner in gelation. Water's presence allows soy lecithin and stearic acid to form worm-like reverse micelles and bilayers, respectively, that structure the oil and make it gel. A reverse micelle occurs when water is on the interior of the micelle with hydrophilic heads directed towards it and hydrophobic tails pointing outwards; the bilayer is a double layer of stearic acid molecules (Figure 1.1).

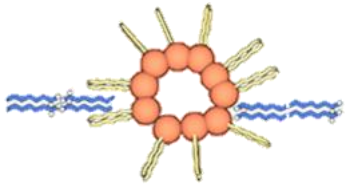

Figure 1.1. Soy lecithin reverse micelle and stearic acid bilayer. From Gaudino et al. (2019).

Lecithin is composed of phospholipids, which are found in both plants and animals (van Nieuwenhuyzen & Tomás, 2008). Soybeans are the primary source of vegetable lecithin, and the predominant phospholipids in soy lecithin are phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. The most prevalent fatty acids found in soy lecithin are linoleic acid (18:2), oleic acid (18:1), and palmitic acid (16:0). The general structure of a phospholipid is shown in Figure 1.2. Stearic acid, the other oleogelator used in this study, is found in both plants and animals and is a saturated long-chain fatty acid with an 18-carbon backbone (Figure 1.3).

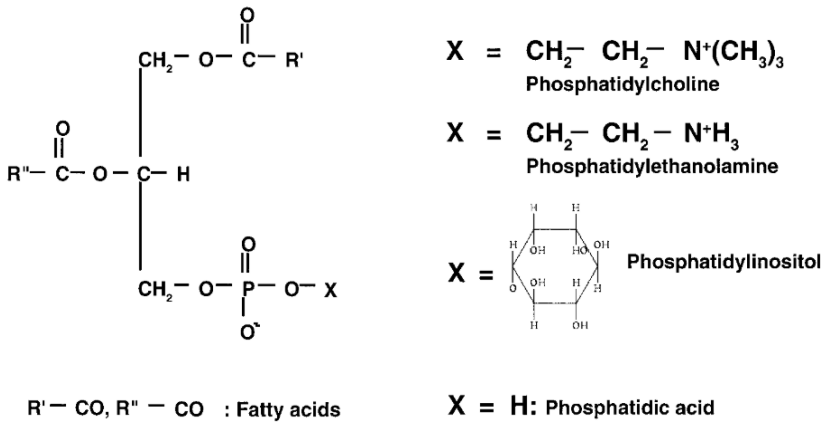

Figure 1.2. General phospholipid structure. Image from Nieuwenhuyzen and Tomás (2008).

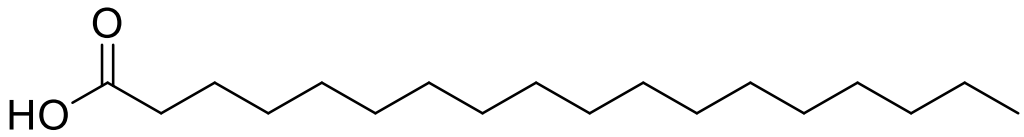

Figure 1.3. Stearic acid structure

Hydrogels

A hydrogel is a gel where the immobilized phase is water (Co & Marangoni, 2012). Common examples of hydrogels include jellies (gelatin), gummy bears (starch), cheese curds (casein), and microbial growth media (agar). There are artificial and natural gelators, and the latter can be further divided into polysaccharides and polypeptides (i.e., proteins) (Ahmed, 2015). Polysaccharides crosslink to form junction zones that ultimately form a gel network structure (Ahmed et al., 2017). Proteins, on the contrary, are more complex because they require some denaturation (unfolding of protein structure which can occur via heat, pressure, enzymes, or chemical means, for example) to expose reactive groups that can participate in intermolecular

interactions (covalent and non-covalent). Additionally, protein gelation requires control of the protein's physico-chemical environment.

Milk proteins are categorized into two main categories: casein and whey (Raikos, 2010). Whey has traditionally been derived from cheese making and is defined as the "proteins remaining soluble at pH 4.6 and 20°C after casein removal from skim milk or whole milk" (Harris, 1990). Whey comprises 80-90% of milk and about 50% of its nutrients (protein, lactose, vitamins, minerals) (Bylund, 2015). The United States produced over 999 million pounds of dry whey in 2018 (National Agricultural Statistics Service, 2019). Whey gelation is well documented in the literature (Bryant et al., 1998; Dickinson & Yamamoto, 1996; Gunasekaran et al., 2007; Gunasekaran et al., 2006). β-lactoglobulin, the primary whey protein (Table 1.2), is 162 amino acid residues long, has two disulfide bridges (between amino acid residues 106-119 and 66-160), and a free thiol group (amino acid 121) that all provide intermolecular and intramolecular disulfide link potential when it undergoes conformational changes during processing (pH, heat, pressure) (Harris, 1990).

Table 1.2. Milk protein composition.

Milk Protein	**g/kg**	**% of total protein**
Total protein	33.0	100.0
Total casein	26.0	79.5
α_{s1}	10	30.6
β	9.3	28.4
κ	3.3	10.1
α_{s2}	2.6	8.0
γ	0.8	2.4
Total whey	6.3	19.3
β-lactoglobulin	3.2	9.8
α-lactalbumin	1.2	3.7
Proteose peptone	0.8	2.4
Immunoglobulins	0.7	2.1
BSA	0.4	1.2
Fat globule membrane proteins	0.4	1.2

From Walstra and Jenness (1984).

Bigels

A bigel is a biphasic system comprised of an oleogel and hydrogel (Almeida, Fernandes, Fernandes, Pena Ferreira, Costa, & Bahia, 2008; Shakeel, Lupi, Gabriele, Baldino, & De Cindio, 2018; Varrato, Di Michele, Belushkin, Dorsaz, Nathan, Eiser, & Foffi, 2012). The first known publication of bigels was in 2008 (Almeida et al., 2008). Bigels can be categorized in three ways: organogel in hydrogel (O/W), hydrogel in organogel (W/O), or bi-continuous (Lupi, Shakeel, Greco, Oliviero Rossi, Baldino, & Gabriele, 2016). Bigels are distinguished from other biphasic systems because both phases are structured, and, hence, no emulsifier is needed. Since both oleogel and hydrogel phases are present, many possible combinations of gelators exist in the final bigel formulation. Furthermore, during bigel preparation many decisions must be made, like homogenization temperature, homogenization shear level, homogenization time, and gelation status of each phase. Each of these can influence the bigel's final properties. Table 1.3 offers a summary of different bigel systems already developed and the breadth of synthesis options.

Bigels have many advantages over a pure hydrogel or oleogel. First, when used for drug delivery, the bigel may offer greater patient compliance (Almeida et al., 2008). Oleogels can deliver lipophilic drugs but are oily and sticky, which is undesirable for patients (Rehman, Amin, & Zulfakar, 2014; Wynne, Whitefield, Dixon, & Anderson, 2002). A hydrogel, on the contrary, cannot deliver lipophilic drugs but is not formidable to handle. Thus, we can exploit the best characteristics of each gel when a bigel is used. Second, bigels are desirable over other biphasic systems because they do not require an emulsifier for physical stability (Almeida et al., 2008). Finally, bigels are advantageous because they are stable for extended periods (>6 months) (Almeida et al., 2008; Rehman et al., 2014; Singh, Banerjee, Agarwal, Pramanik, Bhattacharya, & Pal, 2014).

Bigels, as a drug delivery vehicle, have been explored in several studies. Sahoo et al. (2015), Singh et al. (2014), and Behera, Sagiri, Singh, Pal, & Anis (2014) used bigels to deliver metronidazole. Others have used them to deliver ciprofloxacin (Kodela, Pandey, Nayak, Uvanesh, Anis, & Pal, 2017; Satapathy et al., 2015) or diltiazem hydrochloride (Ibrahim, Hafez, & Mahdy, 2013), for example. Due to success with using bigels to deliver drugs, we hypothesize they can be used to deliver probiotics too. Only one known study (Behera et al., 2014) has explored probiotic delivery via bigels and their survival during digestion. They found that bigels protect probiotics (compared to probiotics not entrapped within a bigel) during digestion; however, the researchers did not subject the bigels to each step of digestion in sequence (they only looked at each phase of digestion separately), and thus the data should be regarded as preliminary.

As discussed above, bigels are a relatively new technology, with the first publication in 2008 (Almeida et al., 2008). By reason of this, little knowledge is known about bigel microstructure. Researchers have used a variety of methods to understand bigel assembly and stability, such as microscopy (brightfield and fluorescence), x-ray diffraction (XRD), rheology, texture analysis, Fourier-transform infrared spectroscopy (FTIR), and thermal analysis (differential scanning calorimetry, DSC, or differential thermal analysis, DTA). Microscopy shows structural arrangement and droplet sizes (Ibrahim et al., 2013; Martins, Silva, Maciel, Pastrana, Cunha, Cerqueira, & Vicente, 2019; Sahoo et al., 2015; Singh, Anis, Banerjee, Pramanik, Bhattacharya, & Pal, 2014; Singh, Banerjee, et al., 2014). XRD shows the presence of certain structures, amorphous material, and crystalline polymorphism (Martins et al., 2019; Satapathy et al., 2015; Singh, Banerjee, et al., 2014). Rheology offers information on the flow and mechanical properties of the bigel. Studies have shown bigels to have non-Newtonian shear

thinning behavior, while others have explored the effect of organogel concentration on viscosity and bigel firmness (Behera, Singh, Kulanthaivel, Bhattacharya, Paramanik, Banerjee, & Pal, 2015; Martins et al., 2019; Singh, Anis, et al., 2014; Singh, Banerjee, et al., 2014). FTIR is used to show functional groups and their interactions, such as C=O stretching, -OH groups, -NH groups, or hydrogen bonding. Hydrogen bonding has been shown in many studies to be a very important intermolecular interaction for bigel formation and stability (Behera et al., 2015; Sahoo et al., 2015; Satapathy et al., 2015). DSC or DTA show melting and/ or crystallization points and how those change with the addition of other components (Ibrahim et al., 2013; Satapathy et al., 2015; Singh, Anis, et al., 2014). Despite all these methods that have been implemented, much more information is still needed about bigel microstructure if they are to be adopted on a large scale. Thus, one of the objectives of this work is to explore bigel microstructure further. This work will be done using many of the methods discussed above, like fluorescence microscopy, rheology, and X-ray scattering.

Many terms are used when describing gels and biphasic systems. Table 1.4 summarizes the main terms and their relationships.

Table 1.3. Summary of bigel formulations and their preparation methods.

Organogelator(s)	Hydrogelator(s)	Homogenization Conditions			Gelation Status – already gelled?		Reference
		Speed (RPM)	Time (min)	Temperature (°C)	Organogel	Hydrogel	
Stearyl alcohol	Agar	200	5	80	No	No	(Kodela et al., 2017)
Stearic acid	Gelatin & Agar	300	10	70	No	No	(Wakhet et al., 2015)
Beeswax	Hydroxypropyl methylcellulose or Sodium alginate	800	10	25	Yes	Yes	(Rehman et al., 2014)
Sorbitan monostearate	Guar gum	1000	Until homogenous	70	Yes, but reheated before homogenization	Yes, but reheated before homogenization	(Singh, Banerjee, et al., 2014)
Span 40	Polyvinyl-pyrrolidone and Polyvinylalcohol	12000	Until "milky-white"	50	No	No	(Behera et al., 2015)
Beeswax	Sodium alginate	600	45	Room temperature	Yes	No	(Martins et al., 2019)
Sorbitan monostearate	Carbopol 934	500	Until homogenous	60	Yes, but reheated before homogenization	No	(Singh, Anis, et al., 2014)

Table 1.4. Summary of terms used to describe gels and biphasic systems. O = oil, W = water, NA = not applicable.

System	Continuous Phase	Dispersed Phase	Aqueous phase gelled?	Organic phase gelled?
Bigel	O, W, or O:W	O, W, or O:W	Yes	Yes
Emulsion	O or W	O or W	No	No
Emulgel	W	O	Yes	No
Oleogel Emulsion	O	W	No	Yes
Oleogel	O	NA	NA	Yes
Hydrogel	W	NA	Yes	NA

Probiotics

The World Health Organization (WHO) and Food and Agricultural Organization (FAO) of the United Nations define probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (2002). Beyond this definition, other attributes of probiotics include: non-pathogenic, able to survive passage through the gastrointestinal (GI) tract, able to withstand processing, and able to adhere to and colonize the intestinal epithelium (Syngai, Gopi, Bharali, Dey, Lakshmanan, & Ahmed, 2016). Consumers can access probiotics through many forms – capsules, powders, yogurt, and fermented milk beverages, for example (de Roos & Katan, 2000). Many bacterial genera are used as probiotics, like *Lactobacillus*, *Bifidobacterium*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*, but *Lactobacillus acidophilus*, *Bifidobacterium* spp., and *Lactobacillus casei* are the primary probiotics (Ashraf & Shah, 2014).

The 2014 sales of probiotics in the US were valued at approximately $300 million, but that is expected to grow to $638.8 million by 2024 (Statista, 2019). One possible reason for this growth is the purported health benefits many consumers see in probiotics. Probiotic use has been explored in treatment for diarrhea, irritable bowel syndrome, dental caries, and gastrointestinal disorders, for example (Bizzini, Pizzo, Scapagnini, Nuzzo, & Vasto, 2012; Hempel et al., 2012;

Indrio et al., 2014; Moayyedi, Ford, Talley, Cremonini, Foxx-Orenstein, Brandt, & Quigley, 2010). It is important to note that there is not one probiotic that confers all these health benefits. Studies have explored different age groups, different treatment levels, and different probiotic strains, so more research is needed to confirm what the exact effects of various strains are, their optimum dosage level, and recommended consumer. For example, looking at the effect of probiotics on dental caries, studies have looked at *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Bifidobacterium lactis*, or *Bifidobacterium animalis* as treatment (Bizzini et al., 2012). They have applied these to many age groups, like young adults (ages 18-35), children (ages 1-6), teenagers (ages 12-16), or young women (ages 21-24) with several delivery vehicles, like milk, cheese, ice cream, or gum. As should be apparent, studies are very specific in their target patient, so more work is necessary to discern the optimum treatment for each unique person.

Even if subsequent research proves the beneficial effects of certain probiotics, one major barrier to their use is survival during processing, shelf-life, and digestion. Processing and storage conditions deleterious to probiotic survival include pH, titratable acidity, oxygen, water activity, salt, sugar, hydrogen peroxide, artificial flavoring, coloring agents, heat treatment, high incubation temperature, product cooling rate, and packaging method (vacuum, oxygen permeability, presence of antioxidant or oxygen scavenger) (Tripathi & Giri, 2014). During digestion, exposure to harsh environmental conditions, such as acid and bile, have proven to be detrimental to probiotic survival. This is why probiotic encapsulation has been explored as a means to protect the fragile microorganisms (Ding & Shah, 2007; Lee & Heo, 2000; Marteau, Minekus, Havenaar, & Huis In't Veld, 1997; Sun & Griffiths, 2000).

Encapsulation of probiotics

Encapsulation of probiotics aims to reduce the number of cells lost during addition to food, shelf-life, and digestion (Heidebach, Först, & Kulozik, 2012). The capsule puts a protective barrier between the bacteria and destructive environmental conditions (Abd El-Salam & El-Shibiny, 2015). A challenge of encapsulation is its potential impact on the product's final sensory properties, like texture, flavor, or appearance (Gandomi, Abbaszadeh, Misaghi, Bokaie, & Noori, 2016; Kailasapathy, 2006; Ribeiro, Chaves, Gebara, Infante, Grosso, & Gigante, 2014). Encapsulation can occur via many methods, such as extrusion (Doherty, Auty, Stanton, Ross, Fitzgerald, & Brodkorb, 2012; Khan, Korber, Low, & Nickerson, 2013), emulsion (Pimentel-González, Campos-Montiel, Lobato-Calleros, Pedroza-Islas, & Vernon-Carter, 2009; Singh et al., 2018), spray drying (Anekella & Orsat, 2013; Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002; Maciel, Chaves, Grosso, & Gigante, 2014), or entrapment within a matrix (Gandomi et al., 2016; Sun et al., 2000). It is important to distinguish between encapsulation and immobilization/ entrapment because they are often interchangeably used in literature but are, in fact, different. Encapsulation is the process whereby a continuous coating around an inner material is created such that the inner material is entirely contained and serves as the center of the material; immobilization/ entrapment is the process whereby the inner material is trapped within or throughout a matrix (Kailasapathy, 2002).

Phospholipids and S-layer proteins

Fatty acids and glycerol esters make up food lipids and can be divided into three main classes: neutral lipids (Triacylglycerols, TAG; Diacylglycerols, DAG; Monoacylglycerols, MAG), polar lipids (phospholipids and glycolipids), and others (sterols, vitamins, etc.) (Cheung & Mehta, 2015; Huppertz, Kelly, & Fox, 2009). Recent research in our lab (publication

forthcoming) suggested that phospholipids (PLs) may enhance probiotic survival in an oleogel emulsion during shelf-life, which is why we explore their effect in chapter 3. A phospholipid (structure shown in Figure 1.2) is made up of four components: a glycerol backbone, fatty acids, a negatively-charged phosphate group, and a nitrogen-containing compound or sugar.

The S-layer proteins of most bacteria are acidic (pI 4-6), while those of lactic acid bacteria are basic (pI >9.4) (Smit, Oling, Demel, Martinez, & Pouwels, 2001), meaning they are cationic. The S-layer is the superimposed surface layer surrounding some bacterial strains and is 25-200 kDa, basic (pI = 9.35-10.4), and made of stable proteins packed into a paracrystalline hexagonal or tetragonal monolayer (Lebeer, Vanderleyden, & De Keersmaecker, 2008; Åvall-Jääskeläinen & Palva, 2005). The proteins bind to the cell wall through noncovalent interaction with cell wall polymers. Within lactobacilli, amino acid residues with a positive charge, like lysine, are prevalent (Åvall-Jääskeläinen et al., 2005). Positively charged amino acid residues can constitute up to 12.5% of the amino acids in lactobacilli (Åvall-Jääskeläinen et al., 2005). The PL head group is anionic, so binding between the cationic S-layer protein and anionic PL may occur.

Further highlighting the importance of S-layer proteins, prior work has shown that S-layer proteins participate in binding to the intestinal mucosal layer (Deepika & Charalampopoulos, 2010). Cleveland (2011) showed that lactic acid bacteria can bind to PLs and that they preferentially bind PLs over other lipid sources. Other work has shown that *L. reuteri* can bind to the milk fat globule membrane (MFGM), whose total lipid content is ~41% PLs, and their binding is strongly associated with the bacteria's hydrophobicity (Brisson, Payken, Sharpe, & Jiménez-Flores, 2010; Fong, Norris, & MacGibbon, 2007). A high number of hydrophobic S-layer protein amino acid residues are found in lactobacilli (31.9-38.7% of amino acid residues

are believed to be hydrophobic) (Åvall-Jääskeläinen et al., 2005). Figure 1.4 offers a summary of a lactobacillus's cell surface architecture and, specifically, where the S-layer proteins are located.

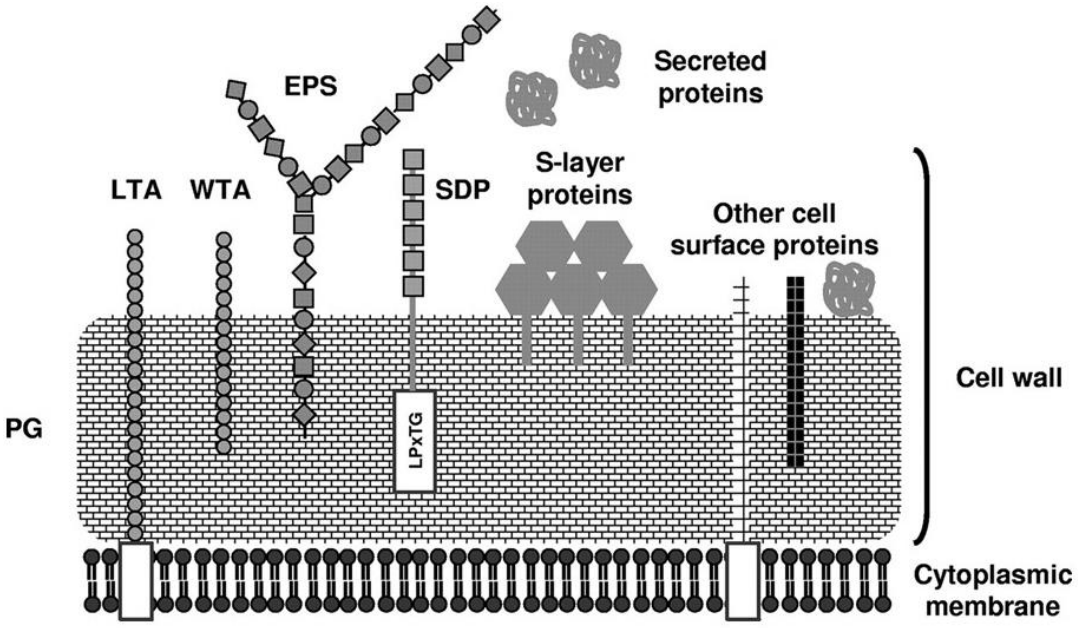

Figure 1.4. Cell surface architecture of lactobacilli showing the location of S-layer proteins. Taken from Lebeer et al. (2008). LTA: lipoteichoic acid. WTA: wall teichoic acid. EPS: exopolysaccharides. SDP: sortase-dependent proteins.

Probiotics, yogurt, and digestion

Although bigels can be used in many disciplines and foods, they were developed herein for application in yogurt. Yogurt is a common delivery vehicle for probiotics. In order to place a "Live and Active Cultures" seal on a package of yogurt, the National Yogurt Association requires the yogurt to have 100 million, or 10^8, cells per gram at the time of manufacture (National Yogurt Association, 2019). Previous studies have shown that probiotic survival in yogurt is low during shelf-life due to intrinsic factors like lactic acid, H_2O_2, and bacteriocins, but after consumption, digestive conditions are also hard on probiotic survival (Afzaal et al., 2019; Ibrahim & Carr, 2006; Mani-López, Palou, & López-Malo, 2014; Marteau et al., 1997; Sun et

al., 2000). For example, Afzaal et al. (2019) found that yogurt pH declined from 4.88 to 4.43 over 28 days of storage (due to acid production), and unencapsulated cells declined from 9.97 log(CFU/mL) at day 0 to 6.12 log(CFU/mL) after 28 days. They also found a decline from approximately 12 log(CFU/mL) to 4 log(CFU/mL) and 10 log(CFU/mL) to 4 log(CFU/mL) for unencapsulated probiotic cells in yogurt during the gastric and intestinal phases, respectively, of *in vitro* digestion. Thus, one of the objectives of this study is to explore probiotic survival after *in vitro* digestion in a probiotic bigel.

Thesis organization

There is rising consumer interest in functional foods, and bigels are a new technology that holds great promise to meet this interest. Bigels hold promise for the pharmaceutical, cosmetic, and food industries. Development of a food-grade bigel to deliver sensitive or bioactive ingredients is one area not well researched and will be explored in this work. The bigel used in these studies was composed of a soybean oil, soy lecithin, stearic acid, and water/ milk oleogel emulsion and a whey protein concentrate 80 and water hydrogel. The overall goal of this research was to elucidate bigel microstructure and their ability to protect probiotics during *in vitro* digestion. Ultimately, the goal is to offer an efficacious means to protect probiotics from harsh processing, shelf-life, and digestive conditions and deliver a higher number of probiotics to the gut.

Chapter two will focus on the characterization of bigel microstructure. Characterization was done through varying the oleogel emulsion:hydrogel ratio, oleogel emulsion water content, and hydrogel protein content. Oscillatory rheology, small angle X-ray scattering, and fluorescence microscopy were used.

Chapter three focuses on understanding bigel breakdown and its ability to protect probiotics during *in vitro* digestion, as well as the role of phospholipids in probiotic survival. Probiotic counts within the bigel were assessed during each phase of digestion, as well as lipolysis during the intestinal phase.

Finally, chapter four will offer a summary of all the findings from this research and offer insights into future research avenues.

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CHAPTER 2. DEVELOPMENT AND CHARACTERIZATION OF A NOVEL SOY LECITHIN-STEARIC ACID AND WHEY PROTEIN CONCENTRATE BIGEL SYSTEM FOR POTENTIAL EDIBLE APPLICATIONS

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Abstract

Bigels are a new technology with great potential in the food industry. Their success with drug delivery suggests they may be able to deliver sensitive compounds in foods, such as probiotics and bioactives. The purpose of this study was to develop and characterize a novel, edible bigel system. The bigel was prepared by homogenizing, at high shear, an oleogel emulsion composed of soy lecithin, stearic acid, soybean oil, and water, and a hydrogel composed of whey protein concentrate and water. Characterization was conducted through small angle x-ray scattering, rheology, and fluorescence microscopy. With the addition of the hydrogel component, the oleogel emulsion retained its basic structural characteristics but lost higher order structuring. The bigels were found to have temperature-dependent G' values. Despite temperature sensitivity, the bigels showed G'>G" at all temperatures from 8 to 98°C. Fluorescence microscopy revealed that a bi-continuous bigel was formed at equal proportions of oleogel emulsion and hydrogel; nevertheless, when either of those phases increased, one of them became the dominant continuous phase. Some interaction between the phases may have occurred at 10 wt% water and 15 wt% protein usage in the oleogel emulsion and hydrogel, respectively, and this synergy improved the bigel's mechanical properties. On the contrary, at protein and water contents outside those listed above, the relationship between phases became antagonistic towards the bigel's mechanical properties.

Keywords:

Bigel; oleogel emulsion; hydrogel; soy lecithin; stearic acid; whey protein concentrate

Introduction

Gels are viscoelastic materials widely used in the food industry in applications like jams and jellies, desserts, and yogurt, with most gelling agents being either a polysaccharide or protein (Banerjee & Bhattacharya, 2012). Either a polar or non-polar phase can be gelled, resulting in a hydrogel or organogel, respectively (Co & Marangoni, 2012; Rogers, 2009). An organogel is called an oleogel when the gelled oil is edible (Marangoni & Garti, 2011). Together, a hydrogel and oleogel can be combined to form a bigel (Shakeel, Lupi, Gabriele, Baldino, & De Cindio, 2018; Varrato et al., 2012). Bigels are a relatively new technology, with the first publication appearing in 2008 (Almeida et al., 2008). Many studies have used bigels for drug delivery, with success, due mainly to the bigel's hydrophilic and hydrophobic components that make it ideal for carrying an array of drugs of various structures (Ibrahim, Hafez, & Mahdy, 2013; Shakeel et al., 2018; Singh et al., 2014a; Singh et al., 2014b).

A bigel is different from other biphasic systems since both phases are structured, which offers superior stability (Varrato et al., 2012). A bigel can be either oleogel in hydrogel (O/W), hydrogel in oleogel (W/O), or bi-continuous (Lupi et al., 2016). Many bigel matrices have been developed, including: agar (hydrogelator) and stearyl alcohol (organogelator) (Kodela et al., 2017), hydroxypropyl methylcellulose or sodium alginate (hydrogelators) and beeswax (organogelator) (Rehman, Amin, & Zulfakar, 2014), agar or gelatin (hydrogelators) and stearic acid (organogelator) (Wakhet et al., 2015), locust bean gum and carrageenan (hydrogelators) and fumed silica (organogelator) (Patel, Manko, Bin Sintang, Lesaffer, & Dewettinck, 2015), or low-methoxyl pectin (hydrogelator) and glyceryl stearate and policosanol (organogelators) (Lupi et

al., 2016). Soy lecithin and stearic acid can act as organogelators, along with either a small or high volume of water to make an oleogel or oleogel emulsion, respectively (Gaudino, Ghazani, Clark, Marangoni, & Acevedo, 2019). Whey protein concentrate can gel within an aqueous solvent, and these hydrogels have even been used for drug release studies (Gunasekaran, Ko, & Xiao, 2007).

Despite the interest in bigels for a variety of applications, minimal information is known about their microstructure. Rehman et al. (2014) found that bigel hardness values increase with an increase in hydrogel content, suggesting that hydrogels play an important role in imparting bigel firmness due to hydrogen bonding. On the contrary, Lupi et al. (2016) and Singh et al. (2014b) found firmness to be most influenced by the oleogel content. To help explain their findings, studies have employed microscopy to show phase interactions and understand continuity, discontinuity, or bi-continuity of the aqueous and organic phases (Behera et al., 2015; Patel et al., 2015; Rehman et al., 2014). To understand microstructure on a smaller scale, other researchers have used X-ray diffraction to show how one phase may change based on the incorporation of another, such as the loss of lactose crystals in whey protein concentrate (WPC) due to solubilization during gelation (Behera et al., 2015). Despite the reported findings, however, very little is still known about bigel systems and the importance of various factors, such as protein content, water content, and oleogel and hydrogel concentrations.

The purpose of this research was to elucidate the factors affecting bigel microstructure in order to improve the gel's physical properties beyond those of a mono-oleogel emulsion or hydrogel. A total of 16 bigel formulations were prepared through five oleogel emulsion:hydrogel ratios, two oleogel emulsion water contents, and two hydrogel protein contents. This is the first

study to date using soy lecithin in combination with stearic acid (organogelators) and whey protein concentrate (hydrogelator) in a bigel.

Materials and Methods

Materials

Soybean oil (SO) was acquired from ADM (Des Moines, IA, USA). Granular soy lecithin (SL, 97% phosphatidylcholine) and Fluorescein isothiocyanate isomer I (FITC, 90% pure) were purchased from Acros Organics (Geel, Belgium). Stearic acid (SA) was purchased from Fisher Chemical (Waltham, MA, USA). Whey protein concentrate 80 (WPC80) was generously donated by Milk Specialties Global (Eden Prairie, MN, USA). Nile Red was acquired from Chem Impex International Inc. (Wood Dale, IL, USA). Glass bottom slides for microscopy were from MatTek Corporation (Ashland, MA, USA).

Sample Preparation

The oleogel emulsion (OE) phase was prepared using SO, SL, SA, and deionized water following a method adapted from Gaudino et al. (2019). SL and SA served as the organogelators and were added at 20 wt% to the OE solution after accounting for water and ratio of 7:3, respectively. The appropriate amount of SL and SA were dissolved in SO in a 95°C oven with stirring at 250 RPM. The OE water (pre-heated) was added, at 10 or 20 wt%, right before homogenization.

The hydrogel (HY) was prepared using WPC80 and deionized water. WPC80 served as the hydrogelator and was added to the HY at 15 or 25 wt%. The hydrogel was prepared following a modified method of de Vries, Jasper, van der Linden, and Scholten (2015). Briefly, the WPC80 was added to deionized water with stirring at room temperature for 2 h. The WPC80

solution was then stored in a 4°C refrigerator overnight to ensure complete protein hydration. The following day, the ionic strength was adjusted to 50mM with NaCl and pH to 7.5 ± 0.01 with 1M NaOH or HCl. The pH and ionic strength-adjusted solutions were placed in an 85°C hot water bath for 30 min to gel.

Sample preparation was timed so that the oleogel solution and HY were completed at the same time, and both were hot when homogenized. The hot, liquid oleogel solution and preheated OE water were poured on the hot, solid hydrogel. Both phases were homogenized at 23000 RPM for 3 min with a preheated homogenizer at 85°C (Ultra Turrax; IKA; Staufen, Germany). Samples were cooled at room temperature for 1 h before storing at 4°C overnight. Five ratios of OE to HY (OE:HY) were prepared: 0:10, 3:7, 5:5, 7:3, and 10:0.

Table S2.1 shows the % mass of each component for each formulation. Samples were labeled as: OE:HY, water usage level (W) in the OE, and protein usage level (P) in the HY. For example, 3:7 10W 25P was formulated with 3 parts oleogel emulsion to 7 parts hydrogel, the oleogel emulsion contained 10 wt% water, and the hydrogel 25 wt% protein.

Analysis Methods

Small Angle X-ray Scattering

Small Angle X-ray Scattering experiments were performed on a Xeuss SAXS System (Xenocs; Sassenage, France) at room temperature. The X-ray source was 30W CuKα (λ=1.54Å), and a Pilatus 300K hybrid pixel detector (Dectris; Baden-Deatwil, Switzerland) was used. The scattering curves were normalized. The sample-to-detector distance was 1221 mm and calibrated with silver behenate. The scattering range used was q 0.05-0.2 with $q = \frac{4\pi}{\lambda}\sin(\theta)$ where λ is

wavelength, and 2θ is the scattering angle. All samples were analyzed in triplicate. Background scattering was collected under the same conditions outlined above. Intensity data of the samples were corrected for background noise. The d-spacing for the obtained Braggs peaks was obtained from patterns on q versus I(q) plots, using: $d=2\pi/q$.

Rheology

Small deformation rheological parameters of the gels were analyzed on a Discovery HR-2 Rheometer (TA Instruments; New Castle, DE, USA) within 24-48 h of preparation. The freshly made bigel (still warm) was poured into circular PVC molds (3.5 cm diameter x 0.3 cm depth) and cooled at room temperature for 1 h before storing in the refrigerator for a minimum of 24 h. A 20 mm crosshatched parallel plate geometry was used. Samples were carefully trimmed to the parallel plate geometry size once on the rheometer. At least four (4-7) discs (20 mm diameter) of each replicate were analyzed, and at least 3 replicates were prepared. Samples for amplitude and frequency sweeps were pulled from the refrigerator immediately prior to analysis.

Amplitude Sweep

An amplitude sweep procedure from Acevedo and Marangoni (2014) with slight modification was used. Briefly, a test from 0.001 to 150% at 1 Hz and 25°C was conducted to determine the linear viscoelastic region (LVR), yield stress, and moduli crossover strain. A 2500 µm gap was used. Yield stress (σ, Pa) was calculated as the oscillation stress when G' had decreased 10% from the G'_{LVR} (Acevedo, Block, & Marangoni, 2012). TRIOS software (TA Instruments; New Castle, DE, USA) was used to determine the moduli crossover.

Frequency Sweep

A frequency sweep procedure from Okuro et al. (2018) was used with slight modification. Briefly, the sample was run from 0.1 to 100 Hz at 0.1% strain and 25°C with axial force control (0.2±0.1N) for the gap. Frequency sweep data were only reported and analyzed up to a frequency of 60 Hz since either slippage or head inertia occurred beyond that point. TRIOS software (TA Instruments; New Castle, DE, USA) was used to find the linear slope of the frequency versus storage modulus (G') curve to understand frequency dependence. Finding a strain offering consistent results, but within the LVR, for all the formulations proved challenging. Thus, for some of the samples 0.1% was outside the LVR, but upon comparing slopes, the standard deviation was very low (<0.02).

Temperature Ramp

A temperature ramp procedure from Patel, Schatteman, De Vos, Lesaffer, and Dewettinck (2013), with minor modifications, was used. A sample that was already prepared and cooled overnight was heated at the base using a Peltier system and immediately run from 98 to 8°C at 5°C/min, 1 Hz, and 0.1% strain using axial force control (0.2±0.1N) for the gap. Oil was dropped on the perimeter of the sample and parallel plates to mitigate moisture evaporation during the run. The temperature of the minimum point on the first derivative of the temperature versus G' curve was found. This temperature corresponds to the temperature at which the maximum rate of G' change occurs. Like the frequency sweep, finding a strain offering consistent results, but within the LVR, for all the formulations proved challenging. Thus, for some of the samples, 0.1% was outside the LVR, but upon comparing the first derivative minimum temperatures, the standard deviation was very low (<2.1°C).

Fluorescence Microscopy

Confocal scanning laser microscopy (CSLM) was performed using FITC and Nile Red to dye the WPC80 and SO, respectively. They were included in each phase prior to gelation at 0.2 and 0.15 g/kg, respectively. A fresh bigel sample was spread on a glass bottom slide. The cover was sealed on the slide with soybean wax after preparation to prevent moisture evaporation prior to imaging. The slide was pulled from the refrigerator immediately prior to analysis. Micrographs were acquired on an SP5 X MP Confocal Microscope (Leica Microsystems Inc.; Buffalo Grove, IL, USA) using a 10X objective magnification with no zoom. Excitation and emission wavelengths for FITC were 495 and 505-540 nm, respectively, and 552 and 565-700 nm for Nile Red, respectively. Images were 1024 x 1024 pixels and acquired at 200 Hz. Signals were overlaid in Leica LAS AF Lite software (Leica Microsystems Inc.; Buffalo Grove, IL, USA) and processed in FIJI (Schindelin et al., 2012), a form of ImageJ (Research Services Branch; National Institutes of Health; Bethesda, MD, USA). A sample with no dye was viewed as well (results not shown) to confirm that no autofluorescence was occurring from the bigel's natural constituents. Multiple images were obtained of each treatment, but a representative one of each is depicted.

Statistical Analysis

All statistical analyses were carried out in JMP Pro 14 software (SAS; Cary, NC, USA). Statistical differences were determined by one-way ANOVA using Tukey's multiple comparisons (with significance set at $p<0.05$). Fulfillment of an ANOVA's underlying assumptions (independence and equal variance) was verified prior to analysis.

Results and Discussion

Bigel Physical Stability

All of the bigels showed immediate structural stability, as they did not separate into layers immediately upon homogenization. The bigels could be inverted; they sustained their own weight and did not flow. Representative images of the 15P 10W treatment are shown in Figure 2.1 (15P 20W, 25P 10W, and 25P 20W, Figure S2.1). The bigels, regardless of protein or water content, appeared opaque and off-white. The more OE present, the more yellow-like the gel appeared, which can be attributed to the presence of liquid oil, whereas when more HY phase was present, the gel appeared more white. No phase separation was observed for at least five months during storage at refrigeration temperature, suggesting that the OE and HY components were compatible with each other.

The 15P bigels were smooth and homogenous, whereas the 25P bigels were non-uniform. This non-uniformity was likely due to the higher protein content causing increased intermolecular interactions. Stronger interactions resulted in a firmer gel that could not be fully broken down during homogenization, and thus solid protein gel fragments were dispersed throughout.

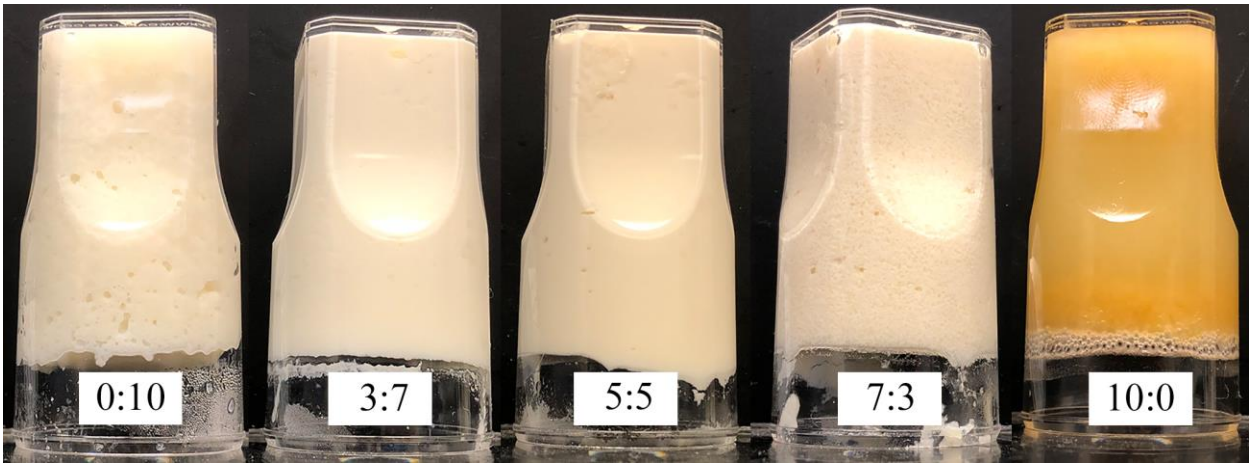

Figure 2.1. Images of 15% Protein, 10% Water bigels showing their appearances.

Small Angle X-ray Scattering (SAXS)

Representative SAXS patterns obtained for the various bigel ratios, protein contents, and water contents are shown in Figure 2.2. The d-spacing values with their corresponding standard deviation and statistical analyses are reported in Table S2.2. The pure HYs, 0:10, did not show any peaks, indicating there was no material present to scatter X-rays.

All of the bigels and pure OE showed a peak with a d-spacing value of 50Å. A previous study (Gaudino et al., 2019) explored OEs with both SL and SA as organogelators and found that SL manifested itself as reverse worm-like micelles at 50.1 to 57.6Å. These results are generally in line with ours, with the SL reverse micelle peak (~50Å) at slightly smaller d-values. Bodennec, Guo, and Rousseau (2016) found that lecithin formed micelles in canola oil oleogels with a diameter of 52 to 53Å. Moreover, Nikiforidis and Scholten (2014) found that micelles in a lecithin sunflower oil organogel had a d-spacing of 52Å and that a single SL molecule is 20 to 28Å. Heeding this finding, a SL micelle (composed of two individual SL molecules) can be anywhere from 40 to 56Å, which our peak fits within. Phospholipids, the primary constituent of

lecithin (van Nieuwenhuyzen & Tomás, 2008), was found to be 25 to 30Å in size by Gupta, Muralidhara, and Davis (2001). The aforementioned authors state that phosphatidylcholine and phosphatidylethanolamine are the primary phospholipids in soybean, their source of phospholipids, which is similar to the present study. In our study, regardless of the bigel's protein or water content, the peak corresponding to a SL reverse micelle always appeared.

We hypothesize that the SL reverse micelle core was water, which can form hydrogen bonds with lecithin's phosphate groups and thereby reduce the interfacial curvature and allow reverse micelle formation (Hashizaki, Taguchi, & Saito, 2009). An increase in water content allows more water to interact with the internal phosphate groups, and thus the entire micelle's size is increased (Gupta et al., 2001). This was most apparent in the 20W pure OE (10:0). Gaudino et al. (2019) explored an OE with 10 and 20 wt% water as well; nonetheless, the water content of the present study's OE within a bigel was higher than that because it is likely that not all of the HY's water was bound to protein. Thus, more water was available to the SL and allowed a larger micelle to form. Only a minor difference was found in micelle size, however, because each phospholipid can imbibe 6 to 10 water molecules (Cevc, 1993; Gupta et al., 2001). The peak at ~57Å of the 10:0 10W may have been a large SL reverse micelle with more water in the core.

Most of the bigels and the pure OEs showed a peak at ~40Å, which corresponds to SA. The SA is believed to be present as bilayers, as evidenced by the similarity in peak d-spacing of Gaudino et al. (2019) and our results for a similar system. The aforementioned authors reported the SA bilayer in a SL and SA oleogel at 39.2 to 39.5Å, while our study found them slightly larger, at 39.9 to 40.1Å. Other works found SA bilayers at 40Å and 38.4 to 41.1Å, which are in line with our results (Blach et al., 2016; Schaink, van Malssen, Morgado-Alves, Kalnin, & van

Der Linden, 2007). The observed results suggest the presence of bilayers. The 3:7 25P samples did not show the d-spacing value corresponding to a SA bilayer, while in the 15P samples of this same ratio, the intensity was very low compared to the other peaks. The SA peak was not present or was very weak in these bigels, probably because of the small amount of SA in the samples, at only about 1.5 wt%, and thus may not have been detectable. However, in higher OE containing gels, the SA was present and detectable since it was at a 5.4 wt% concentration.

Peaks at higher d-spacings, beyond the SL and SA structures (>50Å), were observed in gels with more OE present (7:3, 10:0). Additionally, these higher d-spacing peaks were more intense in the lower water content (10W) bigels. One possible explanation is the worm-like reverse micelles packing into a hexagonal array, which are displayed by the pattern in this region (Figure 2.2). Previous research by Bodennec et al. (2016) found that lecithin and canola oil oleogels formed highly ordered supramolecular assemblies of lecithin micelles in a hexagonal array. It is known that molecular arrangements with hexagonal symmetry have diffraction peaks at ratios of approximately 1, √3, 2, √7, and 3 (Gang, 2015). Consider, for example, the SL reverse micelle peak at 49.8Å in the 10:0 15P 10W bigel. If hexagonal, it would show peaks appearing at 86.3, 99.6, 131.8, and 149.4Å. Of these, the latter two are not visible given our current experimental conditions, but a peak at 99.6Å is clearly visible. Applying this to the 7:3 15P 10W bigel, a similar result is found, with a peak at ~86Å. The disappearance of higher distance spacings as HY content increases is attributed to either dilution or the protein network disrupting SL's hexagonal array formation. The former of these suggestions is less likely as the individual SL reverse micelle peak at ~50Å is still visible in all bigel SAXS curves, despite HY content. The latter suggestion is supported by the CSLM images discussed below.

In 10W bigels with more OE component present, there was a peak with a d-spacing value of ~72 to 73Å. This peak's intensity followed a similar trend to the SA bilayer peak. When the SA bilayer peak disappeared, so too did the peak at ~72 to 73Å. This suggests that the peak is affiliated with the SA bilayer. It is interesting that this peak was not visible in 20W bigels, likely due to a dilution effect. Additional studies should be performed to explore the peak further and draw comprehensive conclusions.

The 20W OE patterns show a peak at 110Å. This peak may be the result of SL incorporating itself into the SA bilayer structure. Work by Uvanesh et al. (2015), who studied SA and Tween 20 oleogels, found that Tween 20 can be incorporated into SA's lamellar structure due to intermolecular hydrogen bonding, and thus create larger SA crystals. Perhaps, instead of Tween 20, SL's reverse micelle is behaving similarly in the present OE system.

Based on these findings, we can conclude that even with the addition of HY, the OE component retained its key structural features. There was no statistically significant difference in peak d-spacings across protein and water contents, suggesting that protein and hydrogel water do not affect soy lecithin reverse micelle and stearic acid bilayer structure sizes significantly under the conditions explored in this study. Therefore, the results indicate the formation of true bigel structures where two discrete network gels contribute to the overall material, and where it seems that the inter-species interactions are weak compared to those of the intra-phase attraction forces.

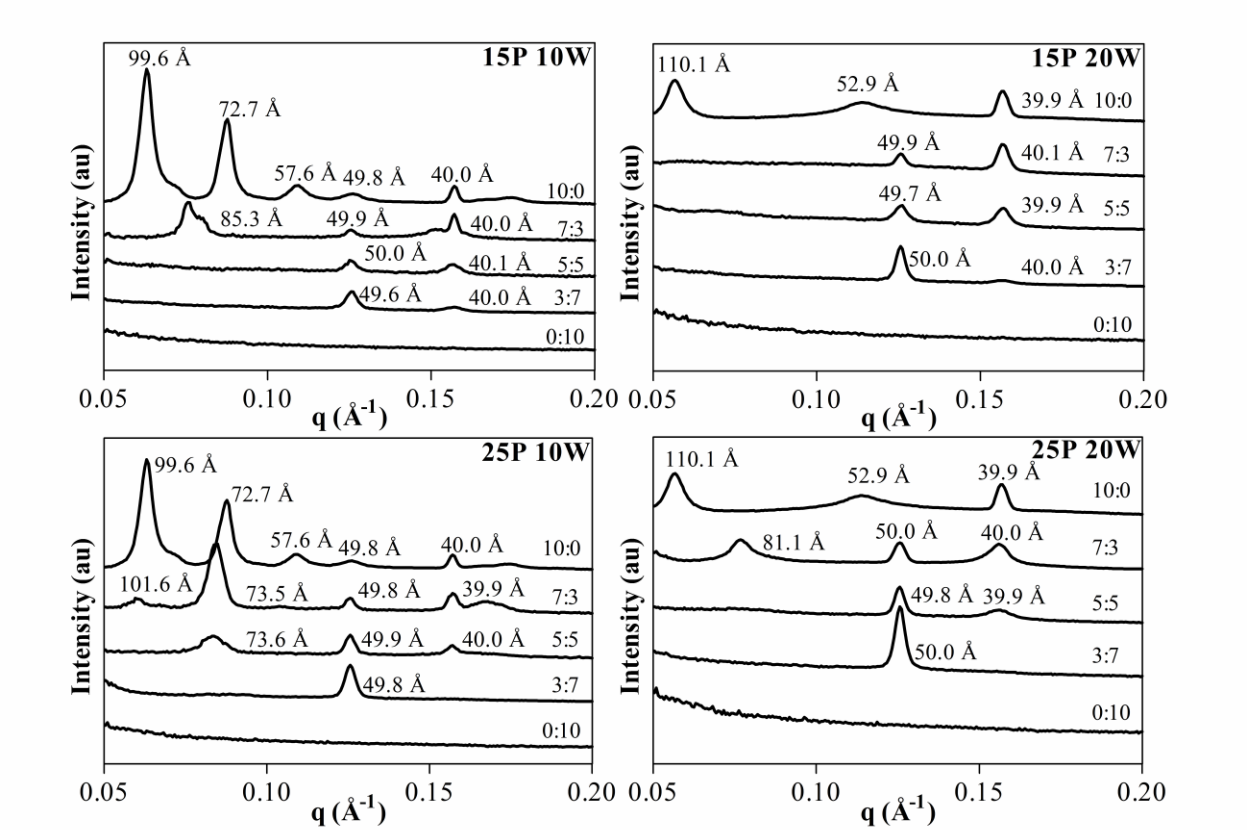

Figure 2.2. SAXS patterns obtained for the bigels formulated with different OE:HY ratios. Average d-spacing values are shown in the patterns.

Rheology

Amplitude Sweep

Amplitude sweeps offered information on the linear viscoelastic region (LVR) (Figure 2.3), yield stress (Table 2.1), and strain at the moduli crossover point (Table 2.1). In all cases, G' was initially greater than G", by 3- to 4-fold, indicating a material with more elastic-like than viscous-like behavior. The OE:HY ratio, protein, and water content had statistically significant effects on the bigels' rheological properties.

Excluding the 25P 10W bigels, where the pure HY (0:10) had greater mechanical strength than the OE-containing gels, the rheological properties of all the other gels were most influenced by the OE phase, where the gels with more OE had the greatest G'. For example, comparing the 7:3 to 5:5 bigel across protein and water contents, the G'_{LVR} for 7:3 was 1.4- to 13.3-fold higher. This is in agreement with previous studies that found bigel rheological characteristics to be strongly dependent on the organogel fraction (Lupi et al., 2016; Singh et al., 2014b).

Nevertheless, it is noteworthy that as the OE:HY ratio increased, the critical strain decreased. At 15P and 25P the LVR length decreased by 30- and 20-fold, respectively, when comparing the 0:10 to the 10:0 samples (Figure 2.3). For the pure OE, 10:0, the critical strain was very low, and the moduli crossover was at a low strain (~0.15%), indicating a shear-sensitive structure that was easily broken down. SL was the primary oleogelator used (70% of the oleogelator concentration), and its shear-sensitivity is in agreement with other research on lecithin based oleogels (Bodennec et al., 2016; Nikiforidis & Scholten, 2014). Gels with more hydrogel component provided superior rheological properties since the critical strain was higher, indicating they can withstand higher shear forces before loss of the elastic component and induction of flow. Despite their lower mechanical strength evidenced by the lower G' values, this is an advantage of using a bigel over a pure OE when a semisolid behavior is desired.

Regarding the effect of HY protein concentration, the 25P samples had greater G' and yield stress than the 15P – about 10 and 6 times greater, respectively. This is in agreement with previous research on whey gels showing that gel strength increased as protein content increased (Chen & Dickinson, 1998). In agreement with our 25P 0:10 results, Rehman et al. (2014), who studied beeswax, hydroxypropyl methylcellulose, and sodium alginate bigels, found that

compared to bigels, the HY component was more adhesive, hard, and had a greater peak stress value, which they attributed to greater intermolecular interactions, such as hydrogen bonding, with the aqueous phase.

When examining the effect of water content, it was observed that regardless of HY protein concentration, the 3:7 and 10:0 experienced an increase in G' when water increased from 10 to 20wt% (10W to 20W). The pure OE (10:0) had the most dramatic increase, at almost a 4-fold G' increase. The increase in OE strength with increase in water content is in agreement with previous studies exploring lecithin-based oleogels (Gaudino et al., 2019; Nikiforidis & Scholten, 2014). Gaudino et al. (2019) hypothesized that water's presence allows the formation of both SL reverse micelles and SA bilayers that synergistically interact to increase hardness.

In the bigel system, mixed storage modulus results were found. In the 25P gels, regardless of water content, the pure HY (0:10) had the greatest mechanical strength (Figure 2.3). In the 25P 10W sample, the 0:10's G' was over 11-fold greater than the 7:3. In the 15P 10W gels, the 7:3 had the greatest mechanical strength (about a 1.5-fold greater G' than the next highest gel). However, increasing the water content to 20W (still 15P) no longer permitted the 7:3 to have the greatest mechanical strength (over a 10-fold decrease in G'_{LVR}); rather, at 15P 20W the 7:3 had the third greatest mechanical strength of the five gels analyzed. This highlights that at the proper protein content, water content, and OE:HY ratio, a bigel can offer superior mechanical properties. Future work is needed to elucidate the exact mechanism for how each component interacts to support the enhanced mechanical properties.

The 7:3 of the 15P 10W bigel samples showed the highest G' and yield stress values suggesting a synergistic effect between phases; nevertheless, its strain when G'=G" was not higher than other OE:HY ratios within the 15P 10W samples (Table 2.1). The low crossover

strain suggests that the OE's sensitive soy lecithin based structure was still dominant, but perhaps the HY was interacting with the OE phase to increase the G' and yield stress beyond that of the other OE:HY ratios within the 15P 10W samples. Gaudino et al. (2019) hypothesized that SA interacted with water through bilayers and the SL reverse micelle. It is possible the protein in the 7:3 15P 10W bigel was performing similarly, but with no significant effect on the OE underlying structure (since no difference was seen in the SAXS findings). Increasing HY concentration beyond 7:3 (in the 5:5 and 3:7) led to a decline in the gel's mechanical properties because G' and yield stress decreased and, as shown in the SAXS figures, no hexagonal array formed. G' and yield stress in the 15P 10W 7:3 bigel were about 13- to 14-fold and about 1.5-fold greater, respectively, than in the 5:5 and 3:7 bigels. CSLM images (discussed below) also showed that a continuous OE network was not formed in gels containing more HY content than the 7:3. Perhaps, in these higher HY containing bigels, the HY concentration is so large that it no longer interacts with the organic phase (because of strong intra-HY interactions), and it interrupts the formation of a continuous OE network. The 5:5 bigels had the lowest G', with their G'_{LVR} being 2- to 59-fold lower than the pure gels (0:10 and 10:0). As will be shown later, CSLM showed a bi-continuous network where neither the HY nor OE were able to form a strong continuous network, and, therefore, a gel with weaker mechanical properties was formed.

Nikiforidis and Scholten (2014) found that the addition of α-tocopherol to lecithin sunflower oil oleogels disturbed the hydrogen bonds between the phosphatidylcholine and water and thus reduced the strength of the interactions. Admittedly, α-tocopherol and WPC80 are very different molecules and were used at different concentrations in the studies, but both contain hydrophilic and hydrophobic portions and can influence lecithin's ability to form a network

structure. This may explain the reduced G' and yield stress of the biphasic systems observed in this study.

The yield stress values of the 25P bigels had high standard deviations; this may be because of the bigels' large and unbroken HY fragments that could not be ruptured by the homogenizer. This non-homogenous structure infringed on a small angle oscillatory rheology assumption. As a result, inconsistencies in gel mechanical strength were acquired and led to high standard deviations. Finally, as will be seen from CSLM, some of the samples had air bubbles, and this may have consequently affected the rheological properties of the sample.

Table 2.1. Yield stress (Pa) and moduli crossover strain (%) as calculated from amplitude sweeps. Different letters signify significantly different results as revealed by Tukey HSD ($p<0.05$). Comparisons were done between bigel ratios within one protein content, water content, and analysis.

OE:HY ratio	% Protein	% Water	Yield Stress	G'=G" Strain
0:10	15	NA	311 ± 85^{a}	120 ± 3^{a}
	25	NA	1955 ± 394^{a}	115 ± 9^{a}
3:7	15	10	19 ± 3^{b}	114 ± 8^{a}
		20	24 ± 2^{b}	118 ± 11^{ab}
	25	10	149 ± 8^{b}	107 ± 10^{ab}
		20	195 ± 64^{b}	77 ± 10^{b}
5:5	15	10	24 ± 4^{b}	87 ± 5^{b}
		20	15 ± 4^{b}	100 ± 4^{b}
	25	10	62 ± 14^{b}	82 ± 9^{bc}
		20	56 ± 5^{b}	84 ± 8^{b}
7:3	15	10	35 ± 4^{b}	59 ± 11^{c}
		20	15 ± 1^{b}	72 ± 4^{c}
	25	10	71 ± 51^{b}	89 ± 20^{abc}
		20	39 ± 20^{b}	74 ± 10^{bc}
10:0	NA	10	14 ± 3^{b}	62 ± 2^{c}
		20	74 ± 8^{b}	49 ± 4^{d}

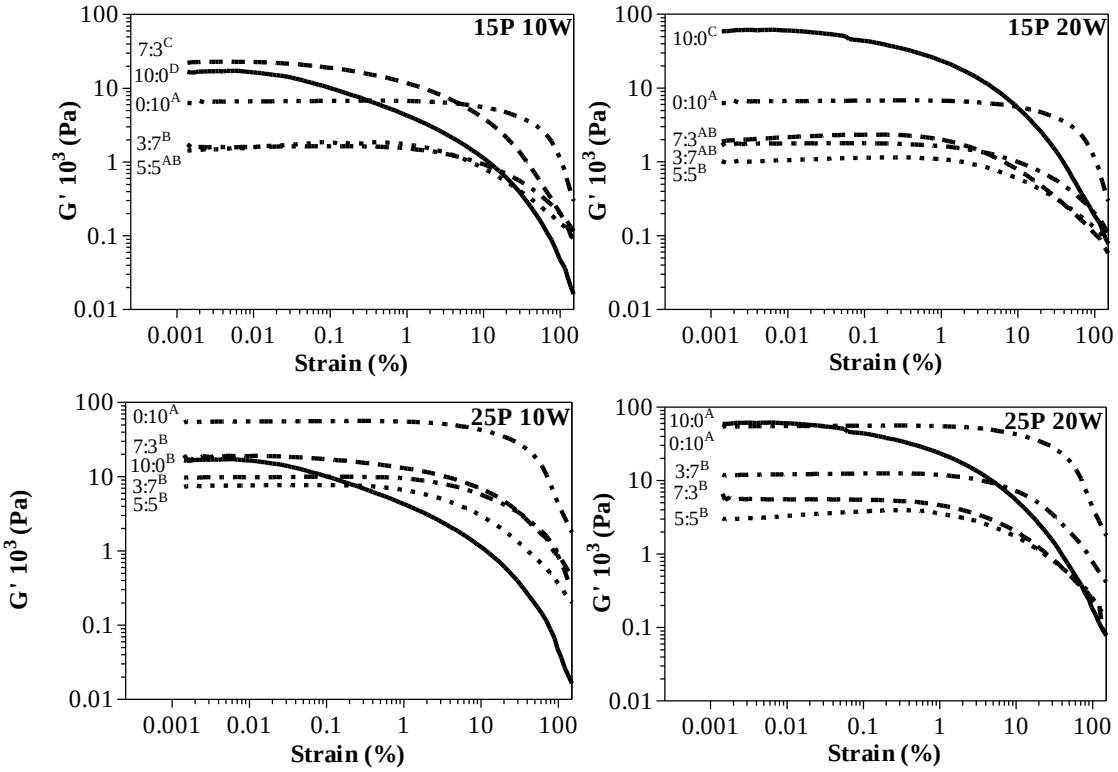

Figure 2.3. Bigel amplitude sweep G' curves. Lines are labeled with OE:HY ratio and different letters indicate significantly different curves within that protein and water content.

Frequency Sweeps

Frequency sweeps were performed to assess the frequency dependence of the formulated samples (Figure 2.4). G' was greater than G" in all gels over the range of frequencies studied, demonstrating the elastic component's dominance. Additionally, a slight frequency dependence was observed for all the samples, suggesting that although the gels were strong, they showed a slightly weak network. Slopes of each frequency curve were calculated to compare frequency dependence numerically. Across the various treatments, the slope values were small, and there was no clear trend in frequency dependence. All samples exhibited a positive slope, and there

was no apparent connection between OE:HY ratio, protein content, water content, and frequency dependence. Patel et al. (2015) also found a slight frequency dependence in fumed silica (organogelator), locust bean gum and carrageenan (hydrogelators), and vegetable oil bigels. The same authors found that a bigel had greater gel strength than the organogel, suggesting that the fat and aqueous phases were interpenetrating synergistically to create a semi-continuous network. Findings from this study are not in agreement with those of the aforementioned authors. This may be the result of two phases that formed two discrete network gels with intra phase attraction forces dominating over inter-species interactions. Patel et al.'s (2015) organogelator was fumed silica which has some silanol groups (Si-OH), which can form hydrogen bonds, and thus offer more hydrophilic behavior and partition into the water phase (creating inter-species interactions). Although the oleogelators of this study have some hydrophilic behavior, they are still mostly hydrophobic and, contrary to Patel et al. (2015), probably did not partition between the bulk water phase (non-OE water) and OE oil. CSLM results, discussed below, highlight that both phases remained mostly separate. As discussed above, the HY was solid (due to thermal treatment) prior to addition of the non-gelled OE. Thus, during homogenization there was minimal non-gelled protein available in solution to interact and gel with the SL and SA synergistically.

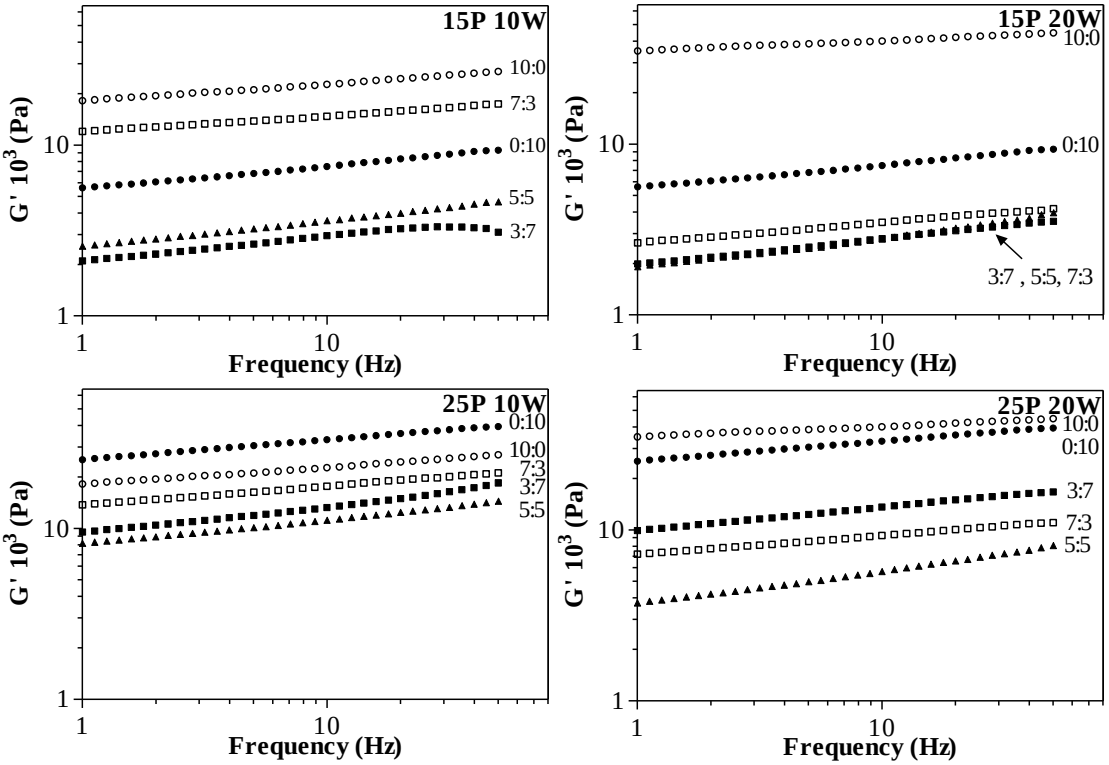

Figure 2.4. Bigel frequency sweep G' curves. Lines are labeled with OE:HY ratio.

Temperature Ramp

Temperature ramps were performed to assess bigel rheological behavior at various temperatures and are shown in Figure 2.5. The selected starting temperature was 98°C since it is slightly above the temperature at which the OE is prepared. The selected ending temperature was 8°C since it is close to refrigeration temperature.

As temperature increased, the bigel's G' decreased, indicating a decline in elastic-like behavior. From the 98°C starting temperature to the 8°C ending temperature, there was anywhere from a 4- to 6114-fold difference in G'. Lupi et al. (2016) reported a similar finding with bigels composed of pectin (hydrogelator), glyceryl stearate and policosanol (organogelators), and olive

oil, where phase angle increased with increasing temperature, suggesting a decrease in solid-like behavior.

For all bigels, G'>G" during the entire test, suggesting the dominance of elastic behavior at all temperatures. At high temperatures, when the OE was likely melted, the HY provided some structure, as whey protein derived hydrogels are thermally irreversible (Damodaran & Paraf, 1997).

In the samples with more OE present, there was a sharp inflection upwards in G' around 30°C. The OE-rich bigel inflections ended at the highest G' values, which was similar to previous findings (Lupi et al., 2016). The gels with more OE phase present (7:3 and 10:0) had the greatest change in G' from the start of the test to the end, with anywhere from a 25- to 6114-fold change in G'. The inflection is believed to be SA crystallizing. Previous research found SA's crystallization temperature in a SA canola oil oleogel to be 36°C (Blach et al., 2016). Another study on SL and SA OEs found a SA crystallization peak at 43-44°C (Gaudino et al., 2019). This same group found that an increase in SA concentration resulted in an increased crystallization temperature. The same trend was found in the present study, where increasing the OE content, and thereby increasing the amount of SA, increased the temperature at which the maximum change in G' occurred. The temperature of transition was significantly different in the 10:0 and 7:3 compared to the 5:5 (no apparent transition temperature observed in the 3:7 and 0:10). The transition occurred ~29°C for the former and ~21°C for the later. The values of the transition were slightly different from those reported in the aforementioned studies, probably due to the different techniques used. Blach et al. (2016) and Gaudino et al. (2019) used differential scanning calorimetry (DSC), whereas this study used rheology.

Similar to what was observed in the present study, previous work found that G' has a strong temperature dependence in SA sunflower oil oleogels, with G' decreasing as temperature increases (Schaink et al., 2007). Schaink et al. (2007) reported that the greatest change in G' occurred between 20 and 30°C, and above 35°C the oleogel effectively had no texture, which is in line with the results from this study. The same group also found that at 15°C and below, the G' was relatively constant. This is not in agreement with our findings, as G' was still increasing when the test ended at 8°C. This can be attributed to the presence of other components, like SL or HY, which, as described earlier, may interact with the SA. Additionally, the temperature ramp progressed rapidly, and the gel may not have had enough time to develop its optimal structure at each temperature before the reading was taken.

SL may have provided some structure at elevated temperatures. Previous work studying lecithin and oil oleogels found that as temperature increased, the gel's firmness, G', and G" decreased, similar to our findings (Bodennec et al., 2016; Nikiforidis & Scholten, 2014). Bodennec et al. (2016), who observed lecithin reverse micelles at 52 to 53Å with SAXS, stated that this peak greatly decreased by 50°C during a heating ramp and completely vanished by 63°C. However, they found that at 63°C a weaker spacing appeared on the SAXS curve at ~67Å, indicating the presence of some structure. This peak's intensity did not change for the remainder of the study, suggesting its temperature independence. The authors hypothesized that the micelles underwent a major structural change during heating and evolved from a gel-network with order to a less-ordered one. Although SAXS data is not available at elevated temperatures for this study, something similar may be occurring and may explain why there was still some structure in the bigels and OE at elevated temperatures. Nikiforidis and Scholten (2014) studied lecithin sunflower oil oleogels and found that the maximum force that could be applied to the gel

decreased as temperature increased. They stated that the packing geometry was dependent on hydrophilic and hydrophobic interactions at various temperatures. At elevated temperatures, the interactions were weakened. Like what the aforementioned authors found, the protein in this study may have affected the hydrophobic and hydrophilic interactions of the SL, SA, and water and thus changed the gel's behavior.

Interestingly, the $G'_{25°C}$ values of the temperature ramp (Figure 2.5) did not match the G'_{LVR} of the amplitude sweep, also conducted at 25°C (Figure 2.3). In nearly all cases, the G' at 25°C from the temperature ramp was greater than the G'_{LVR} from the amplitude sweep. $G'_{25°C,\ temperature\ ramp}$ was 1.1 to 5.8-fold greater than $G'_{amplitude\ sweep}$. One possible reason for this is the samples that were analyzed via an amplitude sweep were done after the structure had 24 h to develop, whereas in the temperature ramp tests the bigel was assessed at each temperature immediately after it reached that point. Stearic acid, like many fats, exists in many polymorphic crystalline forms and, depending on which polymorphic form was present due to the cooling and storage conditions, different mechanical properties would be assessed (Schaink et al., 2007). Additionally, Figure 2.5 shows that G' was still increasing (no plateau) when the test was completed at 8°C, suggesting that an equilibrium state was not reached.

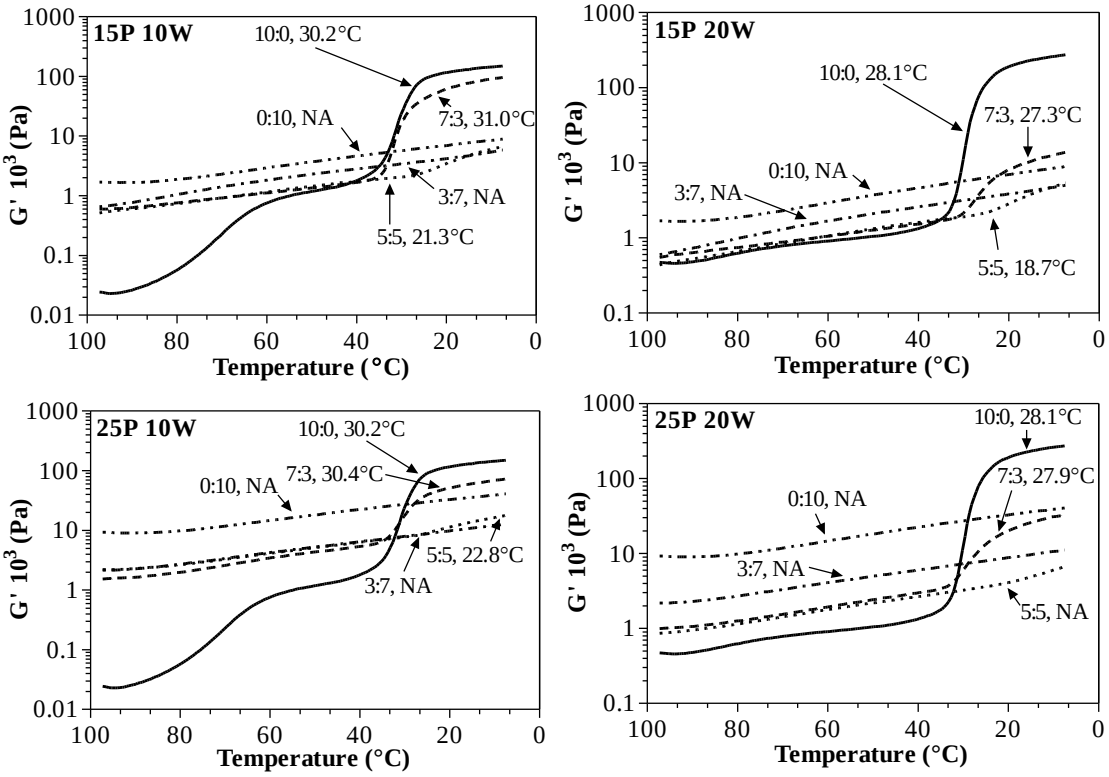

Figure 2.5. Bigel temperature ramp G' curves. Lines are labeled with OE:HY ratio and temperature of greatest G' change, if applicable (NA = not applicable).

Fluorescence Microscopy

A representative image of the various OE:HY ratios at 15P 10W is shown in Figure 2.6. Formulations prepared at 25P and 20W appeared similar in their distribution of each phase and are depicted in Figure S2.5. The protein and lipid phases appear as green and red, respectively.

The 25P samples (Figure S2.5) had larger protein fragments than the 15P, and the protein sections were non-uniformly distributed throughout the system. The 5:5 appeared as evenly distributed spherical, emulsion-like droplets, suggesting both phases interacted in a synergistic fashion. Singh et al. (2014a) also found spherical appearing droplets in their guar gum

(hydrogelator), sorbitan monostearate (oleogelator), and sesame oil bigel CSLM micrographs. The 3:7 appears to have OE particles dispersed throughout a continuous HY network and the opposite in the 7:3 (Figure 2.6) where HY particles are dispersed throughout a continuous OE system. These results are similar to those by Lupi et al. (2016).

There was no clear, distinct barrier between the OE and HY in the bigels, suggesting that the surfactants and phases were interacting to some extent. Hydrophobic parts of the WPC80 may have been partially in the organic phase, while the polar regions of the SL and SA may have been partially in the aqueous phase. In the 10:0 and 7:3 samples, a textured appearance was seen, which is likely SL in its hexagonal array. Beyond these ratios (i.e., at higher HY content), the textured appearance was not visible. This is in accordance with the higher order SL reverse-micelle hexagonal array structures found in SAXS at 10:0 and 7:3.

The CSLM images highlight the continuous nature of the HY and OE in the 3:7 and 7:3, respectively, whereas the 5:5 system appeared bi-continuous. This supports the rheological findings discussed above. If one of the gelled phases is more continuous, it has stronger intermolecular connections, and, thus, a gel with improved mechanical properties is found. On the contrary, a bi-continuous system, such as that seen in the 5:5, could not form strong, long-range intermolecular connections. Thus, a gel with weaker mechanical properties was formed.

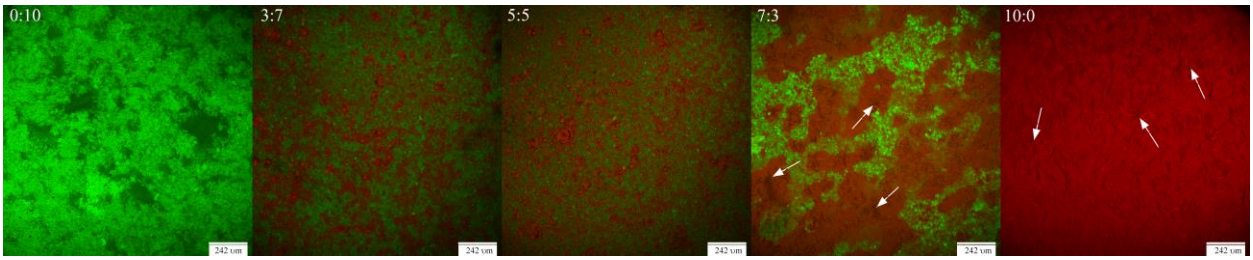

Figure 2.6. CSLM micrographs of 15% protein, 10% water bigels. Arrows highlight a textured appearance that we hypothesize are SL reverse micelles in a hexagonal array.

Conclusion

This study demonstrated successful preparation and characterization of a novel, edible bigel composed of a soy lecithin and stearic acid-based oleogel emulsion and whey protein concentrate 80-based hydrogel. Despite hydrogel addition, an oleogel emulsion still retains its key structural features under a range of protein and water contents. All of the bigels showed solid, gel-like behavior with a slight frequency dependence. The WPC80 appeared to synergistically interact with the oleogel emulsion at low concentrations, but at high concentrations became antagonistic due to intra-species forces dominating over inter-phase forces. Future work can elucidate the mechanism of this interaction. This study found that the addition of hydrogel improved the mechanical properties of an OE and that higher water content increased the OE's G'. Additionally, future studies will be focused on exploring cold-set WPC gels. In these, the hydrogel would not already be set at the time of homogenization, and we hypothesize there would be more opportunity for both phases to interact during the gelation process. This study shows the importance of a continuous network for bigel strength. The bigels show promise for the food industry because of their solid-like behavior and lack of separation over time. One potential application may be inclusion in yogurt to protect probiotics.

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Declarations of Interest

Declarations of interest: none.

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Supplemental Information

Table S2.1. Mass % of each bigel formulation.

Ratio (OE:HY)	Component	15% Protein		25% Protein	
		10% Water	20% Water	10% Water	20% Water
0:10	Soy Lecithin	0	0	0	0
	Stearic Acid	0	0	0	0
	Soybean Oil	0	0	0	0
	Water (OE)	0	0	0	0
	WPC 80	15	15	25	25
	Water (HY)	85	85	75	75
3:7	Soy Lecithin	3.78	3.36	3.78	3.36
	Stearic Acid	1.62	1.44	1.62	1.44
	Soybean Oil	21.6	19.2	21.6	19.2
	Water (OE)	3	6	3	6
	WPC 80	10.5	10.5	17.5	17.5
	Water (HY)	59.5	59.5	52.5	52.5
5:5	Soy Lecithin	6.3	5.6	6.3	5.6
	Stearic Acid	2.7	2.4	2.7	2.4
	Soybean Oil	36	32	36	32
	Water (OE)	5	10	5	10
	WPC 80	7.5	7.5	12.5	12.5
	Water (HY)	42.5	42.5	37.5	37.5
7:3	Soy Lecithin	8.82	7.84	8.82	7.84
	Stearic Acid	3.78	3.36	3.78	3.36
	Soybean Oil	50.4	44.8	50.4	44.8
	Water (OE)	7	14	7	14
	WPC 80	4.5	4.5	7.5	7.5
	Water (HY)	25.5	25.5	22.5	22.5
10:0	Soy Lecithin	12.6	11.2	12.6	11.2
	Stearic Acid	5.4	4.8	5.4	4.8
	Soybean Oil	72	64	72	64
	Water (OE)	10	20	10	20
	WPC 80	0	0	0	0
	Water (HY)	0	0	0	0

Figure S2.1. Bigel appearances. Top row: 15% Protein, 20% Water. Middle row: 25% Protein, 10% Water. Bottom row: 25% Protein, 20% Water.

Table S2.2. d- spacing values (average ± standard deviation) for bigels obtained from SAXS patterns. Different superscripts indicate significant differences in peak d-spacing. All units are Å.

	Peak 1	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**
15P 10W					
0:10	No peak	No peak	No peak	No peak	No peak
3:7	40.0±0^{a}	49.6±0.4^{b}	No peak	No peak	No peak
5:5	40.1±0.1^{a}	50.0±0.2^{b}	No peak	No peak	No peak
7:3	40.0±0.1^{a}	49.9±0.0^{b}	No peak	85.3±2.1^{d}	No peak
10:0	40.0±0.1^{a}	49.8±0.1^{b}	57.6±0.5^{c}	72.7±2.0^{e}	99.6±1.7^{f}
15P 20W					
0:10	No peak	No peak	No peak	No peak	No peak
3:7	40.0±0.1^{a}	50.0±0^{b}	No peak	No peak	No peak
5:5	39.9±0.1^{a}	49.7±0.4^{b}	No peak	No peak	No peak
7:3	40.1±0.1^{a}	49.9±0.1^{b}	No peak	No peak	No peak
10:0	39.9±0.2^{a}	52.9±3.2^{b}	No peak	No peak	110.1±1.6^{c}
25P 10W					
0:10	No peak	No peak	No peak	No peak	No peak
3:7	No peak	49.8±0.2^{b}	No peak	No peak	No peak
5:5	40.0±0.2^{a}	49.9±0.1^{b}	No peak	73.6±2.2^{d}	No peak
7:3	39.9±0.1^{a}	49.8±0.3^{b}	No peak	73.5±0.7^{d}	101.6±2.2^{e}
10:0	40.0±0.1^{a}	49.8±0.1^{b}	57.6±0.5^{c}	72.7±2.0^{d}	99.6±1.7^{e}
25P 20W					
0:10	No peak	No peak	No peak	No peak	No peak
3:7	No peak	50.0±0^{b}	No peak	No peak	No peak
5:5	39.9±0.3^{a}	49.8±0.2^{b}	No peak	No peak	No peak
7:3	40.0±0.1^{a}	50.0±0^{b}	No peak	81.1±1.2^{c}	No peak
10:0	39.9±0.2^{a}	52.9±3.2^{b}	No peak	No peak	110.1±1.6^{d}

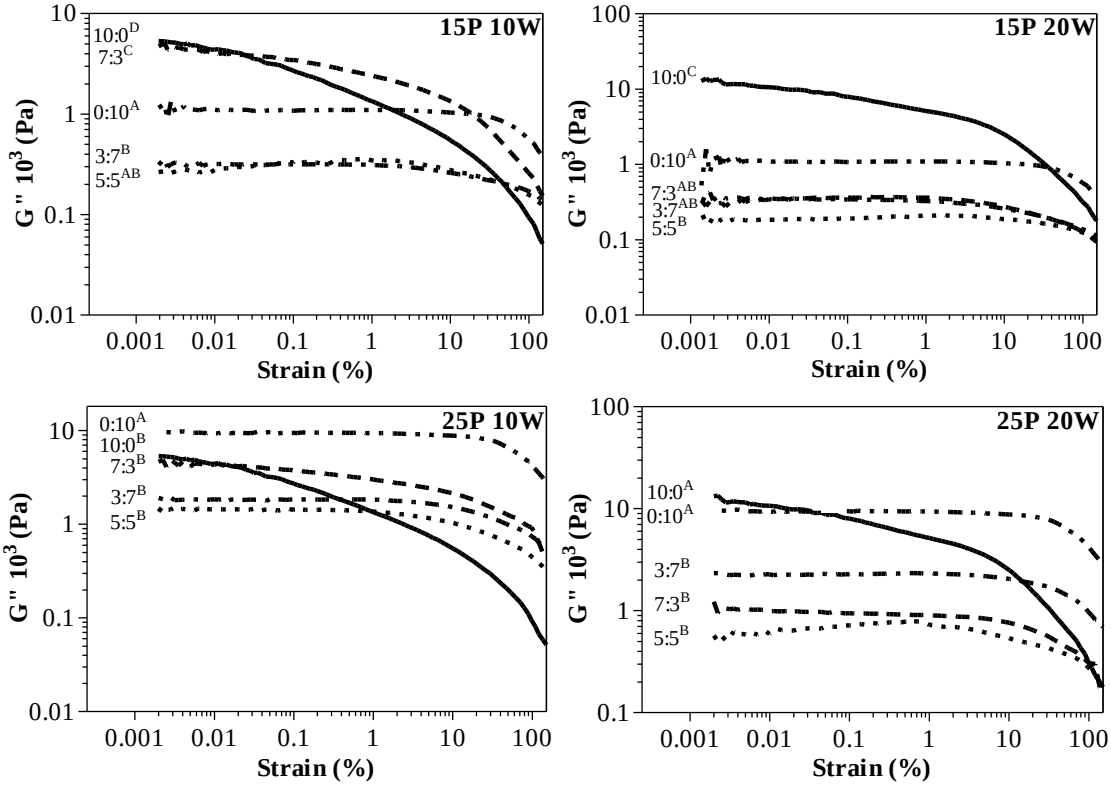

Figure S2.2. Bigel amplitude sweep G" curves. Lines are labeled with OE:HY ratio and different letters indicate significantly different curves within that protein and water content.

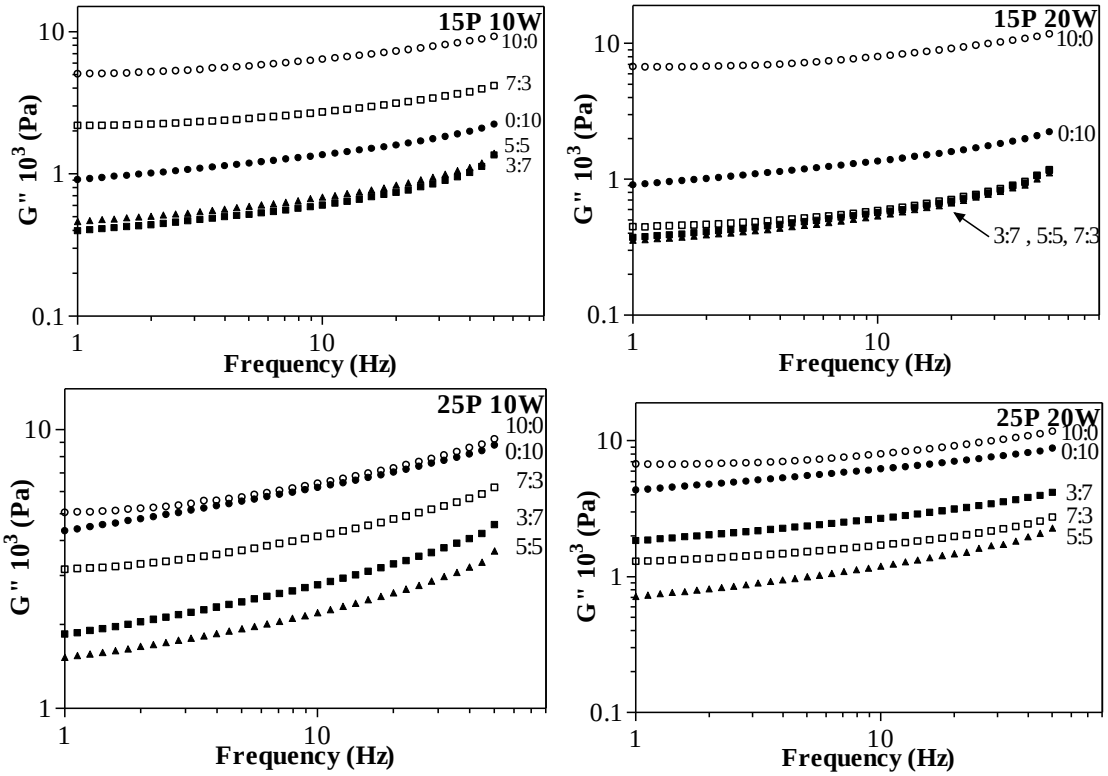

Figure S2.3. Bigel frequency sweep G" curves. Lines are labeled with OE:HY ratio.

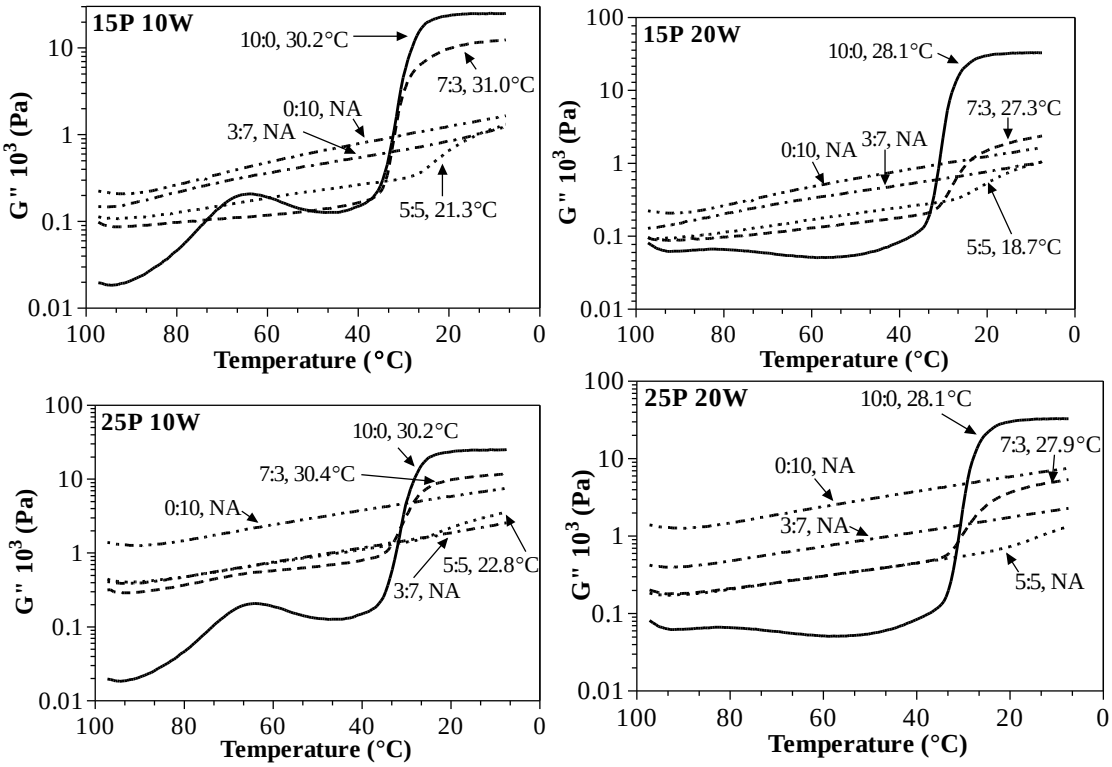

Figure S2.4. Bigel temperature ramp G" curves. Lines are labeled with OE:HY ratio and temperature of greatest G' change, if applicable (NA = not applicable).

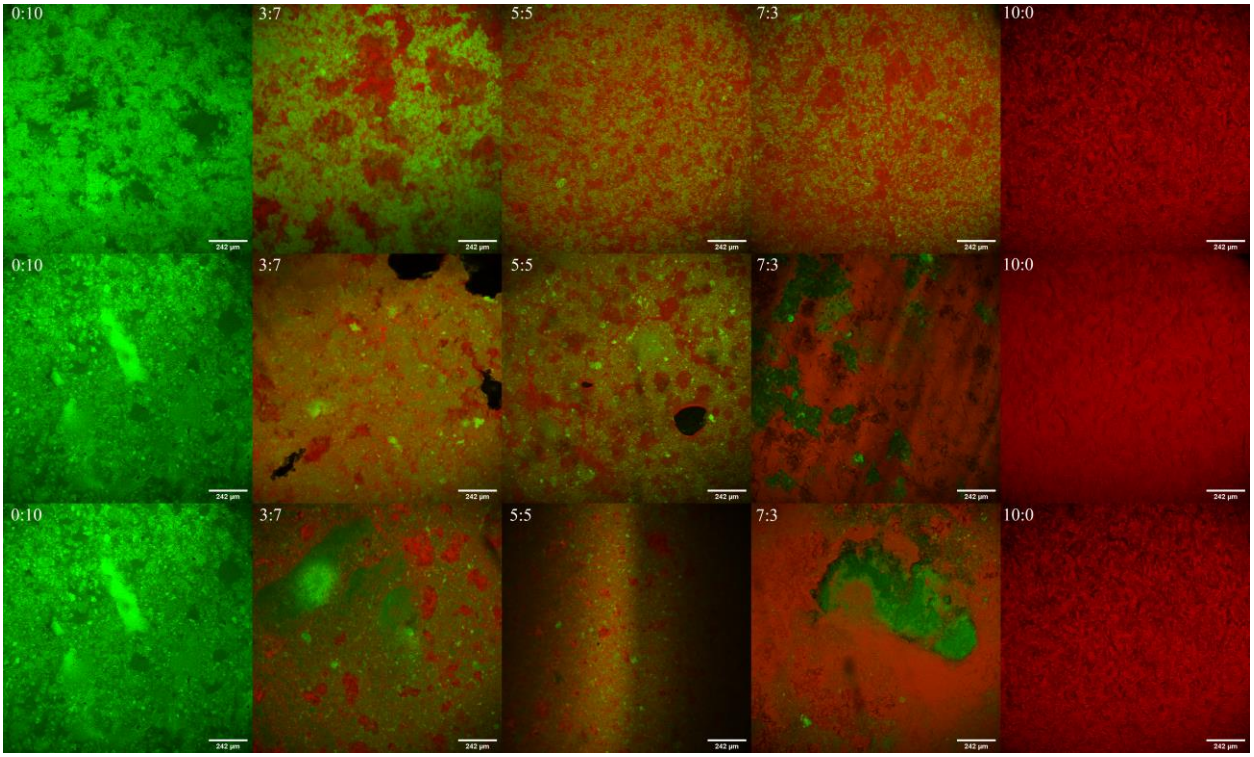

Figure S2.5. Representative CSLM images of bigels. Left column to right: 0:10, 3:7, 5:5, 7:3, and 10:0. Top row: 15% Protein, 20% Water. Middle row: 25% Protein, 10% Water. Bottom row: 25% Protein, 20% Water.

CHAPTER 3. EDIBLE LECITHIN, STEARIC ACID, AND WHEY PROTEIN CONCENTRATE 80 BIGEL PROTECTS PROBIOTICS DURING *IN VITRO* DIGESTION

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Abstract

Bigels are materials with semi-solid organic and aqueous phases and, therefore, have the potential to deliver both lipophilic and hydrophilic compounds to humans. Additionally, because bigels are two-phase systems, a diverse array of synthesis options exist, allowing many unique characteristics to be achieved. We propose herein using a bigel for the first time to deliver a bioactive, specifically probiotics. The purpose of this study was two-fold: first, to assess the ability of bigels to protect probiotics during *in vitro* digestion, and second, to determine the effect of phospholipids on probiotic survival. A standardized INFOGEST *in vitro* digestion system was used, and survival of *Bifidobacterium lactis* and *Lactobacillus acidophilus* was assessed. Gas chromatography was used to ensure the enzymes' lipolytic activity. A control, with no gelators, experienced the greatest lipolysis. The formulated bigel was effective at protecting probiotics from the harsh conditions of the digestive tract compared to the control. *Lactobacillus acidophilus* was more resistant to high shear conditions than *Bifidobacterium lactis* during gel preparation. Probiotics in bigels with phospholipids had greater survival during digestion, but the difference was not significant, and we hypothesize that this is because the phospholipids are enzymatically broken down in the *in vitro* system. Therefore, in order for phospholipids to have a beneficial impact on probiotic survival, they must be in their native form. Overall, this research shows the potential of bigels to protect probiotics during digestion.

Keywords: Bigel; *in vitro* digestion; probiotic viability; phospholipids; lipolysis

Introduction

There are many desirable probiotic attributes, some of which are required by the probiotic definition put forth by the World Health Organization (WHO) and Food and Agricultural Organization (FAO) and others that the scientific community generally agrees on. Desirable attributes of probiotics include: should be non-pathogenic, must be live microorganisms at the time of consumption, must confer a health benefit (when consumed in an adequate amount), should be able to survive the gastrointestinal (GI) tract, should be able to withstand processing, and should be able to adhere to and colonize the intestinal epithelium (Syngai et al., 2016; WHO & FAO, 2002). Two common genera of probiotics found in fermented foods or added to probiotic foods are *Lactobacillus* and *Bifidobacterium*. Delivering viable probiotic cultures to the gut has been a vexing problem for many researchers. The effect of processing, shelf-life, and digestion on probiotic survival must be addressed during product development. Survival can be enhanced through many approaches, with encapsulation being very common. Previous encapsulation techniques include calcium alginate beads or microcapsules, whey protein isolate microbeads, or gellan-xanthan beads, for example (Doherty et al., 2012; Ester et al., 2019; Lee & Heo, 2000; Sun & Griffiths, 2000). Ultimately, the selection of material for encapsulating or entrapping probiotics is important for determining their viability during adverse conditions and, therefore, their ability to impart beneficial function(s). For this reason, bigels should be explored since they may confer greater protection to probiotics than other encapsulation materials, such as those listed above. Ensuring probiotics survive processing and passage through the GI tract is important because many researchers are exploring probiotics as a potential treatment for

antibiotic-associated diarrhea, irritable bowel syndrome, *Helicobacter pylori* infection, and dental caries, for example (Bizzini et al., 2012; Hempel et al., 2012; Moayyedi et al., 2010; Sanchez et al., 2017).

Bigels are biphasic systems comprised of structured organic (called organogel) and aqueous (called hydrogel) phases (Shakeel et al., 2018). When edible, the organogel is called an oleogel (Marangoni & Garti, 2011). Bigels are advantageous because they can carry both lipophilic and hydrophilic compounds. Because of this, numerous drug delivery studies have used bigels (Behera et al., 2015; Rehman & Zulfakar, 2017; Singh et al., 2014). Nevertheless, they have not been explored for food applications, and this will be the first study (to the authors' knowledge) to explore probiotic incorporation into a bigel for food applications.

We recently developed a novel, edible bigel comprised of an oleogel emulsion containing soybean oil, soy lecithin, stearic acid, and water and hydrogel containing whey protein concentrate 80 and water (Bollom et al., 2020). The resulting bigel had improved mechanical properties over a pure oleogel emulsion or hydrogel, depending on the specific ratio of each component. Using bigels to deliver probiotics is a novel concept, and it holds great promise for soft or semi-solid foods, such as yogurt. We hypothesize that the bigel can provide a physical barrier between probiotics and harsh external environmental conditions.

Particularly, bigels containing phospholipids (PLs) are of great interest. Recent work in our lab (publication forthcoming) found enhanced survival of probiotics in an oleogel emulsion containing PLs over a six-week shelf-life (compared to a control without PLs). Prior work showed that *Lactobacillus reuteri* can attach to the milk fat globule membrane (MFGM), and this binding is strongly associated with the bacteria's hydrophobicity (Brisson et al., 2010). The MFGM's total lipid content is ~41% PLs (Fong et al., 2007). Work by Cleveland (2011) showed

that lactic acid bacteria preferentially bind PLs over other lipid sources. This binding may be due to S-layer proteins, which are believed to be important in adhering to the intestinal mucosal layer (Deepika & Charalampopoulos, 2010). S-layer proteins from lactic acid bacteria are basic (pI >9.4), whereas most bacteria have acidic S-layer proteins (pI 4-6), and this means that at neutral conditions the S-layer proteins of lactic acid bacteria are cationic, which allows them to bind with anionic lipid head groups (Smit et al., 2001).

Probiotics face many challenges during their life, and this work focuses on the challenges they face during digestion. Inside the GI tract, obstacles limiting probiotic survival include high acid, bile, heat, water, and oxygen (de Andrade et al., 2019; Liu et al., 2019). It is unknown if bigels can protect probiotics during digestion. Furthermore, it is unknown what effect, if any, PLs have on probiotic survival during digestion.

We hypothesized that a bigel would protect probiotics from the harsh conditions of the digestive tract since the semi-solid material establishes a barrier between the probiotics and the harsh external environment. Additionally, we hypothesized that PLs would enhance probiotic survival during digestion. Therefore, the objective of this study was two-fold. First, to determine the ability of a bigel to protect probiotics from the harsh conditions of the digestive tract. Second, to evaluate the effectiveness, if any, of PLs on probiotic survival throughout digestion.

Materials and Methods

Materials

Soybean oil (SO) was acquired from ADM (Des Moines, IA, USA). Granular soy lecithin (SL, 97% phosphatidylcholine) and sodium propionate were purchased from Acros Organics (Geel, Belgium). Stearic acid (SA), potassium chloride, monopotassium phosphate, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate, calcium chloride dihydrate,

lithium chloride, L-cysteine, lactose monohydrate, peptone, Tween 80, chloroform, methanol, and hexane were purchased from Fisher (Waltham, MA, USA). Anhydrous sodium sulfate, BCl_3-methanol (14%), and bovine bile were purchased from Sigma-Aldrich (St. Louis, MO, USA). De Man, Rogosa, and Sharpe (MRS) and M17 agars were from Oxoid (Hampshire, United Kingdom). 4-bromophenylboronic acid was from Matrix Scientific (Columbia, SC, USA). Pepsin (porcine stomach) and pancreatin (porcine pancreas) were from Alfa Aesar (Tewksbury, MA, USA). 2% fat milk was purchased from a local supermarket. Heptadecanoic acid was from TCI America (Portland, OR, USA). Methyl heptadecanoate and a FAME mixture (Fame #16 mixture) were from Restek (Bellefonte, PA, USA). Milk Specialties Global (Eden Prairie, MN, USA) generously donated whey protein concentrate 80 (WPC80). Dupont-Danisco (Madison, WI, USA) generously donated HOWARU Bifido (*Bifidobacterium lactis*, BL) and HOWARU Dophilus (*Lactobacillus acidophilus*, LA).

Sample Preparation

A bigel containing both structure and phospholipids was made. Both oleogelators, SL and SA, were used, and this bigel is abbreviated SL:SA+WPC henceforth. The SL:SA+WPC bigel was prepared as explained in the following section. A control sample formulated without gelators (SL, SA, WPC80) was used to assess the importance of structure. To test the effect of PLs, a bigel without SL was prepared and is abbreviated SA+WPC hereafter.

SL:SA+WPC bigel preparation

The SL:SA+WPC bigel was prepared following previously published methods with modifications to meet our treatment (Bollom et al., 2020). Briefly, the oleogel emulsion (OE) phase was composed of SO, SL, SA, and milk. The oleogelators were added at 20% w/w and

ratio of 5:5. They were dissolved in SO at 145-150°C. Milk was added at two times (described below) but at a final concentration of 20% w/w. The hydrogel (HY) phase was prepared using 20% w/w WPC80 and 80% w/w deionized water. The WPC80 was added to deionized water with stirring at room temperature for 2 h before storing it in the refrigerator overnight to ensure complete protein hydration. The next day, the pH and ionic strength were modified to 7.5 ± 0.01 using 1M NaOH or HCl and 50 mM using NaCl, respectively. The HY solution underwent gelation in an 85°C hot water bath for 30 min.

Homogenization was conducted in two phases. Each phase was freshly prepared and hot (85°C) for the first homogenization. The oleogel solution (molten) was poured on the thermally set HY and half of the OE's milk on top. They were homogenized at 13500 RPM for 2 min with a preheated homogenizer (Ultra Turrax; IKA; Staufen, Germany). Samples were cooled until the internal temperature registered 55°C. Slightly preheated probiotic milk (described below), comprising the remaining half of the milk weight, was added once the gel's internal temperature reached 55°C. The milk was homogenized in at 8000 RPM for 45 s.

Probiotic milk was prepared by anaerobically growing 0.1 g of LA and 0.1 g of BL in 9.8 mL of MRS broth for 48 h at 37°C. The broth was centrifuged at 18447 x g for 10 min. After discarding the supernatant, the probiotic pellet was rinsed three times with 10 mL of sterile milk and vortexed. Each rinse was added to 69 mL of sterile milk.

SA+WPC bigel preparation

An identical procedure to that described for the SL:SA+WPC bigel was used, except that a SL and SA concentration of 0 and 8% w/w was used, respectively (8% w/w was used as it conferred a similar hardness to the SL:SA+WPC bigel at 37°C (the temperature of digestion)). A TA.XT plus (Texture Technologies; Hamilton, MA, USA) was used to ensure similar hardness

(7 ± 0.04 g) between SL:SA+WPC and SA+WPC bigels. It should be noted that the milk fat likely contributed a small quantity of phospholipids to the SA+WPC bigel.

Control preparation

The same procedure as that described for the SL:SA+WPC bigel was used except no gelators were used, hence the sample lacked structure (was liquid). The Control was comprised only of SO, milk, and water.

Analysis Methods

In Vitro Digestion

An INFOGEST static *in vitro* digestion model based off work reported by Brodkorb et al. (2019), with a few modifications, was used. Table 3.1 describes the composition and concentration of each phase. Briefly, each 50 mL test tube received 5 g of sample, and each sample was run within 12-24 h of preparation. The appropriate amount of simulated salivary fluid (SSF) was added to the test tube containing the sample, and the salivary phase progressed in a shaking 37°C hot water bath (Thermo Scientific) for 2 min. Subsequently, the simulated gastric fluid (SGF), HCl (to reduce pH to 3.0), and pepsin were added following the salivary phase and progressed for 2 h in the shaking hot water bath. For the simulated intestinal fluid (SIF), NaOH (to raise the pH to 7.0), bile, and pancreatin were added to the test tube following the gastric phase, which progressed for 2 h. Each simulated digestion fluid was pre-heated at 37°C before use. Enzymes were prepared fresh for each digestion run and kept on ice to minimize their activity until use. It is worth mentioning that α-amylase enzyme was not used with the SSF in this work since there was no starch in the system.

Samples to assess probiotic survival were withdrawn at the following times: initial, following the salivary phase (2 min), mid-way through the gastric phase (60 min), end of the gastric phase (120 min), mid-way through the intestinal phase (180 min), and end of the intestinal phase (240 min). Fat lipolysis was assessed during the following times in the intestinal phase: initial, 20 min, 40 min, 60 min, 90 min, and 120 min. Samples for fatty acid analysis were transferred to a -80°C freezer until analysis. 4-bromophenylboronic acid was added at 5 mM before placing at -80°C to inhibit residual enzymatic activity during thawing (Williams et al., 2012).

Table 3.1. Composition of digestion fluids.

	Simulated Salivary Fluid (SSF)	**Simulated Gastric Fluid (SGF)**	**Simulated Intestinal Fluid (SIF)**
		Salts (mM)	
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25.0	85.0
NaCl	0.0	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.12	0.33
$(NH_4)_2CO_3$	0.06	0.5	0.0
HCl	1.1	15.6	8.4
$CaCl_2(H_2O)_2$	1.5	0.15	0.6

Probiotic Viability

An 11 g sample was diluted in 99 mL sterile peptone water (0.1% peptone, 10% Tween 80). Serial dilutions were conducted, and enumeration was performed using a pour plate method. LA were grown anaerobically on M17 Agar supplemented with 10% lactose at 37°C for 48 h. BL were grown anaerobically on MRS agar supplemented with 0.2% w/v lithium chloride, 0.3% w/v sodium propionate, and 0.05% w/v L-cysteine at 37°C for 72 h. BD GasPak EZ Anaerobe Container Systems were used to create anaerobic environments. Each replicate was analyzed in

at least duplicate with a total of three replicates completed. Percent survival was calculated as shown in Equation 1.

$$\%\, survival = \frac{\frac{CFU}{mL} at\ that\ time\ point}{initial \frac{CFU}{mL}} * 100. \quad (1)$$

Lipolysis

Sample preparation for fatty acid analysis

The lipids were separated from the digested samples using a Folch extraction based on the method reported by Rodríguez-Alcalá and Fontecha (2010) with a few modifications. Briefly, 3 mL of digestion sample was mixed with 10 mL of 2:1 chloroform:methanol. The mixture was vortexed for 2 min and kept under slight shaking for 1 h under refrigerated conditions. The sample sat overnight to allow for the separation of layers. The chloroform layer was collected and dried under N_2 at 45°C. Heptadecanoic acid was used as the internal standard.

Some of the dried sample was weighed into a vial with 2 mL BCl_3-methanol and heated at 60°C for 10 m to methylate. After cooling, 1 mL of water and 1 mL of hexane were added, and the hexane layer was collected after separation. Anhydrous sodium sulfate was added, and the sample was run through the GC.

Gas Chromatography (GC)

The fatty acids methyl esters (FAMEs) were determined using a gas chromatograph (GC; 6890 Series; Hewlett Packard; Wilmington, DE, USA). A 50 m x 0.25 mm i.d. column (CP-Sil 88; Agilent Technologies; Santa Clara, CA, USA) and flame ionization detector were used. Helium was the carrier gas. The front inlet temperature was 225°C, and the front detector temperature was 200°C. The oven was initially held at 160°C for 2 min, after which the

temperature was raised to 220°C at 3°C min^{-1} and held for 2 min at the end. 1.0 µL of the sample was manually injected with a split ratio of 10:1. A standard curve (62.5 – 8000 ppm) was constructed using methyl heptadecanoate and $R^2 = 0.99$. Fatty acids were identified based on retention time using a purchased standardized FAME mixture. Samples from two duplicates were analyzed in duplicate. Percent lipolysis was calculated (Equation 2) based on O'Sullivan et al. (2017) and accounted for fatty acids derived from the soybean oil, soy lecithin, and stearic acid.

$$\%\ Lipolysis = \frac{FFA(t)[mol]}{FFA\ available\ [mol]} * 100. \quad (2)$$

Statistical Analysis

All statistical analyses were carried out in JMP Pro 14 software (SAS; Cary, NC, USA). The experimental design was a randomized complete block design arranged as a split-plot in time. For the statistical analysis, samples (SL:SA+WPC, SA+WPC, or Control) and time (0 min, 2 min, 60 min, 120 min, 180 min, 240 min) were treated as fixed factors, and replication was a random factor. $p<0.05$ was considered significant. The mean ± SEM (standard error of the mean) is reported for each treatment.

Results and Discussion

The bigels were able to support their own weight and were off-white, with a slightly yellow hue (Figure 3.1), which is similar to the finding of Bollom et al. (2020).

Figure 3.1. Appearance of SL:SA+WPC (left) and SA+WPC (right) bigels.

Effect of different matrices on lipolysis

The % lipolysis (Figure 3.2) shows that, as expected, free fatty acids (FFAs) increased as the intestinal phase progressed. This steady increase confirms the enzymes' activity and ability to digest lipids. Fatty acids originated from different sources, including soybean oil's triacylglycerols (TAGs), SL (or PL) fatty acids (if applicable), SA (if applicable), and milk fat. Since the different gels were formulated with different oleogelators, they contained different initial fatty acid quantities, hence the difference in initial % lipolysis. The FFAs increased as time progressed and did not appear to increase rapidly and plateau. Between the beginning and end of the intestinal phase, the SL:SA+WPC bigel, SA+WPC bigel, and Control experienced a 1.94, 2.04, and 2.85-fold increase in lipolysis, respectively. Previous work on organogels found that the FFA levels after digestion are influenced by the oil gelator (Ashkar et al., 2019). For example, in a β-sitosterol and γ-oryzanol canola oil oleogel, Ashkar et al. (2019) found an inverse relationship between the structuring agent's concentration and FFA release, with the

unstructured sample releasing the most FFA; this agrees with our findings that showed the control (no structuring agent) underwent the greatest lipolysis. We hypothesize that bigel structuring slows lipid digestion. Other work has suggested that structure influences gel breakdown and, thus, enzymatic access to gel constituents, like fat or probiotics (Guo et al., 2014a, 2014b; McClements et al., 2008; O'Sullivan et al., 2017).

In the bigel, greater lipolysis, and therefore greater structure breakdown, exposed the probiotics to harsh intestinal conditions quicker, and perhaps this is related to their demise (see below). O'Sullivan et al. (2017), who studied an ethylcellulose and canola oil oleogel to deliver β-carotene, found that β-carotene transfer from their oleogel was correlated to oleogel lipolysis. Similarly, as the fat, the gel's main component, in our bigel was broken down, probiotics were likely released. Work by Guo et al. (2014b) found that soft whey protein emulsion gels broke down quicker than hard gels, and large quantities of the dispersed oil droplets were released. Additionally, O'Sullivan et al. (2017) found that their firmest oleogel had the least lipolysis due to the gel's greater resistance to breakdown and, therefore, exposure to lipase activity. In our gels, this suggests that a more developed structure slows enzymatic access to the gel constituents and promotes prolonged probiotic survival. Future work in this area can explore if firmer bigels minimize lipolysis and further enhance probiotic survival.

The most prevalent fatty acids were palmitic, stearic, oleic, linoleic, and linolenic (Table 3.2). Stearic acid's concentration experienced the smallest change over the intestinal phase, likely because it was already free at the beginning of digestion, and the small increase that was observed was likely due to its presence in the soybean oil or milk fat. Oleic acid's concentration increased by the largest factor between the beginning and end of the intestinal phase. This increase was likely because, according to the United States Department of Agriculture (USDA)

FoodData Central, one of the most predominant fatty acids in soybean oil is oleic (USDA, 2019), hence its release is more pronounced. Linoleic acid also increased over digestion, but most dramatically in the SL:SA+WPC bigel and Control. Likewise, according to the USDA FoodData Central, linoleic acid is the most prominent fatty acid in soybean oil and soy lecithin (USDA, 2019).

Table 3.2. Fatty acid contents (mg fatty acid/g ± SEM) in samples before and after the intestinal phase.

	Sample (mg/g)					
	SL:SA+WPC		**SA+WPC**		**Control**	
Fatty acid	Initial	Final	Initial	Final	Initial	Final
C16:0	5.42 ± 0.13	12.58 ± 0.24	1.52 ± 0.01	2.85 ± 0.30	4.63 ± 0.35	12.76 ± 1.05
C18:0	46.30 ± 1.13	55.94 ± 2.45	5.77 ± 0.56	9.43 ± 1.34	2.17 ± 0.19	6.03 ± 0.59
C18:1	6.51 ± 0.21	23.02 ± 1.29	2.24 ± 0.11	5.14 ± 0.63	9.91 ± 0.71	29.53 ± 3.48
C18:2	24.04 ± 0.45	64.52 ± 1.74	5.59 ± 0.77	11.95 ± 1.40	26.73 ± 2.70	71.73 ± 6.95
C18:3	3.61 ± 0.01	9.65 ± 0.22	0.99 ± 0.16	1.86 ± 0.18	4.45 ± 0.48	11.53 ± 1.06

C16:0- Palmitic acid, C18:0- Stearic acid, C18:1- Oleic acid, C18:2- Linoleic acid, C18:3- Linolenic acid

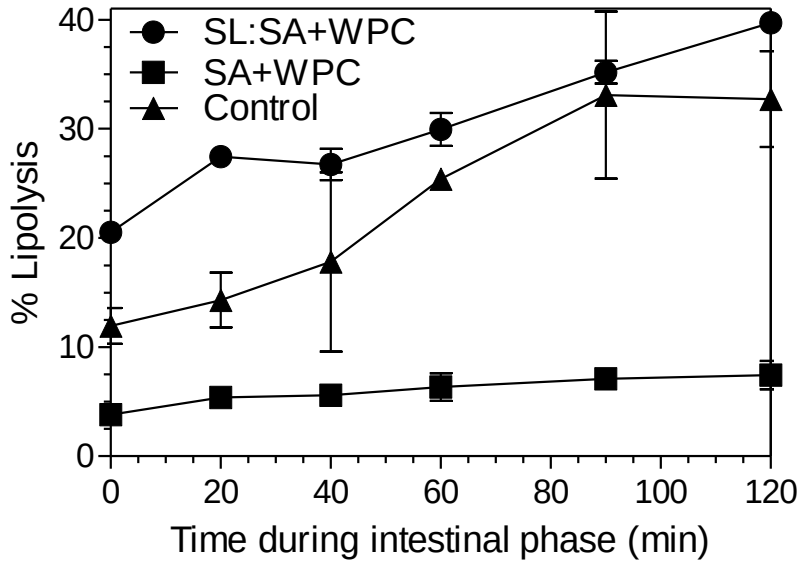

Figure 3.2. % lipolysis of samples at various time points during the intestinal phase. Mean ± SEM are represented.

In vitro tolerance of probiotics

Microbial data were analyzed, and the statistical significance of each factor and interaction was found (Table 3.3).

Table 3.3. P-values of fixed main effects and their interaction.

Dependent Variable	Gel type	Time	Gel-Type x Time
BL survival	0.0005	<0.0001	<0.0001
LA survival	0.2189	<0.0001	0.0001

BL and LA showed similar trends in survival throughout *in vitro* digestion, with decreasing viability as digestion progressed (Figure 3.3). Statistically, there was significant interaction between the gel type and time. The statistical comparison of each time point and gel is found in Tables S3.1-S3.6 and Connecting Letters Reports in Tables S3.7 and S3.8. At the end of digestion, the SL:SA+WPC bigel had 10 and 48% BL and LA viability, respectively; the SA+WPC bigel had 2 and 32% viability, respectively; and the Control had 0% viability for both BL and LA. This finding affirms the ability of both the SL:SA+WPC and SA+WPC bigels to protect probiotics, confirming a hypothesis of this study. Despite the higher probiotic viability found in both bigels relative to the Control, it is worth mentioning that the SA+WPC bigel (PL-free bigel) had slightly lower viability than the SL:SA+WPC bigel (see *Effect of phospholipids* section below).

Homogenization during sample preparation was a lethal step for the BL in the Control. In the Control, no BL survived homogenization, while LA survived. This highlights LA's greater resistance to high shear than BL; LA's initial counts in the Control, however, were lower than in the bigel samples. During homogenization, the Control was completely liquid-like, whereas the

SL:SA+WPC and SA+WPC bigels were higher in viscosity, likely conferring some protection during high shear (gelation mostly occurred during cooling).

Beginning with the salivary phase, both bigels, SL:SA+WPC and SA+WPC, experienced about a 20-55% loss in viability (Figure 3.3). In the Control, the LA lost 1% (as stated above, however, the LA had lower initial counts). Upon reaching the gastric phase (comparing the end of the salivary phase and mid-way through the gastric phase counts), the bigels experienced about a 0-15% loss in viability. In the Control, 99% of LA's viability was lost (putting LA's survival at 0%) upon reaching the gastric phase. Further in the gastric phase (comparing mid-way through the gastric phase and end of the gastric phase counts), the bigels experienced about a 1-15% loss in viability. Similarly, de Andrade et al. (2019), who studied microencapsulated lactic acid bacteria, found that non-protected cells did not survive once exposed to the gastric phase.

The loss in probiotic viability during the gastric phase was likely due to the acidic conditions (pH was adjusted to 3.0), and these results agree with many other studies (de Andrade et al., 2019; Dianawati et al., 2016; Iyer & Kailasapathy, 2005). Dianawati et al. (2016), who studied both *Bifidobacterium animalis* and LA, encapsulated in a spray dried casein-based emulsion, found that their viability decreased over a 2 h exposure to gastric fluid. In one of their analyzed *Bifidobacterium* products, they found 55-60% survival after exposure to gastric conditions for 2 h, which was slightly higher than what we observed in this study (SL:SA+WPC and SA+WPC had 41 and 32% BL viability, respectively). Dianawati et al. (2016) also found that LA had 50-90% survival after a 2 h exposure to gastric conditions. Our results for the SL:SA+WPC and SA+WPC bigels are in line with the aforementioned findings, with 68 and 61% LA survival observed at the end of the gastric phase, respectively. Similarly, Iyer and

Kailasapathy (2005), who studied LA spp. over a 3 h incubation period at low pH (2.0), found that encapsulated probiotic cells had a 1.7 to 3.3 log decrease, whereas non-encapsulated probiotic cells had a 4.2 to 5 log decrease. Liu et al. (2019) suggested that protein can act as a buffering agent. In these bigels, WPC80 in the hydrogel phase could be offering these capabilities and conferring protection to the probiotics during the acidic gastric phase.

Upon reaching the intestinal phase (comparing the end of the gastric phase to mid-way through the intestinal phase counts), both bigels, SL:SA+WPC and SA+WPC, experienced about a 15-35% loss in viability. Further in the intestinal phase (comparing mid-way through the intestinal phase and end of the intestinal phase counts), the bigels experienced about a 0-5% loss in viability. Looking at the intestinal phase as a whole, it appears that probiotic survival declined rapidly at the beginning of the intestinal phase but stabilized by the end.

The decline in probiotic viability upon reaching the intestinal phase was in line with previous research findings (de Andrade et al., 2019; Dianawati et al., 2016; Iyer et al., 2005). Iyer and Kailasapathy (2005), who studied LA encapsulated in sodium alginate, likewise found that encapsulated probiotics declined after exposure to a bile-rich intestinal phase, with a 0.3 to 0.5-log reduction. Free bile acids are believed to damage the membrane, disrupt transmembrane electrical potential, reduce the cell's internal pH, damage DNA, alter protein conformation, and chelate iron and calcium (Kurdi et al., 2006; Urdaneta & Casadesus, 2017). All of these eventually lead to cell death. Bile's primary function in the body is to assist with fat digestion (Begley et al., 2005); thus, the observed trend of decreasing probiotic survival is logical because the main structural component of the bigel, the oleogel emulsion (80% w/w), is composed of fat that can be emulsified/solubilized by the bile. Furthermore, the enzymatic action may degrade bigel structure and make the probiotics more vulnerable to harsh environmental conditions. For

example, the pepsin, added during the gastric phase, may have broken down some of the hydrogel proteins, which provided structural support and protection. GC data, discussed above, support this hypothesis because it shows an increase in lipolysis over digestion.

The kinetics of probiotic survival were assessed over the gastric and intestinal phases for the SL:SA+WPC and SA+WPC bigels (the Control was not assessed as probiotics viability was non-existent). Only the gastric and intestinal phases were assessed because those are where the most lethal factors to the probiotics were found (acid, enzymes, and bile, as described above). Zero, first, and second-order kinetics were examined, and the fit with the highest R^2 was selected. Zero-order kinetics offered the best fit (Table 3.4). Probiotic release, or that of any encapsulated drug, into the external environment is limited by the ability to diffuse out of the gel, structure of the bigel (and its rate of breaking down), the external environment, and interaction between any of these (Fu & Kao, 2010). Thus, to extend probiotic survival even more, one option is to slow the rate of bigel breakdown.

Table 3.4. Zero-order kinetics of probiotic death during gastric and intestinal phases.

	SL:SA+WPC		**SA+WPC**	
	LA	**BL**	**LA**	**BL**
k	632	233	217	369
log(CFU) avg $Population_{start\ of\ gastric}$	5.45	4.66	4.80	4.80
log(CFU) avg $Population_{end\ of\ intestinal}$	5.23	3.96	4.54	3.37
$t_{1/2}$	214	97	220	86
R^2	0.99	0.84	0.99	0.92

Effect of phospholipids on *in vitro* tolerance of probiotics

Overall, the results show there was no significant difference in probiotic viability in the bigels with (SL:SA+WPC) versus those without (SA+WPC) added phospholipids over the entire course of digestion, highlighting the ineffectiveness of PLs for enhancing probiotic survival. Nevertheless, it was observed that higher probiotic viability was found in bigels enriched with

PLs when compared to non-PL bigels (non-significant difference), particularly in the early stages of digestion. For instance, in the case of BL, the SL:SA+WPC (with PLs) bigel had 9-13% greater viability than the SA+WPC bigel during the gastric phase; however, the difference was minimal during the intestinal phase. For LA, the SL:SA+WPC had 7-20% greater viability than the SA+WPC bigels across the gastric and intestinal phases, despite the difference not being significant. Previous work in our lab (unpublished data) demonstrated that a probiotic-enriched OE with PLs had improved viability over a six-week shelf-life study compared to a probiotic OE without PLs.

These findings suggest that PLs may only be effective at increasing viability during stagnant conditions of shelf-life, not the conditions of an *in vitro* digestion system with acid, bile, and enzymes. This may be due to enzymatic action, such as that of pancreatin (added during the intestinal phase), that breaks the PLs down. Liu et al. (2012), who studied soybean-derived PL (mostly phosphatidylcholine) liposomes, found that the liposomes (and therefore PLs) retained their integrity under *in vitro* gastric conditions (low pH and pepsin), but broke down under *in vitro* intestinal conditions (bile and pancreatin). They attributed the breakdown to pancreatin, which often contains phospholipase that can break PLs down and possibly bile, which they say disrupts the PL bilayer on the liposome. Although our study did not involve liposomes, pancreatin was added, which may have broken PLs down, and bile, which may have interfered with probiotic and PL interactions. The combined presence of pancreatin and bile likely minimized any beneficial effects the PLs may have had on probiotic survival. Other work suggested that bile salts and enzymes synergistically interact to hydrolyze PLs (Carey et al., 1983). As described above, the S-layer proteins of lactic acid bacteria are cationic (the *in vitro* system was below their pI, which is >9.4) and, therefore, can interact with anionic parts of lipid

head groups (Smit et al., 2001). However, we hypothesize that if the PL was enzymatically broken down, its charge may be neutralized and thus inhibit binding between the components. We propose that for PLs to have their beneficial effects on probiotics, they must be in their native form.

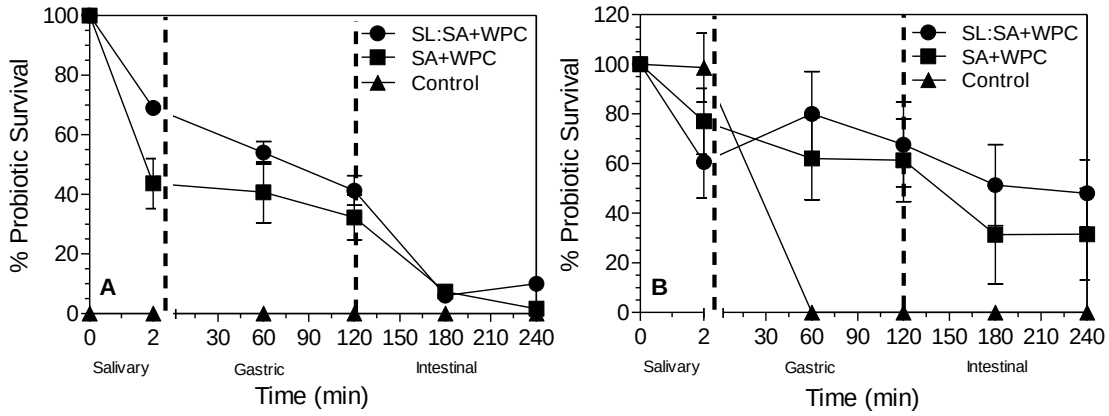

Figure 3.3. *Bifidobacterium lactis* (A) and *Lactobacillus acidophilus* (B) % survival for all three treatments at various digestion time points. Mean ± SEM are represented. For clarity, the connecting letters are shown in Tables S3.7 and S3.8 along with the % survival value.

Conclusions

This work demonstrates that bigels can protect probiotic bacteria in a GI digestion model system. Probiotic survival was observed at the end of digestion, with LA surviving better than BL. When no gelators were included, neither LA nor BL survived the gastric phase. The work also revealed that gelators were critical for BL survival during the homogenization step of bigel preparation because BL did not survive shearing. The effect of PLs on probiotic viability during digestion was not significant, which was attributed to their breakdown by digestive enzymes.

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Supplemental Data

Table S3.1. *Bifidobacterium lactis* p-values when comparing the SL:SA+WPC bigel to the Control.

		SL:SA+WPC bigel					
	Time	**Initial**	**After salivary**	**Mid-way gastric**	**End gastric**	**Mid-way intestinal**	**End intestinal**
Control	Initial	<.0001	<.0001	<.0001	<.0001	0.9995	0.9298
	After salivary	<.0001	<.0001	<.0001	<.0001	0.9995	0.9298
	Mid-way gastric	<.0001	<.0001	<.0001	<.0001	0.9995	0.9298
	End gastric	<.0001	<.0001	<.0001	0.0001	0.9995	0.9298
	Mid-way intestinal	<.0001	<.0001	<.0001	<.0001	0.9997	0.9298
	End intestinal	<.0001	<.0001	<.0001	<.0001	0.9995	0.9515

Table S3.2. *Bifidobacterium lactis* p-values when comparing the SA+WPC bigel to the Control.

		SA+WPC bigel					
	Time	**Initial**	**After salivary**	**Mid-way gastric**	**End gastric**	**Mid-way intestinal**	**End intestinal**
Control	Initial	<.0001	<.0001	<.0001	0.0014	0.9954	1.0000
	After salivary	<.0001	<.0001	<.0001	0.0014	0.9954	1.0000
	Mid-way gastric	<.0001	<.0001	0.0001	0.0014	0.9954	1.0000
	End gastric	<.0001	<.0001	<.0001	0.0025	0.9954	1.0000
	Mid-way intestinal	<.0001	<.0001	<.0001	0.0014	0.9973	1.0000
	End intestinal	<.0001	<.0001	<.0001	0.0014	0.9954	1.0000

Table S3.3. *Bifidobacterium lactis* p-values when comparing the SL:SA+WPC bigel to the SA+WPC bigel.

		SL:SA+WPC bigel					
	Time	**Initial**	**After salivary**	**Mid-way gastric**	**End gastric**	**Mid-way intestinal**	**End intestinal**
SA+WPC bigel	Initial	1.0000	0.0023	<.0001	<.0001	<.0001	<.0001
	After salivary	<.0001	0.0289	0.9118	1.0000	0.0002	0.0008
	Mid-way gastric	<.0001	0.0061	0.7165	1.0000	0.0006	0.0026
	End gastric	<.0001	0.0003	0.0682	0.9797	0.0128	0.0542
	Mid-way intestinal	<.0001	<.0001	<.0001	0.0007	1.0000	1.0000
	End intestinal	<.0001	<.0001	<.0001	<.0001	1.0000	0.9900

Table S3.4. *Lactobacillus acidophilus* p-values when comparing the SL:SA+WPC bigel to the Control.

		SL:SA+WPC bigel					
	Time	**Initial**	**After salivary**	**Mid-way gastric**	**End gastric**	**Mid-way intestinal**	**End intestinal**
Control	Initial	1.0000	0.8621	0.9998	0.9658	0.6087	0.5081
	After salivary	1.0000	0.9112	0.9999	0.9760	0.6491	0.5480
	Mid-way gastric	0.0064	0.2824	0.0653	0.1591	0.5280	0.6289
	End gastric	0.0064	0.2824	0.0502	0.1931	0.5280	0.6289
	Mid-way intestinal	0.0064	0.2824	0.0502	0.1591	0.5799	0.6289
	End intestinal	0.0064	0.2824	0.0502	0.1591	0.5280	0.6772

Table S3.5. *Lactobacillus acidophilus* p-values when comparing the SA+WPC bigel to the Control.

		SA+WPC bigel					
	Time	**Initial**	**After salivary**	**Mid-way gastric**	**End gastric**	**Mid-way intestinal**	**End intestinal**
Control	Initial	1.0000	0.9988	0.8888	0.8758	0.1457	0.4113
	After salivary	1.0000	0.9996	0.9122	0.9009	0.1638	0.4466
	Mid-way gastric	0.0064	0.0673	0.2987	0.2682	0.9737	0.8449
	End gastric	0.0064	0.0673	0.2546	0.3134	0.9737	0.8449
	Mid-way intestinal	0.0064	0.0673	0.2546	0.2682	0.9804	0.8449
	End intestinal	0.0064	0.0673	0.2546	0.2682	0.9737	0.8713

Table S3.6. *Lactobacillus acidophilus* p-values when comparing the SL:SA+WPC bigel to the SA+WPC bigel.

		SL:SA+WPC bigel					
	Time	**Initial**	**After salivary**	**Mid-way gastric**	**End gastric**	**Mid-way intestinal**	**End intestinal**
SA+WPC bigel	Initial	1.0000	0.8621	0.9998	0.9658	0.6087	0.5081
	After salivary	0.9988	1.0000	1.0000	1.0000	0.9961	0.9868
	Mid-way gastric	0.8888	1.0000	1.0000	1.0000	1.0000	1.0000
	End gastric	0.8758	1.0000	0.9999	1.0000	1.0000	1.0000
	Mid-way intestinal	0.1457	0.9853	0.6087	0.9176	0.9999	1.0000
	End intestinal	0.4113	0.9999	0.9182	0.9976	1.0000	1.0000

Table S3.7. % survival with connecting letter(s) based on Tukey's HSD for *Bifidobacterium lactis*.

Time point during digestion	**SL:SA+WPC Bigel**	**SA+WPC Bigel**	**Control**
Initial	100^{A}	100^{A}	0^{E}
After salivary	69^{B}	44^{C}	0^{E}
Mid-way gastric	54^{BC}	41^{C}	0^{E}
End gastric	41^{C}	32^{CD}	0^{E}
Mid-way intestinal	6^{E}	8^{E}	0^{E}
End intestinal	10^{DE}	2^{E}	0^{E}

Table S3.8. % survival with connecting letters based on Tukey's HSD for *Lactobacillus acidophilus*.

Time point during digestion	**SL:SA+WPC Bigel**	**SA+WPC Bigel**	**Control**
Initial	100^{ABC}	100^{AD}	100^{ABCDF}
After salivary	$61^{ABCDEFG}$	77^{ABDE}	99^{ABCDF}
Mid-way gastric	$80^{ABCDEFG}$	$62^{ABCDEFG}$	0^{EG}
End gastric	$68^{ABCDEFG}$	$61^{ABCDEFG}$	0^{EG}
Mid-way intestinal	52^{DEFG}	31^{CFG}	0^{EG}
End intestinal	48^{DEFG}	32^{BCEFG}	0^{EG}

CHAPTER 4. GENERAL CONCLUSION

General Conclusion

A novel, edible bigel was developed for implementation into foods. The bigel was composed of an oleogel emulsion comprised of soybean oil, soy lecithin, stearic acid, and water/milk/probiotics and hydrogel comprised of whey protein concentrate 80 and water.

The first phase of this research focused on understanding the bigel's structure. The nano-, micro-, and macro-structure were analyzed using small angle X-ray scattering, fluorescence microscopy, and rheology, respectively. SAXS revealed that the OE phase retained basic structural units, a reverse micelle from the SL and bilayer from the SA, despite HY addition. The hexagonal array of SL reverse micelles was observed but disappeared at higher HY usage levels. CSLM showed the interaction of phases – specifically o/w, w/o, or bi-continuous. Rheology revealed that an OE's critical strain was improved with HY addition. Additionally, at certain OE:HY ratios, water contents, and protein contents, a bigel can have improved mechanical properties over an OE or HY on their own.

The second phase of this research had two objectives. The first objective was to assess the bigel's ability to protect probiotics during *in vitro* digestion conditions. The second objective was to assess the ability of phospholipids, naturally found in soy lecithin, to augment probiotic survival. These objectives were fulfilled using a standardized *in vitro* digestion method, microbial plating, texture analysis, and gas chromatography. The two probiotics used were *Lactobacillus acidophilus* and *Bifidobacterium lactis*. The bigel was able to successfully protect probiotics during *in vitro* digestion compared to a control with no structure. The *Lactobacillus acidophilus* was more resistant to homogenization conditions during gel preparation and the

digestive tract's harsh conditions. Phospholipids did not have a significant effect, and this is likely because they are broken down during digestion.

Limitations

An unofficial, preliminary sensory panel (conducted by a different graduate student for their research) revealed that the oleogelators (especially stearic acid) released strong flavors. It is unknown whether the bigel created in this study would be acceptable to consumers. Future work may want to consider alternative oleogelators or methods to mask their flavor.

The INFOGEST *in vitro* digestion system used in this study was static, but that is not necessarily representative of actual human digestive tract conditions. Similarly, a limitation of using this system is its many inherent assumptions. For example, the method does not account for whether the system is in the fed or fasted state. *In vivo* studies may want to be considered in the future.

The *in vitro* studies described herein only used one OE:HY ratio, protein content, and water (milk) usage level. As revealed in Chapter 2, the amount of each of these components is important for determining the gel's macroproperties. Thus, the findings described here are only for one particular bigel and are not representative of all possible bigel formulations.

Future Direction

Future researchers may want to consider alternative gelators; specifically, ones that can simultaneously gel. A bigel made using a cold set-WPC80 gel was successfully developed in some preliminary studies, but no significant difference was found between the bigel explored in this study and that one. It should be noted, however, that only one OE:HY ratio, OE water content, OE gelator concentration, and HY protein concentration was explored. Furthermore,

many other variables, such as ionic strength, are very important when making cold set gels, so a bigel made using a cold set-WPC80 gel still holds promise, but more work is necessary to elucidate the important factors.

Future investigators may want to explore a formal shelf-life stability test of the bigels. In Chapter 2 it was shared that the bigels were stable in the refrigerator for at least 5 months. These gels, however, would likely have been stable for many more months. Sterile technique was not used when preparing those gels, and, thus, they became moldy and could no longer be kept; however, if they had been made with aseptic technique, they likely would have been stable for much longer.

Other work should also consider understanding the exact mechanism of interaction between organic and aqueous phases. As was observed in this study, at one particular OE:HY ratio, OE water content, and HY protein content, improved mechanical properties were found. It should be studied why improved mechanical properties were only found at this one particular concentration.

Other investigators may want to consider the addition of an emulsifier, such as Tween 80, because this may change phase behavior and mechanical properties. When selecting an emulsifier, its HLB value and ratio of organic and aqueous phases should be considered.

For probiotic survival, future researchers may want to explore the addition of a prebiotic, such as lactose, to help improve probiotic survival even more. Also, a reducing agent, like cysteine, may be added to help remove oxygen and enhance BL survival. Investigators may also want to explore counting injured cells using a non-selective media.

Finally, future work may want to consider why a hexagonal array, not cubic mesophase, was observed.