The Effects of Lite Salad Dressing Formulation on *Zygosaccharomyces parabailii* Growth and Rheological Behavior

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By

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Authorization to Submit Thesis

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Abstract

Zygosaccharomyces parabailii (*Z. parabailii*) is a common spoilage yeast that is known to grow in high-acid foods such as wine, juices, soda, and salad dressings. The objectives of this work were to 1) reduce the growth of *Z. parabailii* in lite salad dressing through adjusting storage conditions, acidulant type, and stabilizer composition and 2) determine changes in salad dressing rheological properties due to these parameter adjustments. Lite salad dressing formulations were prepared with different combinations of acidulants and/or stabilizers and inoculated to a *Z. parabailii* concentration of 10^4 CFU/mL. Inoculated salad dressings were stored at 4°, 10°, or 25°C. *Z. parabailii* concentration in the dressings was monitored over a 45-day storage period. Formulations were evaluated every 15 days for changes in viscosity and viscoelastic properties. Storage conditions significantly impacted the growth of *Z. parabailii* in the salad dressings. Samples stored at 4°C had downward trends in growth. Storage at 10° or 25°C was unable to inhibit growth of *Z. parabailii*. The type of acidulant used had varied results on *Z. parabailii* growth depending on storage temperature. Overall, the formulation that had acetic acid as the sole acidulant showed the greatest inhibition of *Z. parabailii* growth at all storage temperatures. Acidulants, either alone or in combination with other acids, had different effects on the viscosity and viscoelastic properties of the salad dressings. When polysaccharide-based stabilizers were replaced by milk protein-based stabilizer (milk protein isolate, whey protein isolate, and micellar casein) and acidified by acetic acid, *Z. parabailii* growth was significantly inhibited at all storage temperatures. The use of protein stabilizers also had a significant effect on the linear and nonlinear viscoelastic properties of dressings. In general, lite salad dressings with milk protein-based stabilizers acidified with acetic acid, and stored in temperatures at 4°C had the best inhibitory effect on *Z. parabailii* growth.

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Dedication

I would like to dedicate this to my loving wife and family, whom have been very supportive of me and my decision to pursue a higher education.

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CHAPTER 1: INTRODUCTION

Lite dairy-based salad dressings are oil-in-water emulsions commonly used as a condiment. Dressings sold to consumers can either be a shelf-stable product or a refrigerated product. Many salad dressings have preventative measures to reduce or prevent spoilage and pathogenic microorganism growth to extend their shelf life. Altering salad dressing pH is usually preferred to thermal processing for reducing the microbial load in the dressing. Dressings often include microbial inhibiters in their formulation, such as acids to lower the pH below 4.6 and antimicrobial compounds to inactivate microorganisms directly. The most common form of antimicrobials used in salad dressings are weak organic acids for pH reduction below 4.6. This pH is adequate for preventing the growth of target pathogens such as *Clostridium botulinum*, but does not prevent the growth of a select group of acid-tolerant spoilage microorganisms.

Food is an ideal environment to grow diverse types of microbial flora due to composition and bioavailability of nutrients within the food matrix. Some of these nutrients include amino acids, carbohydrates, and vitamins (Meyer 1989). Microorganisms have beneficial properties in yogurts, cheeses, and other fermented products. In salad dressing, microbial growth is generally unwanted and is considered to be a contaminant. If allowed to grow, these microbial contaminants can be harmful to human health or destructive to food product quality (Smittle 1982). Spoilage microorganisms, compared to pathogenic microorganisms are relatively harmless to humans but produce fermentation products that can cause unwanted sensory attributes in food, e.g. sour, bitter, and ethanolic flavors. This spoilage can be a financial burden on food manufacturers due to decrease product quality and shelf-life (Yang 2003).

Spoilage microorganisms such as bacteria, yeast, and molds, have been known to cause unappealing changes in food appearance, aroma, flavor, and texture. To prevent growth of these spoilage microorganisms, most foods are preserved through pasteurization, salting, or pH reduction. However, some spoilage organisms have developed tolerances to salt, antimicrobials, and/or low pH. Notable spoilage organisms found to grow in salad dressings, soda, wines, and other high pH foods are those found in the *Zygosaccharomyces* genus (Thomas 1985). Because *Zygosaccharomyces sp.* have high tolerance to low-pH environments (Stratford 2013), traditional salad dressing formulations provide an environment for *Zygosaccharomyces sp.* to grow with little competition.

Salad dressings are oil-in-water emulsions made using at least 30% w/w vegetable oil, 4% w/w egg yolk, and enough vinegar to lower the pH below 4.6 (US, FDA, 21CFR169.150 2017). A dressing formulation containing 30% fat yields a high-calorie product, which may cause concern for consumers trying to reduce fat and calorie intake. Thus, health concerns prompt consumers to switch from foods that are high in calories from fat to lower-calorie alternatives. Manufacturers have been looking for ways to reduce the amount of fat in salad dressings to meet this consumer demand. The most common way to reduce fat in salad dressings is through the replacement of vegetable oil with water, starch, gums, and sugar. However, the absence of fat in the dressing can lead to changes in mouthfeel, flavor, and texture (Peressini 1998). To increase palatability of reduced-fat dressings, stabilizers are added to the formulation to mimic the mouthfeel and texture of fat in a full-fat dressing. Acids can also be added to the dressing to compensate for flavor loss. Adjusting the formulation of the dressing in this manner can lead to improved consumer acceptance but may also cause dramatic changes to the dressing structure and antimicrobial capabilities (Turgeon 1996).

Zygosaccharomyces parabailii (*Z. parabailii*) is a *Zygosaccharomyces* strain found in salad dressing products (Suh 2013). When *Z. parabailii* grows in salad dressing, it produces gas and sour flavors through fermentation (Thomas 1985). The gas production can cause packaging failures by pressure-induced expansion. *Z. parabailii* growth is hard to control due to its high acid and antimicrobial tolerances (Stratford 2013). The main methods to prevent microbial growth in salad dressing is lowering the pH below 4.6 and using antimicrobials. However, low-pH salad dressing formulations create an environment where *Z. parabailii* has little to no competition for growth. The ability to grow in a low-pH environment where other organisms cannot is the main contributing factor to the prevalence of *Z. parabailii*-induced spoilage in salad dressing. This study aims to reduce *Z. parabailii* growth by changing both formulation and storage conditions.

Changing the formulations of a product can result in the changes in the physical properties (Franco 1997); changes in salad dressing physical and mechanical properties can result in altered consumer texture perceptions (Liu 2007). Thus, it is important to be aware of how formulation changes affect both *Z. parabailii* growth and physical properties.

The overall goal of this study was to determine the effects of milk protein stabilizers, different acidulants, and storage conditions on *Z. parabailii* growth and rheological behaviors in lite salad dressing formulations. The information in this study can be used as a guide to increase salad dressing shelf life by reducing the incidence of spoilage while maintaining dressing rheological properties.

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CHAPTER 2: LITERATURE REVIEW

Consumers concerned with health complications related to a high-fat diet are looking for ways to reduce fat consumption. This prompts some consumers to make dietary substitutions such as lite salad dressings for full-fat salad dressings. However, consumers want the same quality in a lite salad dressing that a full fat dressing provides. Thus, formulations for lite salad dressings should maintain acceptable textures and flavors. It should also inhibit growth of spoilage microorganisms, specifically *Zygosaccharomyces parabailii*, a yeast that has caused significant revenue loss for salad dressing manufacturers through product loss. This review will explore 1) salad dressing ingredients and their contribution to the overall dressing matrix, 2) *Zygosaccharomyces parabailii* and its growth factors, and 3) the effects of dressing formulations on viscosity and viscoelastic behaviors of salad dressings.

2.1 Standard of identity for salad dressings

Salad dressings are oil-in-water emulsions that meet the specified criteria set by the Food and Drug Administration (FDA) (Riscardo 2003). According to the FDA, salad dressings are intended to be used as a condiment (Manni 2017; US, FDA, 21CFR169.150 2017). Salad dressings formulations are intended to be shelf stable at 25°C, but some manufacturers do produce salad dressings that are intended to be stored at refrigeration temperatures.

Per the United States Food and Drug Administration, for a condiment to be labeled as "salad dressing," the formulation must contain water, oil, an acidifying ingredient, starch, and egg yolk (Liu, 2007; US, FDA, 21CFR169.150 2017). Each of the previously mentioned ingredient plays a critical role in the development of salad dressing texture, mouthfeel, and

flavor. Oil is used for emulsion development, texture, and flavor (Wendin 2001). Salad dressing formulations contain at least 30% w/w oil derived from a vegetable source (US, FDA, 21CFR169.150 2017). Egg yolks are primarily used as an emulsifier in the product and comprise at least 4% w/w of the formulation (US, FDA, 21CFR169.150 2017). Egg yolk contains amphiphilic lecithin, an emulsifier. The lecithin arranges itself at the oil/water interface to form a steric barrier around the oil droplets, preventing coalescence. (Peressini 1998). Egg yolk also contributes to salad dressing flavor (Carrillo 1988). The incorporation of starch increases dressing viscosity and further helps stabilize dressing formulations. After the salad dressing is stabilized, acidulants are added to the dressing to reduce the pH below 4.5 to prevent growth of pathogens and spoilage microorganisms (Smittle 2000). The acidifying ingredient(s) must include a vinegar (acetic acid) but can also contain other acidulants verified for use in food such as citric, gluconic, and lactic acid (US, FDA, 21CFR169.150 2017).

In addition to the previously discussed ingredients, salad dressing formulations may also include ingredients to increase stability and promote flavor development. These ingredients include hydrocolloids, salt, spices, and sequestrants. Hydrocolloids increase the shelf life of the salad dressing by giving the emulsion more colloidal stability (Abedinzadeh 2016). The most common hydrocolloids used to stabilize dressings are gum arabic, xanthan, and carboxymethylcellulose (Parakevopoulou 2005). Hydrocolloids such as gaur gum and xanthan increase the water-holding capacity of the aqueous phase stabilizing the oil droplets preventing coalescence (Santiago 2002). Salt, spices (e.g. mustard, black pepper), and ethylenediaminetetraacetic acid (EDTA) are often included in formulations for their antimicrobial properties. Further, salt and spices are added to the salad dressing for flavor and to create a verity of products (e.g. ranch, Caesar, French).

A dressing labeled as full fat usually consist of 70-80% w/w fat (Smittle 2000). To be called “lite,” the product must have 50% less fat or 33% fewer total calories from the original formulation (US, FDA, 21CFR101.56 2017). Further, more than 50% of the calories removed from the original formulation must be from removal of fat (US, FDA, 21CFR101.56 2017). Fat substitutes in salad dressings include carbohydrates (gums, modified starches, and polydextrose), proteins (modified whey proteins), or fat-based replacers (Olestra™, Salatrim®) (Ma 2013). Water can also be added to salad dressing to further reduce caloric and fat content, but an increase in water content can reduce the stability of the dressing, allowing for easier separation of the water and oil phases. To compensate for the loss in stability, more starch and hydrocolloids can be added to the finished formulation (Peressini 1998).

2.2 Stabilizers and emulsifying agents used in salad dressings

A well-stabilized salad dressing prevents the separation of the oil and water phases through stabilizers and emulsifying agents (Santiago 2002). The traditional emulsifying agent in salad dressing is egg yolk. The lipoproteins and phospholipids naturally found in egg yolk form a complex coating on the outside edge of oil droplet. The hydrophobic end of the lipoproteins and phospholipids stabilizes the oil droplets and the hydrophilic end of the lipoproteins and phospholipids prevents the oil droplets from flocculating (Riscardo 2003). Egg yolks can be used in combination with other emulsifiers to increase emulsion stability and reduce production costs (Parker 1995). These other emulsifiers can be low molecular weight surfactants such as Tween 20 or derived from vegetable, dairy, or meat sources. The viscosity, viscoelasticity, oil droplet size and oil droplet stability of the salad dressing are influenced by the blend of emulsifiers used in the salad dressing (Franco 1997). Generally, the

salad dressing stability increases as the concentration of emulsifying agents increases (Parker 1995).

Other factors that can affect the stability of the oil-in water-emulsion in salad dressings are temperature, pH, salt content, droplet size, droplet dispersion, and hydrophilic–lipophilic balance. If these factors are not balanced correctly, the emulsion can destabilize (Meyer, 1989). Mechanisms of destabilization include droplet dissolution/growth, Ostwald ripening, coalescence, aggregation, and creaming (Walstra 2002). Ostwald ripening is a phenomenon during which a smaller oil droplet defuses into a larger oil droplet over time (Walstra 2002). This leads to slow coarsening of the dressing (Walstra 2002). Coalescence happens when two separate oil droplets become one after their outer barrier breaks and reforms around them. This phenomenon can lead to phase separation. Aggregation is agglomeration of oil droplets which are held together by van der Waals forces or depletion interactions (Walstra 2002). Creaming in salad dressing is caused by density differences between the water and oil phases that allow oil, the less dense material, to rise to the surface. During creaming, larger droplets will rise to the surface faster than smaller droplets (Santiago, 2002).

One of the ways to reduce the rate of destabilization in salad dressing is by increasing the viscosity of salad dressings. This relationship between viscosity and stability can be explained using Stokes' law (Eqn. 1), which calculates the velocity of a sphere through a viscous liquid (Walstra 2002). In Eqn. 1, frictional force, dynamic viscosity, sphere radius, and flow viscosity are represented by F_d, η, R, and ν respectively.

$$F_d = 6\pi\eta R\nu \quad (1)$$

The most common way to increase dressing viscosity is by using stabilizers such as polysaccharides (Wendin 2001). Addition of polysaccharides to salad dressings increases their stability (Franco 1997). Common stabilizers used in salad dressings include xanthan gum, galactomannans, native or modified starches (corn or wheat), propylene glycol alginate, pectin, and carboxymethylcellulose (Liu, 2007). Starch is required to make salad dressing per the CFR (21CFR169.150) and is commonly used in combination with xanthan gum as an emulsifying and suspending agent, and to increase oil droplet dispersion. (Garcıa-Ochoa, 2000). Xanthan gum is an exopolysaccharide produced by the bacteria *Xanthomonas campestris* with a primary backbone consisting of D-glucose, D-mannose, D-glucuronic acid, pyruvate, and acetate (Garcıa-Ochoa 2000). The concentration of xanthan gum needed to stabilize a full fat salad dressing is 0.1 to 0.5% w/w (Parker, 1995); the optimal concentration to stabilize lite salad dressing is approximately 0.5% w/w (Abedinzadeh 2016).

2.3 Acidulants used in salad dressing formulations

Acidulants inhibit the growth of pathogens and spoilage microorganisms in salad dressings and can affect the flavor of the dressing (Smittle 2000). The most common acidulants used are organic acids such as citric, acetic, benzoic, and malic acid (Meyer, 1989). Lite salad dressings usually have a pH between 3.2-4.2, which prevents the growth of pathogens and most spoilage microorganisms by having a low pKa (Smittle 1982). The pH can be lowered further for better inhibition of spoilage microorganisms but the additional acidulant may result in unfavorable sour flavors (Smittle 2000). Low pH inhibits microbial growth by slowing down metabolic pathways and fermentation processes (Rodrigues 2012). The antimicrobial potential of an acid is determined by is chemical properties such as hydrophobicity, volatility, and pK_a (Mira 2010). For example, acids can diffuse through the

cell membrane if the internal pH of the cell is greater than the pK_a of the acid (Guerreiro 2012). When weak undissociated acids diffuse through the cell membrane, they dissociate into H^+ and their conjugate base (Guerreiro 2012). The H^+ and conjugate base accumulate inside the cytoplasm; they are no longer able to pass through the cell membrane because they are charged. The accumulation of H+ ions is toxic to the cell, leading to eventual cell death (Mira, 2010).

2.4 Commercial production of salad dressing

Commercial production of salad dressing involves 1) bulk scaling and ingredient addition, 2) mixing, and 3) packaging; the full process is shown in Figure 2.1. During bulk scaling and ingredient addition, dry and wet goods such as spices, flavors, and stabilizers are separately weighed as designated by a formulation (Riscardo 2003). At this point in the process, stabilizers that do not dissolve easily into water are dissociated into an oil slurry to ensure complete dispersal of hydrocolloids during the mixing process (Riscardo 2003). Next, water-soluble ingredients, excluding acidulants and occasionally spices, are mixed in a commercial mixer with water at high shear. After the water-soluble ingredients are mixed, the oils and oil slurries are slowly added into the mix to create an oil-in-water emulsion. A rotor-stator turbine or colloidal mill can be used at this point to decrease the oil droplet size to help stabilize the emulsion. After the emulsion is formed, the acidulant is added to the salad dressing to achieve the target pH. The resulting product is packaged and stored. Salad dressings do not typically undergo a heat treatment step because the typical salad dressing formulation has a pKa<4.75 which is sufficient for stopping most pathogens and spoilage organisms from growing (Smittle 2000). Shelf-stable dressings are aseptically packaged and

sealed; refrigerated dressings are usually not aseptically packaged. Dressings may be packaged in an atmosphere where air is replaced in whole or in part by carbon dioxide or nitrogen (21CFR169.150). If the product is intended to be refrigerated, the dressing is packaged and stored at 4°C. Refrigerated dressings tend to contain ingredients that will oxidize if stored at room temperature.

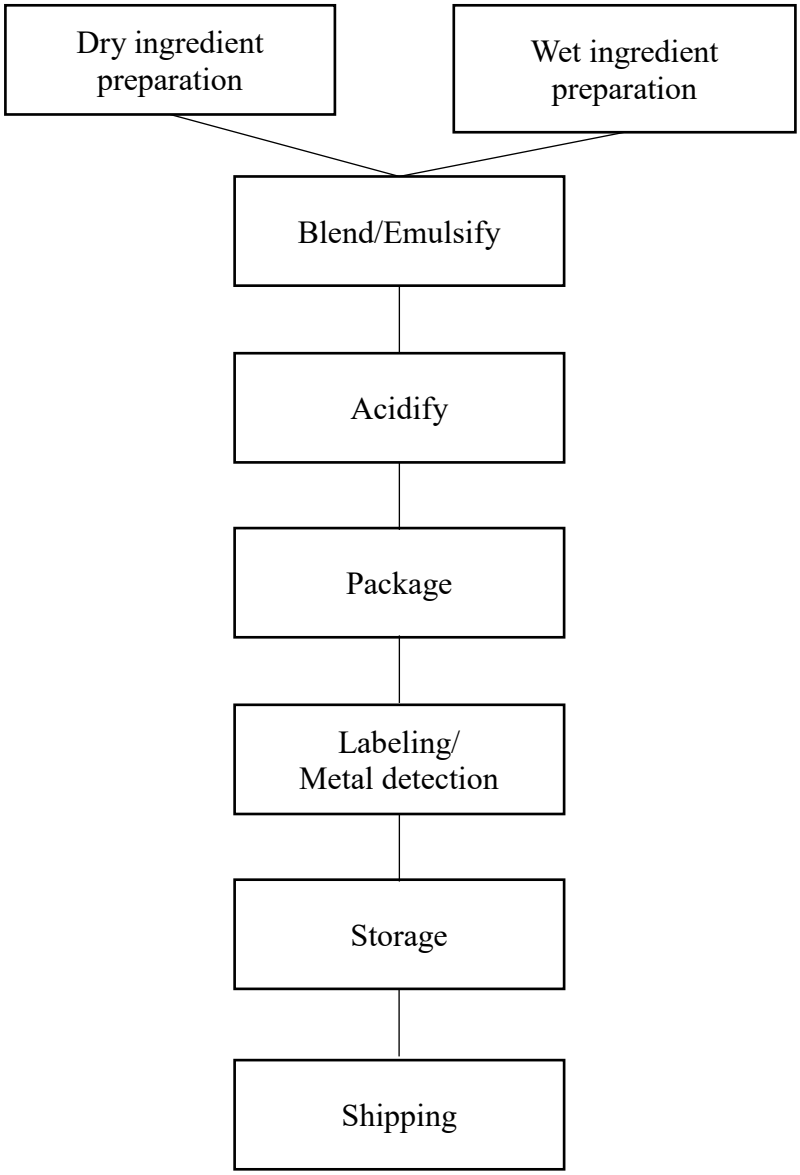

Figure 2.1 Flow diagram for commercial production of salad dressing products

2.5 *Zygosaccharomyces parabailii* distinction from other *Zygosaccharomyces* sp.

Zygosaccharomyces sp. is a group of yeasts that cause spoilage in food products. The two most common of these are *Zygosaccharomyces ruxii* and *Zygosaccharomyces bailii* (*Z. bailii*) (Thomas 1985). This review will focus mainly on *Z. bailii* and *Z. bailii*-like species such as *Zygosaccharomyces parabailii* (*Z. parabailii)* as they are predominant source of spoilage in the salad dressing industry (Smittle 1982). *Z. bailii* is an acid-tolerant organism that can cause product spoilage due to packaging damage, discoloration, and production of yeasty smells and other off-flavors caused by alcohol production (Thomas 1985). *Z. bailii* was known as *Saccharomyces bailii* until the development of better detection methods to further classify yeasts were developed (Stratford 2013). Like *Saccharomyces bailii, Z. bailii* has also been further analyzed and additional species have been distinguished from *Z. bailii*. One of these species of note is *Z. parabailii,* as it has recently been linked to salad dressing spoilage outbreaks in the United States and United Kingdom (Suh 2013).

Zygosaccharomyces has been isolated from contaminated dressing bottles worldwide (Meyer, 1989). Contamination in dressings can be attributed to either the *bailii* or *parabailii* strains. *Z. parabailii* and *Z. bailii* are difficult to distinguish from each other by conventional physiological tests or microscopy (Suh 2013). The ability to use certain metabolites varies within strains of both species of yeast, making it difficult to categorize them through physiological tests. The two species of yeasts also look identical underneath a microscope (Suh 2013). Thus, *Z. parabailii* was named as such due to its phylogenetic likeness to *Z. bailii* (Suh 2013). *Z. bailii* and *Z. parabailii* mainly differ in terms of their genetic markers (Suh, 2013). As of the time of publication, no studies have been published on the effects of the genetic difference between *Z. parabailii* from *Z. bailii*.

Z. parabailii grows in shelf-stable foods prepared with acids as well as products high in fermentable sugars. Foods that can contain *Z. parabailii* include fruit juice, soda, grape must, wines, salad dressings, pickles, vinegar, and tomato sauces (Stratford 2013). The main sources of *Z. parabailii* are fermented or dehydrated fruits, tree exudates, and parts of the sugar refinement process (Kurtzman 1971). These sources are a great medium for *Z. parabailii* growth due in part to the presence of fructose, the preferred sugar for fermentation by *Z. parabailii*, and limited competition for growth.

Table 2.1 *Z. parabailii* fermentation table comparing sugars that can be fermented (+) to those that cannot be fermented (-)

Sugar	Fermentation (+/-)
Glucose	+
Fructose	+
Galactose	-
Sucrose	-
Maltose	-
Lactose	-
Raffinose	-
Starch	-

2.5.1 Growth factors for *Z. parabailii*

Z. parabailii is a slow-growing yeast that can ferment simple sugars such as fructose and glucose (Table 2.1). in both aerobic and anaerobic conditions (Sousa-Dias 1996). *Z. parabailii* is unusual as it favors fructose for fermentation; most microorganism prefer

glucose for fermentation (Guerreriro 2012). However, *Z. parabailii* has a greater ability to bring fructose into the cell than glucose, so will preferentially ferment fructose.

The intrinsic factors that affect the growth of microorganisms are pH, redox potential, water activity, and presence of antimicrobials. *Z. parabailii* has high tolerance to the ranges of these intrinsic factors that typically inhibit the growth of microorganisms. For example, *Z. parabailii* has resistance to weak acids as well as sulfur dioxide (Guerreiro 2012). It has been reported to grow in wines with an ethanol concentration as high as 15% v/v (Thomas 1985). *Z. parabailii* has also been reported to grow in pH between 2.0-7.0 and water activity between 0.8-0.99 (Guerreiro 2012).

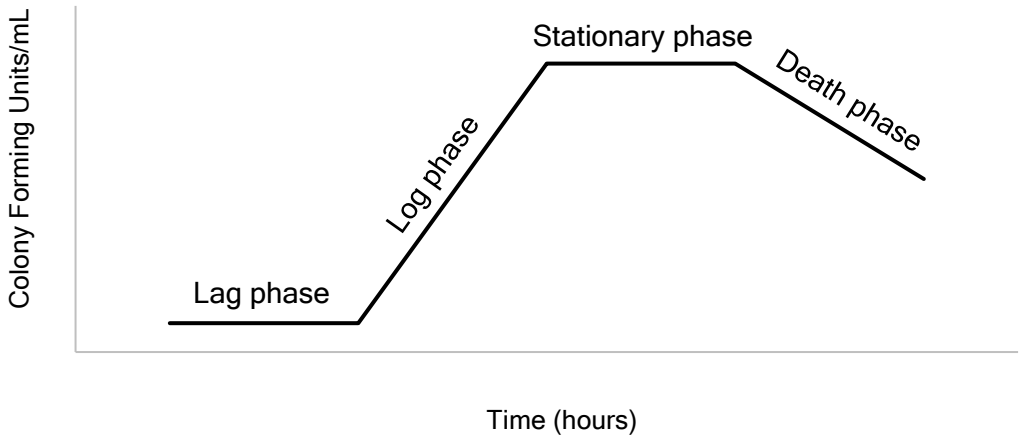

Figure 2.2 Example of a growth curve for microbes under ideal conditions

The growth curve of *Z. parabailii* follows the typical microbial growth curve (Montville 2007). The growth curve contains four phases: lag, log, stationary, and death phase (Figure 2.2). The lag phase is the time needed for the organism to adapt to its environment and start replicating. Cell growth is at equilibrium with cell death until they aclimate to their

environment (Montville 2007). After the organism has aclimated to its environment, it enters the log phase and starts to grow at an exponential rate (Montville 2007). During the log phase, the amount of time it takes for a population of cells to double is called the doubling time. The doubling time of *Z. parabailii* is approximantly 3 hr. The log phase ends when cell death is equal to the rate at which new cells are formed. This phase is known as the stationary phase (Montville 2007). The final phase, death phase, occures when rate of cell death is greater than the rate of creation of new cells. The death phase is usually caused by a lack of bioavailabile nutrients (Montville 2007).

2.5.2 Morphology of *Z. parabailii*

On yeast mold agar over a period of 3 to 7 days, *Z. parabailii* will grow into smooth, round, convex, white colonies 2-3 mm in diameter. The cells in the colonies are ellipsoidal in shape and are between 2.0-4.0µm wide and 2.5 x 7.5µm long (Stratford 2013). Cells can be seen in a single unit, pairs, or small clusters. *Z. parabailii* reproduce asexually through multilateral budding. The yeast will also form ascospores, or sexual spores for reproduction formed by meiosis in the ascus, through conjugation with an independent cell or a bud to form to an ascus (Figure 2.3) (Kutzman 2011). There is no clear distinction between *Saccharomyces, Torulaspora,* or *Z. parabailii* based on microscopy. To distinguish between the different yeast types, rRNA- or DNA-based methods are required (Kurtzman, 2006).

Z. parabailii has a high tolerance to weak organic acids such as acetic acid (Guerreiro 2012). The mechanism behind these tolerances has been attributed to the ability of *Z. parabailii* to use acetic acid and/or other acids as a carbon source for the cell. In the case of acetic acid, *Z. parabailii* catabolizes acetate, the dissociated form of acetic acid, through the

TCA cycle (Rodrigues 2012). Yeasts like *Saccharomyces cerevisiae* are unable to catabolize acetate; *Saccharomyces cerevisiae* acetate catabolism is repressed by glucose (Warth 1977).

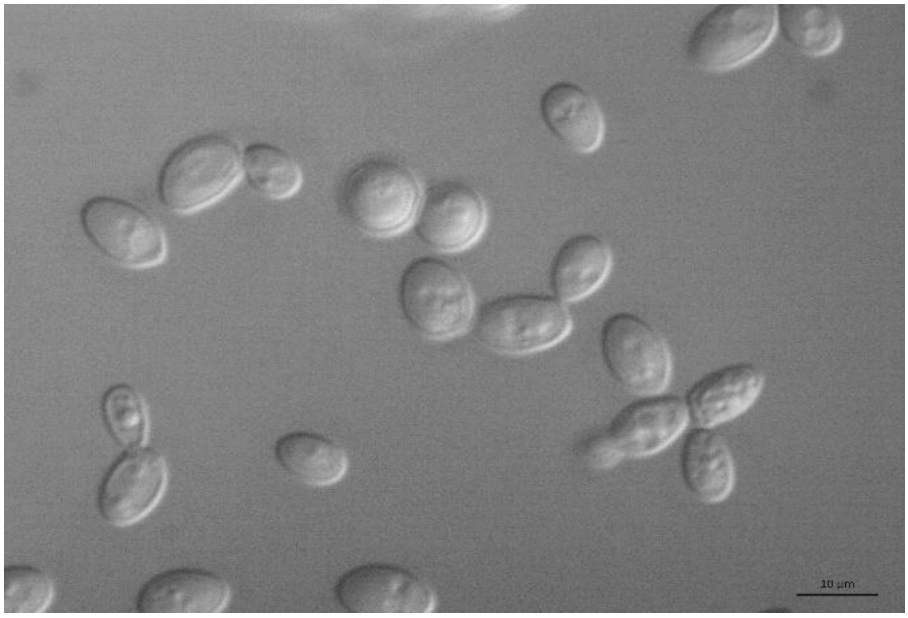

Figure 2.3 *Zygosaccharomyces parabailii* at 100x taken with a Zeiss bright field microscope (Gottingen, Germany). Yeast budding circled in red.

This ability of *Z. parabailii* to catabolize acetate allows it to reduce osmotic pressure buildup inside the cell. As for the H^+ ions remaining from the dissociated acetic acid, *Z. parabailii* has ATPase pumps to remove H^+ ions and maintain cell pH (Guerreiro 2012). Startford et al. 2013 showed that acetic acid alone or in combination with lactic or benzoic acid may stimulate more alcoholic fermentation of glucose for *Z. parabailii*. Additionally, if *Z. parabailii* has been grown in harsh osmotic conditions, the resulting cell will have an increased resistance to mitochondrial oxidative stress through the development of faster metabolic pathways (Guerreiro 2012).

2.5.3 Transport and intercellular metabolism of fructose

As previously stated, *Z. parabailii* prefers fructose as a nutrient source over glucose (Sousa-Dias 1996). When *Z. parabailii* is grown in media with an elevated level of fructose, it shows different metabolic outputs as compared to similar yeasts such as *Saccharomyces cerevisiae*. For example, *Z. parabailii* produces ethanol at a higher rate in a fructose-based medium than in a glucose-based medium (Sousa-Dias 1996). This preference for fructose may partly be due to *Z. parabailii* having separate intermembrane transporters for fructose and glucose. However, fructose can compete for use of the glucose membrane transporter (Rodrigues 2012). If fructose is present, the high affinity for fructose transport will decrease the amount of glucose brought through the cell membrane. On the other hand, if fructose is bound to glucose to make sucrose, *Z. parabailii* cannot utilize the sucrose as a sole source of carbon due to its affinity for pure fructose (Yang 2003). *Z. parabailii* grown in sucrose-based mediums requires 2-4 weeks for *Z. parabailii* to hydrolyze sucrose. This causes the lag phase *Z. parabailii* to be extended for 2-3 months before it can proliferate. (Yang 2003).

2.5.4 Media based identifications of *Z. parabailii*

Although *Z. parabailii* has a high affinity for fructose, glucose-containing media is the established standard for counting yeast (Kurtzman 2011). Media commonly used for *Z. parabailii* enumeration include Sabouraud medium, malt extract agar (MEA), tryptone glucose yeast extract agar (TGYE), and yeast glucose chloramphenicol agar (YGC) (Thomas, 1985). To detect acid-resistant yeasts like *Z. parabailii*, an acidified media is recommended, such as MEA or TGYE with 0.5% (v/v) acetic acid added (Zuehlke 2008). *Z. parabailii* optimal growth conditions after plating are at 25 °C with access to oxygen for a period of 4-7 days (Suh, 2013). When using agar media for plate counting yeasts, the surface spreading

technique is recommended over the pour plate method. In spread plating, a thin layer of microorganisms is applied to the top of a set agar plate. The pour plating technique involves pouring a cool but molten agar over an aliquot (usually 1 mL) of an inoculated broth. Spread plating gives a better recovery of cells with lower dilution errors (Kurtzman 2011).

2.5.5 Potential risk associated with *Z. parabailii* to humans

There are three major grouping of spoilage yeasts. Group 1 yeasts are categorized as preservative resistant, osmotolerant, and highly fermentative. These spoilage organisms will cause excessive gas to form in containers, which can lead to rupture of product and possible physical injury. Group 2 yeasts grow due to food handling errors such as poor sanitation or cross contamination. This group also lacks the preservative resistances that Group 1 displays. Group 3 comprises mainly harmless yeasts that are usually used as an indicator of hygienic conditions. Group 3 yeasts do not cause flavor changes or create gas in food. *Z. parabailii* is classified as a Group 1 yeast because of its high fermentation rates and acid resistance (Stratford 2013). It does not cause illness, but its fermentation has been known to create minor injuries (Grinbaum 1994). Any danger that is caused by *Z. parabailii* is not the microbe itself, but the high pressure developed in packaging through gas production during fermentation. The pressure can cause the packaging to explode, creating a potential hazard (Grinbaum 1994).

2.6 Rheology

Quality parameters of lite salad dressings can be evaluated through the use of rheology. Rheology is the study of material flow and deformation. Rotational, small-strain, and large-strain data collected from rheological tests can help explain perceived textural differences and

distinguish flow and viscoelastic behaviors of different food products (Liu 2007). Small strain and rotational tests can be used to observe viscosity, yield stress, dynamic, and viscoelastic properties (Steffe 1996, Wendin 2001). Large-strain tests can be used to differentiate samples that share similar small-strain behaviors but exhibit differing sensory attributes (Melito 2013). By matching the mechanical properties, reduced-fat products should have similar textures to full-fat products (Liu 2007). Rheological testing can also be used to evaluate food structural integrity during a long shelf life study (De-Cássia 2009).

2.6.1 Steady shear tests

Steady shear rheology is commonly used to evaluate the quality of salad dressings (Ma, 2013). Shear rate sweeps allow for understanding of viscosity profiles, yields stresses, and time-dependent shear behaviors (Diftis 2005). Most manufacturers use a Brookfield viscometer for rapid single-point viscosity measurements, giving limited empirical data about viscosity profiles. Although the information it provides is limited, the Brookfield offers an inexpensive alternative to a rheometer, a tool that can perform a wide variety of rheological tests. Rheometers yield more fundamental information about the mechanical behavior of the product as compared to a Brookfield. Salad dressings can be stored and used at different temperatures; viscosity is inversely related to temperature (Steffe 1996). Thus, a refrigerated salad dressing's viscosity will be greater than a dressing stored on the shelf. A rheometer can be used to determine the degree at which viscosity changes in these situations. Another small advantage that a rheometer has over a Brookfield is the amounts of sample required to test viscosity of a sample. The sample size required to test on a rheometer is significantly less than the sample sized need for a Brookfield viscometer.

Shear rate sweeps are rotational tests that can be used to calculate the viscosity of a sample over a range of different shear rates. A shear rate sweep imparts a shear rate to a sample and gives a shear stress response. Shear stress can be converted into apparent viscosity (Eqn. 2) (Franco, 1997). Viscosity is the resistance to flow under a shear stress where apparent viscosity(η) is equal to shear stress (σ) divided by shear rate (γ).

$$\eta = \frac{\sigma}{\gamma} \tag{2}$$

Apparent viscosity can be plotted against shear rate to create a viscosity profile, which can be used to determine a material's shear dependence. Viscosity profiles typically fall into the following behaviors: Newtonian, power law, Bingham plastic, or Herschel–Bulkley (Steffe 1996). A Newtonian fluid shows linearly proportionality between strain rate and shear stress. Power law fluids have shear-dependent viscosity, but no yield stress. A yield stress is the stress required for a material to flow (Steffe 1996).A Bingham Plastic fluid has Newtonian behavior after a yield stress has been overcome. A Herschel-Bulkley fluid has both shear-dependent viscosity and a yield stress.

Salad dressing tend to exhibit Herschel-Bulkley behavior (Chiralt 1992; Abedinzadeh 2016). Salad dressings stabilized with hydrocolloids or starches have a small yield stress, or the amount of applied stress needed before the dressing begins to flow. The yield stress can be affected by the particular stabilizers and emulsifiers in the salad dressings formulation. For example, yield stress increases with increased starch concentration (De Cássia da Fonseca 2009). Some stabilizers such as xanthan gum do not affect the yield stress (Chiralt 1992). Nevertheless, all stabilizers will increase salad dressing viscosity based on their concentration

and molecular weight (Franco 1995). Salad dressing will also thin over time when a constant shear is applied to it, which is called thixotropic behavior (Franco 1997).

2.6.2 Oscillatory dynamic tests

Oscillatory tests measure the viscoelastic properties of materials and are typically performed in the linear viscoelastic region (LVR) (Figure 2.4) (Franco 1997). The LVR is the region where the material responses are independent from the magnitude of applied strain or strain rate. Typically, the LVR is determined using a strain sweep. In a strain sweep, an increasing oscillatory strain is placed on the sample at constant frequency. A strain sweep generates a storage modulus (G´) and loss modulus(G´´), which are indicators of viscoelastic behaviors. G´ relates to the energy stored or the elastic-type behavior, and G´´ relates to energy dissipated or the viscous-type behavior (Steffe 1996). G´ and G´´, are also related through G* (Eqn. 3).

$$\sqrt{(G')^2 + (G'')^2} = G^* \tag{3}$$

G* can be used to calculate the LVR by determining the strain at which G* deviates by greater than a certain percentage, typically by more than 2-10%, of the previous value. Determining the LVR is important for rheological testing because the viscoelastic properties are independent to strain. Testing outside LVR is more complex, as it violates the assumptions of strain-independent properties, and requires specific data processing methods to provide physical meaning to the data. Thus, rheological tests such as frequency sweeps, creep, and stress-relaxation tests are generally performed within the LVR.

Frequency sweeps can be used to evaluate frequency-dependent behaviors. In a frequency sweep, samples are oscillated at a constant strain within the LVR over increasing frequencies.

This can be used to categorize the viscoelastic behaviors of semisolid foods such as salad dressing (Franco, 1997). The viscous response allows the dressing to be poured from the bottle and the elastic response allows the dressing to cling to the salad ingredients. Elastic-dominated behavior versus viscous dominated behavior is determined by the phase angle. A phase angle under 45° indicates elastic-dominant behavior; a phase angle over 45° indicates viscous-dominant behavior. A phase angle of 0° indicates an ideal elastic material and a phase angle of 90° indicates an ideal viscous material (Steffe 1996). Generally, salad dressings with starch or polysaccharides tend to have phase angles > 45° in the LVR for both frequency and strain sweeps, indicating elastic-dominated behaviors (Dolz 2006).

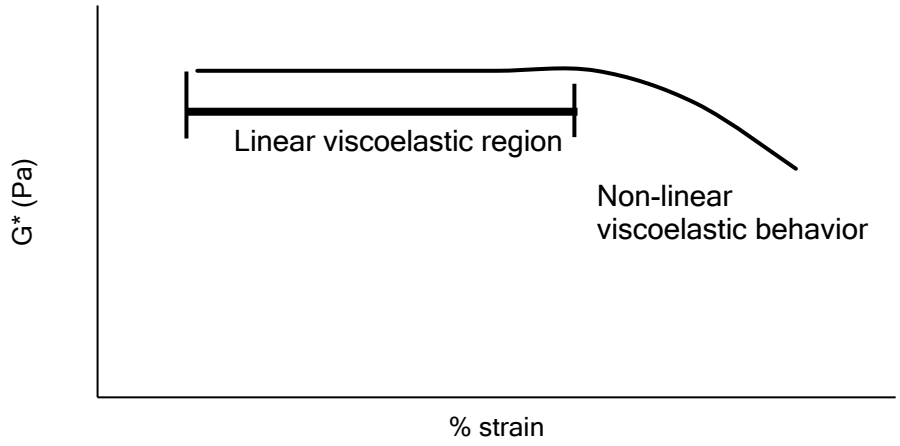

Figure 2.4 Example of linear viscoelastic region for a strain sweep indicated on the graph as the area between the two vertical lines

Many changes in dressing rheological behaviors can be attributed to their formulation (Dolz 2006). The effects of hydrocolloids, starches, and emulsifiers on salad dressing structures can be altered by other ingredients in the formulations such as acid and salt content (Martinez 2007). Some dressing ingredients work synergistically, such as egg yolk and salt; others affect dressing rheological behaviors independently.

2.6.3 Effect of salt on rheology or dressing

Salt concentration can increase salad dressing emulsion stability and also plays a significant role in dressing rheological properties (Martinez 2007). When salt is added to the dressing, the proteins from egg yolk are influenced by the change in the ionic strength of the solution as this change helps the development of calcium bridge networks. This causes the overall dressing stability and viscosity to increase if the salt concentration is below 2.5%, leading to an increase in elastic behaviors (Carrilli 1998; Martínez, 2007). This increased stability has been seen with other protein substitutes such as pea protein and soy proteins (Diftis 2005). However, addition of salt does not influence the size of the oil droplets (De Cássia da Fonseca 2009).

2.6.4 Effect of fat and stabilizers on rheology of dressings

Hydrocolloids are often used in the food industry to help stabilize products. Gum arabic, used for its surface activity, acts as an emulsifier in salad dressings forming a film of high surface shear viscosity (Dolz 2006). Gum arabic solutions, unlike other polysaccharides, have low viscosity and Newtonian-like behaviors below 40% w/w concentration (Parakevopoulou 2005). The gum stabilizes against flocculation and coalescences. However, in salad dressing products the sedimentation becomes more apparent during storage if gum arabic is used a sole stabilizer (Dickinson 2009). Therefore, gaur gum is typically paired with another hydrocolloid such as xanthan gum to improve its stability over time (Parakevopoulou 2005).

Xanthan gum stabilizes salad dressing formulations by increasing the continuous phase viscosity (Parker 1995). The addition of xanthan gum increases the viscosity and viscoelastic properties of the salad dressing (Dolz 2006). Additionally, it helps to further stabilize the

dressing by preventing coalescence and aggregation (Parker 1995). When combined with starch and locust bean gum in salad dressing, xanthan gum has a synergistic effect that increases the elastic behavior of the salad dressing (Dolz 2006).

A commercial full fat dressing compared to a lite dressing can be differentiated through dynamic testing. Lite dressing tends to have a higher elastic response than the full fat dressing (Chiralt 1992). This is partially due to the increase in stabilizers used to increase the stability of the lower fat food matrix. The role that fat plays in consumer acceptance of a product is more related to flavor than it is to rheological behaviors (Charles 2000). Consumer acceptance of salad dressing products has been found to be directly linked to the amount of fat in the dressing. The amount of stabilizer and the type of emulsifiers were not significant for dressing acceptability (Wendin 2001).

2.7 Potential areas for study

2.7.1 *Zygosaccharomyces parabailii* reduction in salad dressings

There have been many studies on *Z. bailii,* but there are few studies on *Z. parabailii*, particularly in salad dressings. Numerous studies have evaluated the growth of *Zygosaccharomyces sp*. for wine applications, but studies about *Zygosaccharomyces sp*. growth in wine may not translate to *Zygosaccharomyces sp*. growth in salad dressing, as wine contains ethanol, does not contain fat or hydrocolloids, and has a higher water activity. Dressings can be created with a larger variety of acids which have different inhibitory effects on microorganisms. Studies tend to focus on acetic acid as the sole source for preventing *Zygosaccharomyces sp*. growth, not on concentrations or combinations of acid to prevent growth. There have also been no studies on the ability of yeasts in general to grow in aqueous

gluconic acid. Further, the growth of *Z. parabailii* in salad dressings could be prevented by removing potential food sources (i.e. starches and stabilizers) and replacing them with protein stabilizers and sucrose to increase shelf stability.

2.7.2 Rheology

The effects of salt content, emulsifiers, and stabilizers on rheological behavior have all been studied, but there is little information on large-strain behaviors of salad dressing. One study found that combining whey protein with a stabilizer can increase emulsion stability (Sun 2009). Other studies have examined the stability of whey-based dressings (Turgeon 1996; De Cassia 2009) but little research has been done on the incorporation of other types of milk proteins and their small- and large-strain behavior in emulsions. By testing both small- and large-strain behavior, this study can give some insights into viscoelastic properties of dressings made with milk protein and how they change over time (Duvarci 2017).

2.8 Conclusions

Z. parabailii is a spoilage yeast that causes salad dressing spoilage and has high resistance to antimicrobials. This study explored the reduction of *Z. parabailii* growth in lite salad dressings through the use of different acid combinations, storage conditions, and dairy protein stabilizers. Additionally, the impact of formulation on the rheological behavior of the salad dressings was determined. The information from this work can provide valuable information in regard to preventing *Z. parabailii* growth in lite salad dressings.

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CHAPTER 3: THE EFFECT OF ORGANIC ACIDS AND STORAGE TEMPERATURE ON LOW-CALORIE SALAD DRESSING RHEOLOGY AND *ZYGOSACCHAROMYCES PARABAILII* GROWTH

3.1. Abstract

Zygosaccharomyces parabailii (*Z. parabailii*) causes spoilage in salad dressings due to its high osmotic tolerance and resistance to weak organic acids. The objective of this study was to determine the effect of organic acid combinations and storage temperature (4, 10, and 25°C) on *Z. parabailii* growth and salad dressing mechanical properties. Acetic, lactic, and gluconic acids were used alone and in combination to acidify a lite salad dressing formulation. Formulations with acetic acid alone tended to have lower CFU/mL of *Z. parabailii* compared formulations with the other acids alone or in combination. Storage at 4°C was most effective at preventing *Z. parabailii* growth. Storage at 4°C in combination with acetic acid as the sole acidulant reduced *Z. parabailii* CFU/mL by 2 logs, the greatest reduction of any acid–storage combination. Overall, controlling storage temperature was the most effective way to reduce *Z. parabailii* growth over the 45-day storage period. Acidulant type and combination also impacted salad dressing mechanical properties. During the 45-day storage period, all formulations showed increased viscosity, a Herschel-Bulkley viscosity profile, and elastic-dominant viscoelastic behavior. The degree of change in rheological behaviors over time was dependent on the type of acid used in the formulation. While acidulant type and combination affected dressing rheological behaviors and inhibitory effects against *Z. parabailii*, certain acidulants may not adequately prevent *Z. parabailii* growth.

3.2. Introduction

A typical salad dressing is an oil-in-water emulsion with a formulation composed of oil, egg yolk, acidulants, and starch (US, FDA, 21CFR169.150 2017). Standard salad dressing formulations contain at least 30 % fat by weight. The amount of fat in salad dressings may prompt health concerns for consumers, so lite salad dressings have been developed to make a healthier reduced-fat option. To be considered "lite," a salad dressing with > 50% calories from fat needs a 50% fat reduction by weight from the original formulation. For a salad dressing with ≤ 50% calories from fat to be considered "lite," the final product needs a 33.33% reduction in all calories (Chiralt 1992; US, FDA, 21CFR110.68 2017). Modifying dressing formulas to meet lite specifications usually involves increasing starch and water content (Peressini 1998). Additional starch along with hydrocolloids are needed to increase dressing emulsion stability. However, the increased starch levels cause lite dressing formulations to be susceptible to the growth of undesirable spoilage organisms, as starch is a potential food source (Kurtzman 1971).

A notable spoilage microorganism in salad dressings is *Zygosaccharomyces parabailii (Z. parabailii). Z. parabailii* is a yeast that shares similar characteristics and genetic expression with *Zygosaccharomyces bailii* (Suh 2013). As there few published studies on *Z. parabailii*, many of the assumptions made about *Z. parabailii* are based on *Zygosaccharomyces bailii* metabolic and osmotic responses. *Z. parabailii'*s tolerance to low pH, salts, and antimicrobial compounds allows it to grow in dressings with little competition (Sousa-Dias 1996). It can grow in pH between 2.0 and 7.0, as well as in products with up to 12.5% w/w salt content (Thomas 1982). Spoilage from *Z. parabailii* has caused significant economic loss in the food industry (Smittle 1996; Fleet 2007). Unchecked growth and

fermentation will produce flavors, colors, and odors which are not palatable to consumers. Additionally, the rapid production of CO_2 during fermentation can cause damage to packaging containers by creating high pressures in the bottle, potentially resulting in explosion of the container.

Food producers commonly use chemical preservatives to control the growth of pathogens and spoilage organisms in salad dressings (Warth 1977). These preservatives consist of antimicrobial compounds, such as ethylenediaminetetraacetic acid (EDTA), and organic acids, such as benzoic, sorbic, acetic, lactic, and gluconic acids (Stratford 2013). These organic acids defuse through the microorganisms' lipid bilayer and dissociate into their salt forms. This lowers the internal cell pH, creating a high osmotic pressure that can cause cell death. Unfortunately, organic acids are not effective against acid-tolerant microorganisms such *Z. parabailii.* Several studies have investigated the mechanisms behind the high acid tolerance in *Z. parabailii* (Guerreiro 2012; Startford 2013; Macpherson 2005). The high resistance to weak acids was attributed to the ability of *Z. parabailii* to utilize organic acids and preservatives as carbon sources combined with the use of an H^+ pump to remove H^+ ions from within the cell (Macpherson 2005). Because control of *Z. parabailli* with weak organic acids can be difficult and because the acid selected may impact dressing flow behaviors, the objective of this study was to determine the effects of using different combinations of organic acids and storage temperatures on *Z. parabailii* growth, as well as how these acid–temperature combinations affected dressing mechanical properties during storage.

3.3 Materials and Methods

3.3.1 Materials

Litehouse Inc donated the ingredients to make the salad dressing including soybean oil (ADM, Cheney, WA, USA), enzyme modified egg yolk (Michael Foods, Minnetonka, MN, USA), MIRA-SPRESE (Tate & Lyle, Hoffman Estates, IL, USA), buttermilk powder (All American Foods, Inc, North Kingstown, RI, USA), sugar (National Sugar, Boise, ID, USA), maltodextrin (Tate & Lyle, Hoffman Estates, IL, USA), gum arabic (TIC gums, White March, MD, USA), Fastir xanthan (Tate & Lyle, Hoffman Estates, IL, USA), acetic acid (99.98% w/w Fischer, Hampton, NH), lactic acid (50% w/w Fischer, Hampton, NH), and gluconic acid (50 % w/w Fischer, Hampton, NH). Freeze-dried *Z. parabailii* cultures (ATCC® 36947™) were purchased from ATCC (Manassas, VA, USA) and tryptic glucose yeast extract agar (TGYE) and buffered peptone water were purchased from VWR (Radnor, PA, USA).

3.3.2. Lite salad dressing preparation

Each formulation of lite salad dressing was prepared in a blender (Waring Commercial; Torrington, Connecticut, USA) at 8,000 rpm to promote formation of a stable emulsion. Water and water-soluble ingredients were first mixed together as follows: 22.6% w/w DI water, 21.4% w/w sugar, 4.7 % w/w egg yolk, 2.6 % w/w buttermilk powder, 4.2% w/w starch, 0.4% w/w xanthan gum, 0.4% w/w gum arabic, and 8% w/w maltodextrin. After blending these ingredients for 30 s, 35.7% w/w soybean oil was added to the mixture with the blender running. over a period of 60 s to create an oil-in-water emersion. After emulsification, the formulations were acidified to a final pH, verified by a FiveEasy pH meter (Columbus, OH, USA), of either 3.2 or 4.2 based on the formulations in Table 3.1. Formulations were

made in triplicate. Each finished batch of salad dressing was separated into 500 mL lots and stored at 4°, 10°, or 25°C. The lots were allowed to reach the desired storage temperature before inoculation with Z. *parabailii.*

Table 3.1 Food grade acid combinations used as potential mitigators of *Z. parabailii* growth in lite salad dressing

Acidulant	Ratio	pH	% w/w	Abbreviations
Gluconic acid	-	4.2 3.2	1.00 2.00	GDL GDL2%
Acetic acid	-	4.2	0.50	AC
Lactic acid	-	4.2	0.50	LA
Gluconic + Lactic acid	1:1 1:2	4.2	0.75	GL GL2
Gluconic + Acetic acid	1:1 1:2	4.2	0.75	GA GA2
Gluconic + Acetic + Lactic acid	1:1:1	4.2	0.50	GAL
Acetic + Lactic acid	1:1	4.2	0.75	AL

3.3.3. Inoculation of salad dressings with *Z. parabailii*

An inoculum was prepared directly from *Z. parabailii* cells that were kept as frozen stocks (-80°C). The 500 mL lots of previously prepared salad dressings were inoculated with ~$1x10^4$ CFU/mL. The inoculated lots of dressing were distributed in 10 mL aliquots to 15 mL fermentation tubes. Inoculated 15 mL fermentation tubes were stored in 4°, 10°, or 25°C for 45 days.

Three fermentation tubes from each batch were removed from storage every 5 days for plate counts and discarded after use. The salad dressing from the fermentation tubes was serial diluted in buffered peptone water and spread plated on solid TGYE agar with 0.5% acetic acid for *Z. parabailii*. The dilution factor was predetermined from preliminary studies and ranged between 10^{-1} and 10^{-7}. Inoculated plates were incubated at 25°C and colonies were counted after 72 hr. Plating was done in triplicate for each sample. All work was conducted in a biological hood to prevent sample contamination.

3.3.4. Small strain and rotational rheology

All rheological measurements were performed on a DHR3 (TA Instruments; New Castle, Delaware, USA) with a cone and plate system (1°, 40 mm diameter) at 25°C. Each sample was conditioned at 25°C for 30 s, then presheared at 10 rad/s for 20 s. The sample equilibrated for 60 s before the test started. Each test was done in triplicate.

Shear rate sweeps were conducted from 0.01 to 100 1/s and 100 to 0.01 1/s to evaluate viscosity profiles and hysteresis. Strain sweeps were conducted from 0.01 to 100% strain at 1 rad/s to determine critical strain and parameter values at critical strain. Critical strain and stress, or the strain and stress at the end of the linear viscoelastic region (LVR) were determined as the strain–stress pair at which the complex modulus deviated from the previous value by more than 3%. Frequency sweeps were performed at 75% of the smallest critical strain for all formulations to evaluate small-strain viscoelastic behaviors. Frequency sweeps were conducted at 0.0775% strain from 0.1 to 100 rad/s.

3.3.5. Data analyses

Microbial counts were analyzed for statistical differences with SAS software version 9.1 (SAS, Cary, NC) using a two-way analysis of variance (ANOVA) followed by Tukey's test. Significant differences were recorded at $P < 0.05$. Viscosity curves for each formulation were averaged together and the resulting average curves fitted to a Herschel Bulkley model, $\sigma = \sigma_o + K\dot{\gamma}^n$, where shear stress, consistency coefficient, shear rate, flow index, and yield stress are represented by σ (Pa), K (Pa.s^n), $\dot{\gamma}$ (1/s), n, and σ_o (Pa) respectively.

3.4. Results and Discussion

3.4.1. The effects of organic acids on *Z. parabailii* growth in lite salad dressings

The type of acid used in the formulation resulted in significant differences in the concentration of *Z. parabailii* in different formulations of salad dressing stored at 25°C (Table A.3.1). Comparing formulations with a single acidulant, AC had the lowest concentration of *Z. parabailii* after 45 days of incubation. GDL and GDL2% had the highest concentration of *Z. parabailii* after 45 days of storage. It should be noted that even though the use of different acids had rates of growth and CFU/mL counts over time, *Z. parabailii* counts in all formulations reached 10^5 CFU/mL by Day 5. At this concentration of *Z. parabailii*, the salad dressing develops off-flavors and other noticeable spoilage characteristics due to fermentation (Kurtzman 2011). All formulations, regardless of acidulant or acidulant combination, showed signs of spoilage.

Comparing formulations stored at 25°C with multiple acidulants, GA formulations had the lowest concentration of *Z. parabailii* and GL2 had the highest concentration of *Z.*

parabailii after 45 days of incubation. Like the single-acidulant formulations, all formulations with multiple acidulants had rapid growth during the first several days of storage, which resulted in dressings with concentrations of *Z. parabailii* over 10^5 CFU/mL after 5 days of incubation (Figure 3.1). Differences in *Z. parabailii* growth among formulations may have been related to differences in the chemical properties of the weak organic acids used in the formulation. Each organic acid used in this study varied in size, hydrophobic/hydrophilic properties, pKa, and other chemical properties. These differences would have impacted the degree to which each weak acid could affect the cellular pH of *Z. parabailii.*

Overall, the concentration of any weak organic acid used was not high enough to inhibit the growth of *Z. parabailii* at 25°C (Figure 3.1). Interestingly, the GDL2% formulation did not have the expected impact on reduction of *Z. parabailii* at 25°C when compared to GDL (1% gluconic acid). The higher concentration and lower pH of GDL2% were expected to have a greater inhibitory effect on *Z. parabailii* growth but GDL and GDL2% had no significant differences in terms of *Z. parabailii* growth (Table A.3.1.). Thus, the concentration of gluconic acid must be greater than 2% to have an inhibitory effect on *Z. parabailii.*

Weak acid can damage yeast cells by entering the cytoplasm of the cell through simple diffusion (Warth 1989; Stratford 2013). The rate of diffusion of a weak acid into the cell usually starts rapidly but slows to an equilibrium rate over time. Along with simple diffusion, weak acids have their own pH-dependent equilibrium that causes the weak acid to dissociate. The rate and degree of dissociation is related to the pKa of the weak acid. Since the internal pH in the cytoplasm is typically higher than the environment, weak acids dissociate to their conjugate acids and bases in the cytoplasm. This disassociation can lead to a reduction in the

cytoplasm pH, which can cause damage to key enzymes used for *Z. parabailii* cellular function (Stratford 2013).

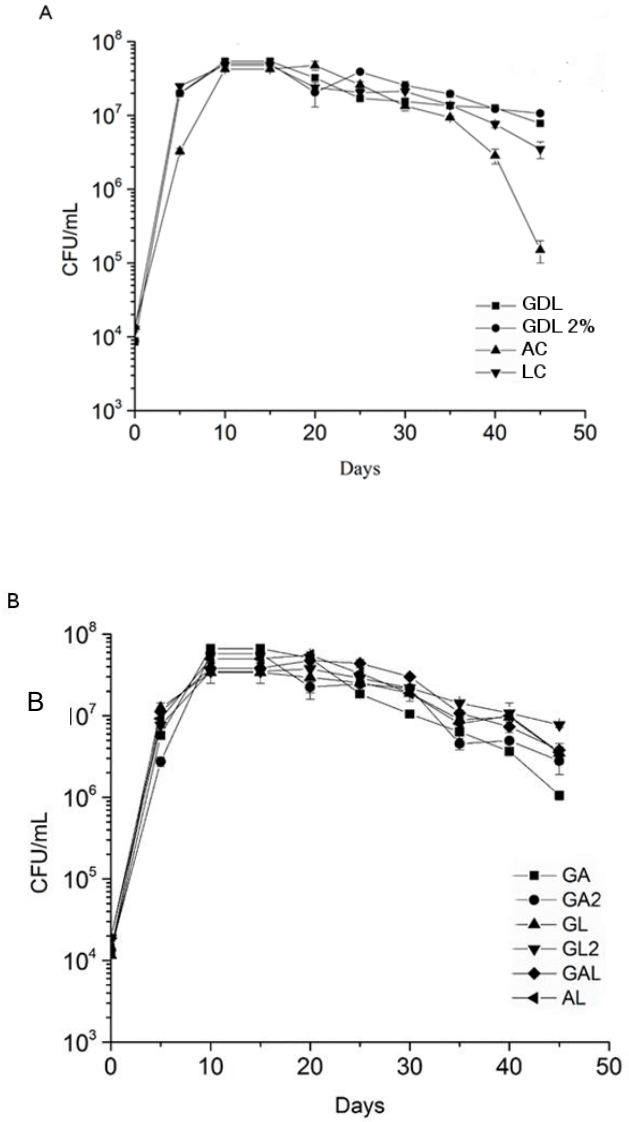

Figure 3.1 *Z. parabailii* growth in lite salad dressings prepared with (A) individual acids and (B) combinations of acids over a 45-day storage period at 25°C. Error bars represent standard error.

Damage to the enzymes can stop the enzyme from functioning, leading to cell death.

However, the death phase was not observed in the samples stored at 25°C. This may be due to

Z. parabailii resistance to dissociation of weak organic acids in its cytoplasm. One mechanism through which *Z. parabailii* may have maintained its internal pH is metabolization of the dissociated forms of different organic acids used in the salad dressing formulations. Other studies have shown that acetate and glucono delta-lactone, the dissociated form of acetic acid and gluconic acid, respectively, can be catabolized through the TCA cycle in *Z. parabailii* cells (Guerreiro 2012; Macpherson 2003). Another possible mechanism for defense against weak organic acids is the ability of *Z. parabailii* to pump H^+ ions out of the cell through its H^+–ATPase pumps (Macpherson 2003), which would prevent cell pH from dropping too low to support life.

The rate of *Z. parabailii* growth at 10°C was significantly lower than the rate of growth at 25°C (Figure 3.1, 3.2). This change in growth rate from 25°C was probably due to the decrease in the rate which enzymes used for cellular respiration and replication could react in the cytoplasm at lower temperatures (Kurtzman 2011). However, no formulation was able to completely inhibit the growth of *Z. parabailii* at 10°C: all samples reached concentrations of *Z. parabailii* $\geq 10^5$ CFU/mL between 5-10 days growth, indicating spoilage. The type of acid used in the formulation resulted in significant differences in *Z. parabailii* concentration in formulations stored at 10°C (Table 3.A.1). When the concentration of *Z. parabailii* was $<10^5$, AC and GDL2% formulations had the lowest concentration of *Z. parabailii* (Figure 3.2 A). Before the formulations with a combination of acidulants reached *Z. parabailii* concentrations $>10^5$ CFU/mL (i.e. during the first few days of storage), GA had the lowest concentration of *Z. parabailii* (Figure 3.2 A). As the concentration of *Z. parabailii* increased in the formulations stored at 10°C, the ability of organic acids to affect the growth of *Z. parabailii* altered. At the end of the 45-day storage period, formulations containing acetic acid generally

had lower concentrations of *Z. parabailii* compared to formulations that did not. Overall, GDL and GDL2% had higher concentrations of *Z. parabailii* over time compared to the other formulations (Figure 3.2). These results agreed with the growth observed at 25°C. *Z. parabailii* was shown to have the ability to assimilate and metabolize d-glucono-1,5-lactone (Suh et al., 2013). Therefore, the increased growth of *Z. parabailii* in formulations containing gluconic acid was attributed to its ability to ferment the cyclic ester of gluconic acid, glucono delta-lactone.

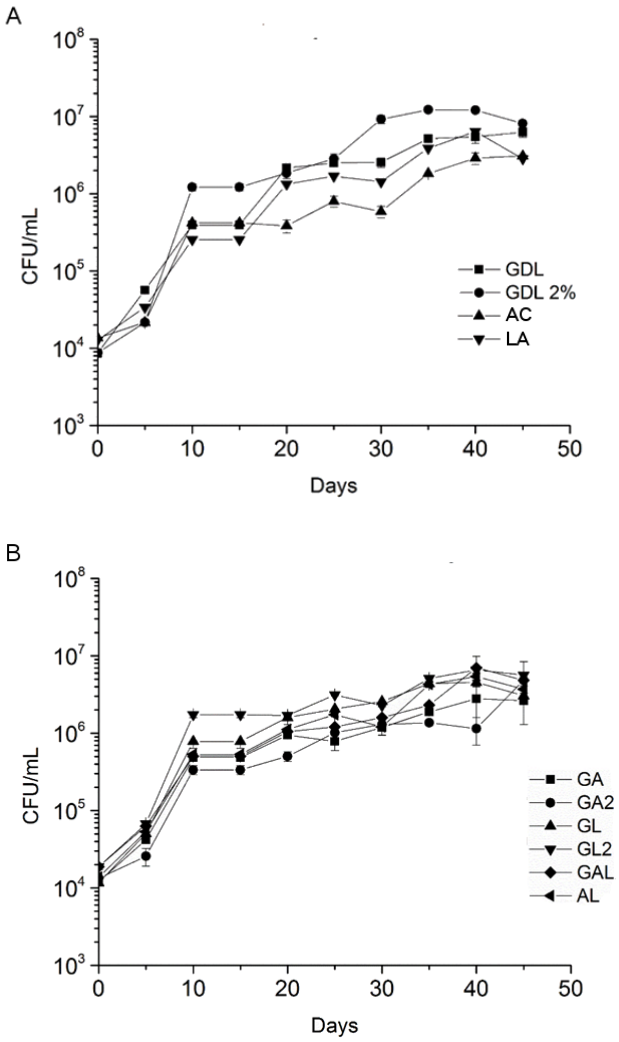

Figure 3.2 *Z. parabailii* growth in lite salad dressings prepared with (A) individual acids and (B) combinations of acids over a 45-day storage period at 10°C. Error bars represent standard error.

Dressings stored at 4°C were the only samples to show inhibitory effects on *Z. parabailii* growth. All formulations showed downward trends of *Z. parabailii* counts starting around 20 days of storage (Figure 3.3). For the first 20 days GAL had the highest concentration of *Z. parabailii*, LA had the lowest, and all the other formulations showed no significant differences in concentration (Table A.3.1). After 45 days, AC had the most impact on *Z. parabailii* growth, as it reduced the concentration of *Z. parabailii* by 2 logs. GDL and GDL2% reduced the concentration by approximately 1 log after 45 days, and the other formulations reduced the concentration of *Z. parabailii* growth by 0.5 log.

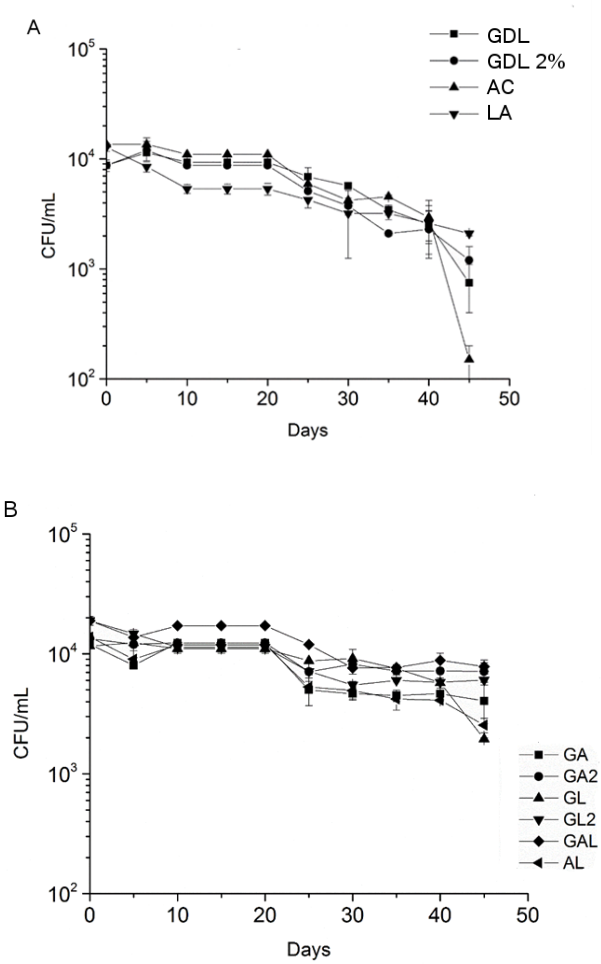

Figure 3.3 *Z. parabailii* growth in lite salad dressings prepared with (A) individual acids and (B) combinations of acids stored at 4°C over a 45-day storage period at 4°C. Error bars represent standard error.

Temperature played a critical role in the growth of *Z. parabailii*. For any microorganisms, the temperature range suitable for growth is dictated by enzyme kinetics and the ability of molecules to move through the cytoplasm. At lower temperatures (e.g. 4°C), *Z. parabailii* cellular respiration could be affected by low enzyme activity and reduced availability of other molecules within the cell. *Z. parabailii* was best inhibited at 4°C as all formulations used in this study had concentrations of *Z. parabailii* below 10^5 CFU/mL. An elevation in temperature from 4°C to 10°C was adequate for *Z. parabailii* to proliferate to spoilage levels after only a few days of storage. Thus, 4°C seemed to be the threshold temperature for inhibition of *Z. parabailii* growth and preventing growth-induced spoilage.

3.4.2 Viscosity of lite salad dressing with multiple acidulants

All lite salad dressing formulations had Herschel-Buckley behavior (Table 3.2). Herschel-Buckley fluid behavior is similar to a power law fluid but also includes a yield stress (Peressini 1998). All samples showed shear-thinning behavior ($n<1$) across all days (Table 3.2). However, each formulation had significant differences in extent of shear thinning, yield stresses, and consistency coefficients (Table 3.2). Differences in the viscosities can be attributed to the hydrophobic/hydrophilic properties of the weak acids used in the formulations. Both the ability for acids to interact at the oil/water interface and effects of the different pka of the acids on the starch may have resulted in changes to the viscosity behaviors (Romero 2009).

The consistency coefficient, K ($Pa.s^n$), has a strong relationship with viscosity. GDL formulation had the highest K value for all timepoints. Gluconic acid can interfere with electrostatic interactions by influencing the charges on the polysaccharide.

Table 3.2 Viscosity profiles, where $\sigma = \sigma_o + K\dot{\gamma}^n$, for low-calorie salad dressings

Day 0				Day 15			
Sample	**N**	**K (Pa.s^n)**	**σ_o(Pa)**	**Sample**	**n**	**K (Pa.s^n)**	**σ_o (Pa)**
GDL	0.46	22.2	14.7	GDL	0.46	22.6	16.8
AC	0.32	16.1	13.2	AC	0.32	18.7	15.4
LA	0.46	21.1	15.4	LA	0.47	19.9	17.0
GA	0.42	19.6	17.2	GA	0.45	22.0	18.4
GA2	0.44	19.2	15.4	GA2	0.48	20.3	16.1
GL	0.48	18.5	18.0	GL	0.49	20.9	18.0
GAL	0.46	17.0	15.4	GAL	0.49	18.6	21.0
AL	0.49	16.4	16.6	AL	0.51	17.3	17.2

Day 30				Day 45			
Sample	**N**	**K (Pa.s^n)**	**σ_o(Pa)**	**Sample**	**n**	**K (Pa.s^n)**	**σ_o(Pa)**
GDL	0.47	28.2	22.1	GDL	0.46	29.7	23.3
AC	0.47	26.8	15.2	AC	0.45	27.2	18.3
LA	0.53	22.4	20.3	LA	0.55	24.0	24.0
GA	0.47	24.3	25.1	GA	0.52	24.4	29.9
GA2	0.49	26.9	30.6	GA2	0.47	20.9	17.1
GL	0.50	25.1	28.2	GL	0.37	26.8	31.2
GAL	0.51	19.5	22.0	GAL	0.52	21.1	24.0
AL	0.53	19.7	17.4	AL	0.53	19.5	17.7

The degree to which any weak acid can affect the charges on polysaccharides can be associated with its pKa. Gluconic acid has a pKa of 3.86, which is lower than the pKa of acetic acid (pKa= 4.76) but equal to the pKa of lactic acid. Both GDL and LA had similar viscosity parameters on Day 0. Therefore, the differences between formulations were attributed to the differences in the pKas of the acids used (Hamdine 2005). For individual

acids, K decreased as pKa increased. AC, which had the lowest K, n, and σ_o, had the highest pKa.

When the acids were used in combination, their effects on viscosity were determined by the particular combination of acids. GA, GA2, GAL, GL, and AL had higher K and n values than AC, but smaller K and n values than GDL and LA on Day 0. The presence of acetic acid had a larger effect on K and n values on Day 0 for formulations containing multiple acidulants as compared to the presence of gluconic or lactic acid. The presence of multiple acids seemed to have a synergistic effect on σ_o, as those values were all higher compared to formulations with a sole acidulant on Day 0. Yield stress represents the amount of stress that is needed for a material to flow; higher yield stress indicates more energy is needed to break down the internal structure and induce flow (Steffe 1995). The combination and dissociation of different acids used in AG, AG2, AL, GAL, and AL may have altered the charge distribution in the salad dressing structure, leading to an increase in van der Waals forces between polysaccharides and proteins. This in turn would lead to increased yield stress for salad dressings formulations with acid combinations.

The K values increased over time for all formulations. Starches and hydrocolloids create internal structures during quiescent storage through entanglement of long polysaccharide chains, which would cause an increase in K over time (Felix 2017). These internal forces can create resistance to flow, subsequently increasing K and σ_o. The amount of change in the K values was influenced by the specific acid(s) used. For example, AC had an 70.0% increase in K over 45 days compared to the 13.7% increase in K in LA (Table 3.2). Lactic, gluconic, and acetic acid may be affecting the degree of polysaccharide rearrangement in the formulations over time. Other than a general increase in K values, the particular

acidulant(s) used did not have any definitive trends over time. The K values were expected to increase during storage as starches, gums, and proteins undergo structural rearrangements over time, resulting in a higher consistency coefficient.

Both n and σ_o values also increased significantly over time for all samples, indicating reduced shear-thinning behavior and increased yield stress over time. The decrease in shear-thinning behavior over time may have been related to breakdown of the polysaccharide microstructures in the salad dressing formulation. The increased yield stress was likely due to an increase in van der Waals forces and increased polysaccharide entanglement over time, requiring more energy to induce flow. Samples containing an equal ratio of gluconic acid plus another acidulant had greater increases in σ_o over time compared to the other samples. The equal ratio of gluconic acid to acetic or lactic acid possibly had a greater effect on the charge distribution between polysaccharides over time. The pKas of acetic acid and gluconic acid are not equal; this might affect charge distribution between polysaccharides and proteins. A lower net charge would increase the electrostatic interaction between polysaccharides, which in turn increased the σ_o (Table 3.2).

Overall, acid type had a notable impact on K, σ_o, and n values. Viscosity profiles give foods a certain mouthfeel; changes in salad dressing viscosity profiles may lead to textural differences (Liu 2007). However, differences in the K, σ, and n values among the formulations were small and may not have created noticeable texture differences. Sensory studies such as descriptive analysis would need to be conducted to provide conclusive textural data.

3.4.3. Strain sweep for lite salad dressings composed of different acids

Table 3.3 Critical values from strain sweep for formulations stored for 0 and 45 days[a]

Day	Sample	G' (Pa)	G'' (Pa)	Strain (%)	G* (Pa)	Phase angle (deg)
0	GDL	255^{B}	64.2^{D}	0.251^{A}	262^{CB}	14.1^{F}
	AC	236^{C}	67.8^{DC}	0.250^{A}	245^{CD}	16.0^{A}
	LA	250^{B}	66.1^{DC}	0.250^{A}	259^{CB}	14.7^{E}
	GA	294^{A}	80.8^{AB}	0.249^{A}	304^{A}	15.3^{D}
	GA2	253^{B}	71.6^{BC}	0.251^{A}	263^{B}	15.7^{BC}
	GL	221^{DC}	60.6^{D}	0.251^{A}	229^{ED}	15.3^{D}
	GL2	283^{A}	81.7^{A}	0.250^{A}	295^{A}	16.0^{A}
	GAL	216^{D}	61.4^{D}	0.251^{A}	224^{E}	15.8^{AB}
	AL	189^{E}	53.3^{E}	0.251^{A}	196^{F}	15.7^{C}
45	GDL	270^{A}	62.2^{A}	0.251^{A}	277^{A}	12.9^{ED}
	AC	165^{DC}	43.8^{BC}	0.250^{A}	171^{ED}	14.8^{A}
	LA	202^{B}	46.1^{BC}	0.250^{A}	207^{C}	12.8^{E}
	GA	212^{B}	49.4^{BC}	0.251^{A}	218^{C}	13.1^{D}
	GA2	175^{C}	44.4^{BC}	0.250^{A}	181^{D}	14.2^{C}
	GL	254^{A}	56.9^{AB}	0.250^{A}	261^{B}	12.6^{F}
	GL2	210^{B}	48.7^{BC}	0.250^{A}	216^{C}	13.0^{D}
	GAL	137^{E}	34.4^{C}	0.250^{A}	141^{F}	14.0^{C}
	AL	151^{D}	38.9^{BC}	0.250^{A}	156^{E}	14.4^{B}

[a] For each day, letters in each column that are different indicate significant differences ($p<0.05$).

Strain sweep data were used to calculate critical strains to ensure that frequency sweeps were conducted within the linear viscoelastic region (LVR) (Franco 1997). All samples exhibited elastic-dominated behavior at critical strain (Table 3.3). The elastic modulus G' (Pa), loss modulus G'' (Pa), complex modulus G*(Pa), and phase angle (degrees)

values of most samples significantly decreased during the 45-day storage period. The only exception was GL, which had a significant increase in G' values over the 45-day storage period. No differences were found among the critical strain values for all formulations; thus, all formulations had similar LVRs. The critical strains of all formulations did not show significant differences between Day 0 and Day 45. This was not expected based on the viscosity results. The viscosity curves showed an increase in the yield stress over time, so it was expected that the critical strain values would similarly increase. A possible explanation for this may be that the yield stress changes over time as measured by shear rate sweeps were relatively small and had insignificant impact on critical strain. The decrease in phase angle over time indicated that samples had increased elastic-dominated behaviors with increased storage time. This behavioral change was attributed to the rearrangement of polysaccharides in the salad dressing formulations. Polysaccharide entanglement in the dressing could have given additional structure to the salad dressing, causing an increase in elastic-type behavior (Santiago 2002).

G* values generally decreased over time. The differences in G', G'', and G* values among the formulations, although statistically significant, were slight and may not result in noticeable differences in terms of processing behavior or textural attributes. As previously suggested, sensory studies are needed to determine whether differences in moduli result in different texture perceptions.

3.4.4 Frequency sweeps for lite salad dressings formulated with different acidulants

All formulations showed weak gel viscoelastic behavior (Figure 3.4). A weak gel is an intermediate between a solid and a liquid which shows mechanical rigidity. The gel in the salad dressing consisted of polysaccharide polymers, which entangle in the aqueous phase of

the dressing giving it a network structure and a yield stress (Saha 2010). A weak gel is indicated by G' > G" combined with frequency dependence as indicated by an increase in G' with frequency. All formulations showed weak gel and elastic-dominated behavior (G' > G"), which aligned with the strain sweep data. Many of the formulations showed a decrease in both the storage and loss modulus values after 45 days (Figure 3.4.). The decrease in these values did not necessarily indicate a decrease in elastic behavior; rather, the decrease in the moduli vales were associated with the ability of the formulation to store and dissipate energy (Vianna-Filho 2013). Over time, the carboxyl groups of the polysaccharide chains become ionized by the H^+ ions and conjugate bases of the dissociated weak acids in the food system. Polysaccharide ionization may affect the way it interacts with the lecithin in the egg yolks, which in turn affects the macroscopic properties of the salad dressing (Myers 1990).

Polysaccharides interact with themselves through physical association of their polymer chains, hydrogen bonding, hydrophobic association, and cation mediated cross-linking (Saha 2010). The ability for polysaccharides to interact with emulsifying agents, other polysaccharides, and themselves was impacted by the acids used in formulation. For example, AC and GL, unlike the other formulations, showed no significant changes in G' and G"over the 45-day storage period. Most formulas showed a decrease in moduli values over the 45-day storage period. GDL moduli values, on the other hand, were statistically similar on Day 0 and Day 15, significantly decreased at 30 days of storage, but increased after 45 days of storage. The increase in moduli values for GDL at Day 45 may have been due cross-linking of long

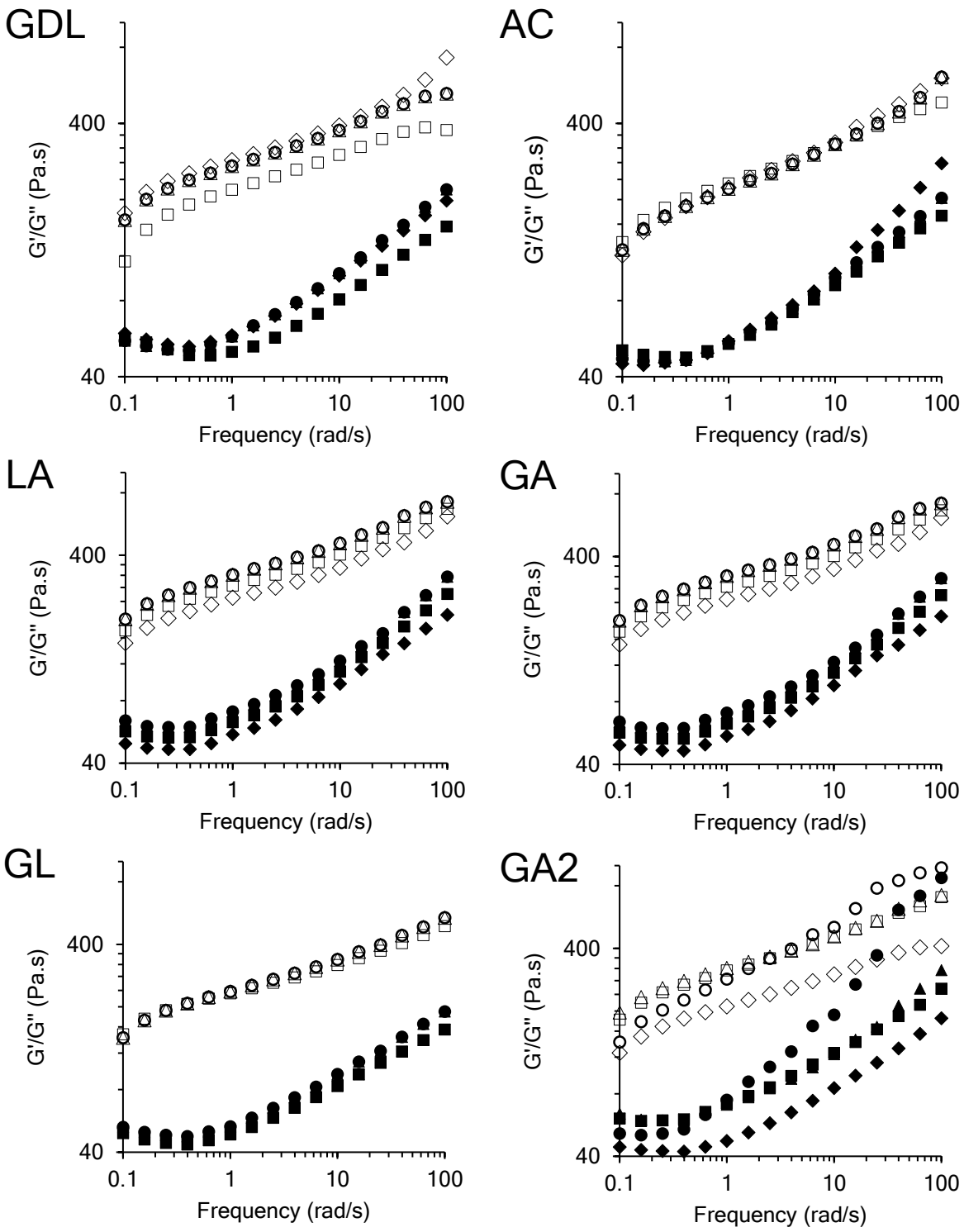

Figure 3.4 Frequency sweep for lite salad dressing formulations made with different acid combinations. Formulations were tested on Day 0 (○), Day 15 (Δ), Day 30 (□), and Day 45 (◊). Open symbols represent G' closed black symbols represent G"

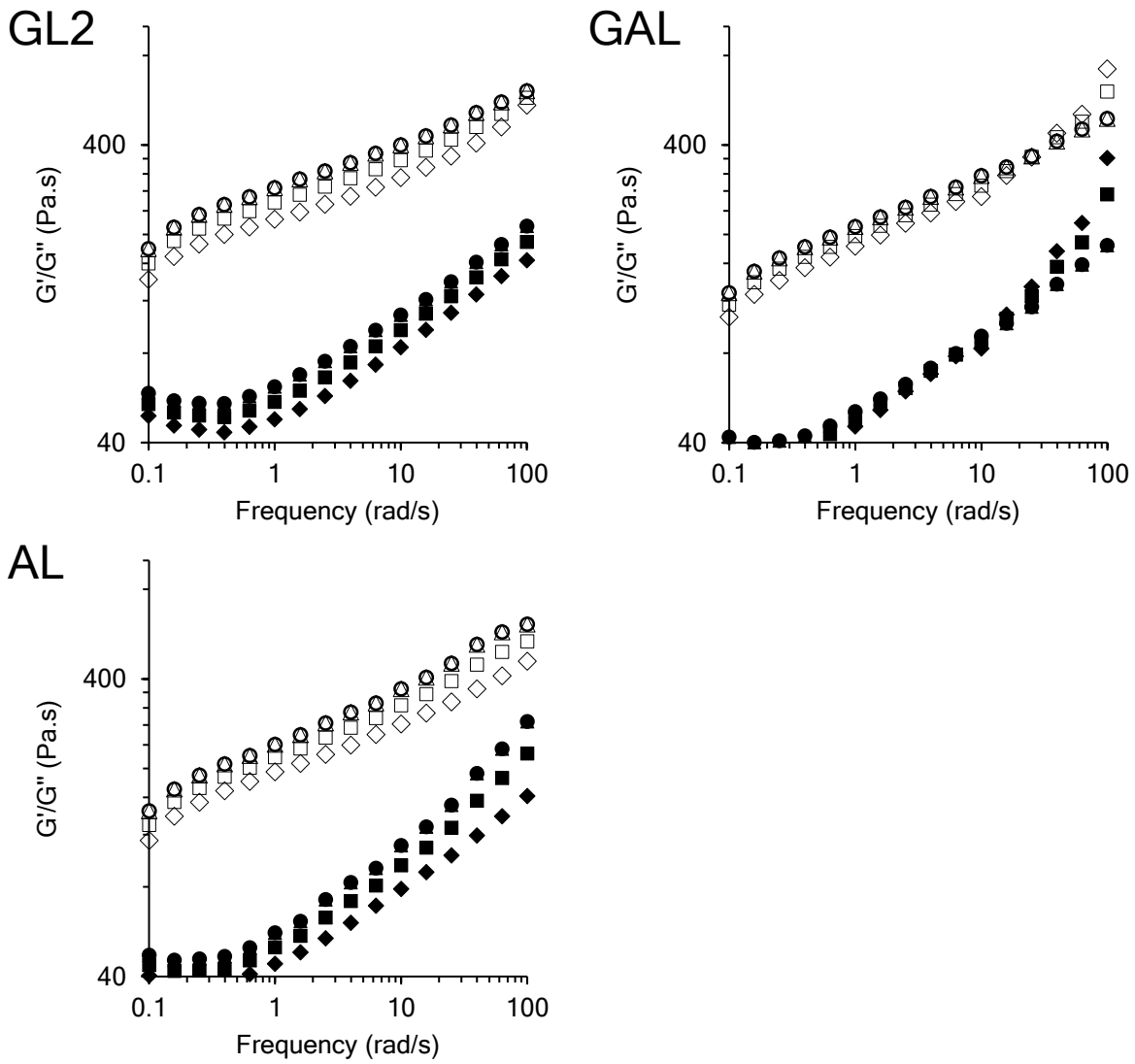

Figure 3.4 (continued from the previous page)

polymer chains in the dressing formulation. The long chains may have difficulty in sliding past each other, resulting in a greater extent of elastic-type behavior.

In general, different acid combinations significantly impacted the viscosity, small-strain, and inhibition of *Z. parabailii* growth. All formulations used in this study were unable to stop the growth of *Z. parabailii* at 25° and 10°C, but the formulation that had the lowest concentrations of *Z. parabailii* at the end of the 45-day storage at either of these temperatures

was AC. The use of acetic acid as a sole acidulant in AC also had the lowest counts of *Z. parabailii* in 4°C as well. Changes in the viscoelastic properties and viscosities in all formulations were slight and may not affect sensory attributes. While sensory studies would need to be conducted for conclusive information on perceived textures, GA2 had the most consistent viscosity behavior over time, and GL had the most consistent viscoelastic properties over time, indicating better formulation stability during storage compared to the other formulations. However, these two formulations were not as effective as AC at reducing *Z. parabailii.* The use of acetic acid as a sole acidulant may not yield formulations with the same stability over time as other combination of acidulants used, but the ability of acetic acid to inhibit growth of *Z. parabailii* more effectively makes it a better choice of acidulant when formulating salad dressing for *Z. parabailii* growth.

3.5. Conclusions

Using various combinations of weak acids resulted in significant differences in *Z. parabailii* concentration over a 45-day storage period. However, storage temperature had a notably larger effect on the growth of *Z. parabailii* than the type of acidulant(s) used. Of the organic acids evaluated in this study, acetic acid was the most effective for reducing *Z. parabailii* growth. Although the use of organic acids did not stop *Z. parabailii* growth at 10 or 25°C, combining acidulant use with use of refrigeration temperatures (4°C or lower) inhibited the growth *Z. parabailii* over 45 days of storage. Even though *Z. parabailii* growth rates were significantly reduced at temperatures around 10°C as compared to 25°C, dressings formulations still showed CFU/mL $>10^5$ at 10°C, which indicated spoilage. Viscoelastic behaviors and changes to these behaviors over time were dependent on the acids used in the formulation. Changes in salad dressing acid composition impacted viscosity and viscoelastic

properties. However, the differences in rheological behavior between formulations were small and may not give a significant difference in textural perception. Overall, acetic acid was considered the most effective acidulant of the acids studied, even though GA2 and GL formulations showed a lesser degree of rheological changes over time. The most effective method of reducing *Z. parabailii* in salad dressing was by storing the dressing at 4°C with acetic acid as a acidulant.

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CHAPTER 4: DAIRY PROTEIN STABILIZERS AFFECT BOTH RHEOLOGICAL PROPERTIES AND GROWTH OF *ZYGOSACCHAROMYCES PARABAILII* IN LITE SALAD DRESSINGS

4.1 Abstract

Lite salad dressings were evaluated for the effects of dairy protein stabilizers on *Zygosaccharomyces parabailii* (*Z. parabailii*) growth and rheological properties. Salad dressing formulations were prepared with 12.5% w/w milk protein isolate, whey protein isolate, or micellular casein. Formulations were inoculated with 10^4 CFU/mL of *Z. parabailii* and stored at 4°, 10°, or 25°C for 45 days. The viscosity and small- and large-strain viscoelastic properties of the formulations were also determined via rheometry. Formulations containing milk proteins were compared to a control lite salad dressing containing 4.2% w/w starch. Formulations containing a milk protein stabilizer and acidified with acetic acid showed no detectable growth after 5 days of storage at any temperature. The viscoelastic properties of the milk protein-stabilized samples were not as consistent over time as the viscoelastic properties of the polysaccharide-stabilized formulation. While milk protein-stabilized lite salad dressings showed significantly reduced *Z. parabailii* growth compared to the control, the changes in viscosity and viscoelastic properties of these dressings indicated destabilization of the dressing over time.

4.2 Introduction

Salad dressings have a nutrient-rich environment that promotes the growth of spoilage organisms, including *Zygosaccharomyces parabailii (Z. parabailii)* (Kurtzman 2006). While the low pH (≤4.6) of a typical salad dressing prevents the growth of most pathogenic and

spoilage microorganisms (Smittle 1982), *Z. parabailii*, an acid-tolerant spoilage yeast, can grow in high-acid products such as salad dressing. Since few microorganisms can grow at pH<4.6, salad dressings naturally create an environment with little competition for *Z. parabailii* (Stratford 2013). As *Z. parabailii* grows, it ferments the sugars in the product, resulting in sour and bitter flavors that are unfavorable to consumers (Fleet 2007). Fermentation also results in rapid production of CO_2. Because dressings are usually packed in a sealed container (Stratford 2013), the pressure in the bottle increases during gas production until the container bursts. Not only is a leaking salad dressing bottle unappealing to consumers, packaging failures in salad dressing containers can cause injures to workers from a bottle explosion (Grinbaum 1994).

Salad dressings are oil-in-water emulsion composed of a vegetable oil, an acidifying ingredient, egg yolk, and a starch (US, FDA, 21CFR169.150 2017). By removing ingredients that include easily fermentable sugars (e.g. glucose chains in starches and gums), *Z. parabailii* growth in salad dressings may be reduced (Yang 2003). Protein stabilizers such as milk proteins can be used in place of polysaccharides to maintain emulsion stability without providing a nutrient source for *Z. parabailii*. In addition to their nutritive and functional properties (Walstra 2006), milk proteins also contain bioactive peptides that can provide antimicrobial effects (Haque 2008). However, substituting milk proteins for polysaccharide stabilizers will likely impact the stability and rheological properties of the dressings both immediately after manufacture and over time. Because these effects are dependent on protein type and concentration as well as environmental conditions, the rheological behaviors and stability of protein-stabilized dressings need to be assessed. Therefore, the objective of this

study is to determine the impact of protein stabilizers on 1) *Z. parabailii* growth in salad dressing and 2) rheological behaviors of lite salad dressing systems.

4.3. Materials and Methods

4.3.1 Materials for microbiology and salad dressing

The following ingredients for the lite salad dressing formulations were donated by Litehouse Inc.: soybean oil (ADM, Cheney, WA, USA), enzyme modified egg yolk (Michael Foods, Minnetonka, MN, USA), MIRA-SPRESE starch (Tate & Lyle, Hoffman Estates, IL, USA), buttermilk powder (All American Foods, Inc, North Kingstown, RI, USA), sugar (National Sugar, Boise, ID, USA), maltodextrin (Tate & Lyle, Hoffman Estates, IL, USA), gum arabic (TIC gums, White March, MD, USA), Fastir xanthan (Tate & Lyle, Hoffman Estates, IL, USA), acetic acid (Fischer, Hampton, NH), and gluconic acid (Fischer, Hampton, NH).

Milk protein isolate was donated by Idaho Milk Produces (Jerome, Idaho, USA). Whey protein isolates and micellular casein were donated by Glanbia Foods (Twin Falls Idaho, USA). *Zygosaccharomyces parabailii* cultures (ATCC® 36947™) were purchased from ATCC (Manassas, VA, USA). Both tryptic glucose yeast extract agar (TGYE) and buffered peptone water were purchased from VWR (Radnor, PA, USA).

4.3.2 Preparation of lite salad dressing

A lite salad dressing was created using either milk protein- or polysaccharide-based stabilizers (Table 4.1). First, 22.6% w/w water, 21.4% w/w sugar, 4.7% w/w egg yolk, 2.6 % w/w buttermilk powder, 4.2 % w/w starch, 0.4 % w/w xanthan gum, 0.4 % w/w gum arabic, and 8% % w/w maltodextrin were mixed in a benchtop blender (Waring Commercial;

Torrington, Connecticut, USA) at a speed of 8,000 rpm until all ingredients were dissolved. Oil was slowly added to the water phase over a period of 60 s with the mixer running. Care was taken to prevent oil from pooling at the top. The resulting dressing was acidified with acetic acid to a pH of 4.2.

The above steps were repeated to make three different protein-stabilized formulations, except the MIRA-SPERCE, xanthan gum, maltodextrin and gum arabic were removed from the formulation and replaced with an amount of milk protein isolate, micellular casein, or whey protein isolate that was specified on Table 4.1. All formulations were acidified to a pH of 4.2 using the acids specified in Table 4.1. Formulations were equilibrated at 4°, 10°, or 25°C before inoculation with *Z. parabailii.* Each formulation was made in triplicate.

Table 4.1 Stabilizers and acidulants used in lite salad dressing formulations

Stabilizer	Acidifier(s)	pH	% w/w protein	Abbreviation
Whey protein isolate	Acetic acid	4.2	12	WPI
Milk protein isolate	Acetic acid	4.2	12	MPI
	Gluconic acid	4.2	12	GLU
Micellular casein	Acetic acid	4.2	12	CAS
Starch	Acetic acid	4.2	-	CON

4.3.2 Dilution plating and incubation

Tryptic glucose yeast extract agar (TGYE) was prepared by boiling 23 g TGYE powder in 1L of deionized water for 1 min. The agar was autoclaved at 121°C at 15 psi for 20 min, then 5 mL of acetic acid was added to the TGYE for selectivity for *Z. parabailii*.

The inoculum for the salad dressing was prepared directly from *Z. parabailii* cells stored as frozen stocks at (-80°C). Frozen stocks were added to YM Medium (Becton Dickinson, Franklin Lakes, NJ) to be used in challenge studies. Each lot of salad dressing (Table 4.1) was inoculated with *Z. parabailii* to get a concentration of ~$1x10^4$ CFU/mL. After inoculation, the lot was distributed to 15 mL fermentation tubes in 10 mL aliquots. The fermentation tubes were stored at 4°, 10°, or 25°C for 45 days.

Three fermentation tubes per sample replication were used for each timepoint and discarded after testing. The fermentation tubes were serial diluted between 10^{-1} and 10^{-7} in buffered peptone water then plated on TGYE with 0.5% acetic acid. The dilution factors used were based on preliminary studies on the expected growth rates of *Z. parabailii* during storage. TGYE plates used for plate counts were incubated at 25°C for 72 hours before plates were inspected for growth and colonies were counted. Plating was done in triplicate for each sample.

4.3.3 Small Strain and Rotational Rheology

Rotational, small- and large-strain tests were performed on a DHR3 (TA Instruments; New Castle, Delaware, USA) with a cone and plate system (1°, 40mm) at 25°C. Before testing samples were equilibrated at 25°C for 30 s, presheared at 10 rad/s for 30 s, and equilibrated at rest for 60 s. All rheological tests were performed in triplicate.

Shear rate sweeps were conducted from 0.01 1/s to 100 1/s and 100 to 0.01 1/s. Strain sweeps were conducted from 0.01 to 100% strain at 1 rad/s and the results used to calculate the critical strain and linear viscoelastic region (LVR). The critical strain was calculated as the strain at which the complex modulus (G*) deviated by 3% from its previous value. Frequency

sweeps were performed from 0.1 to 100 rad/s at 75% of the smallest critical strain for all formulations (0.0775% strain).

Large amplitude oscillatory shear (LAOS) testing was conducted to determine the nonlinear viscoelastic behavior of the dressings. Samples were measured at 1, 10, 25, 50, and 100% strains at each of three frequencies (0.2, 2, and 20 rad/s).

4.3.4 Data analyses

A two-way analysis of variance (ANOVA) followed by Tukey's test was used to determine differences in microbiological data ($\alpha = 0.05$). Analyses were completed using SAS version 9.1(SAS, Cary, NC). Critical values and LAOS parameters were tested for statistical differences using ANOVA followed by Tuckey HSD (SAS 9.1). MITlaos (Version 2.1 beta, freeware distributed from MITlaos@mit.edu), developed by Ewoldt et al. (2007), was used to evaluate the stress and strain waveform data in MATLAB (MathWorks; Natick, Massachusetts, USA)

The shear rate curves for each formula were averaged together and the average curves were fitted to a Herschel Bulkley model (Eqn. 1) using the DHR3 TRIOS software. The Herschel Bulkley formula comprises a shear stress (σ, Pa), shear rate ($\dot{\gamma}$, 1/s), consistency coefficient (K, Pa.s^n), yield stress (σ_0, Pa), and flow behavior index (n, unitless).

$$\sigma = \sigma_o + K\gamma^n \quad (1)$$

4.4. Results and Discussion

4.4.1 Effects of protein stabilization on *Z. parabailii* growth

After 25 days of incubation, the concentrations of *Z. parabailii* at 25°C in CON and GLU were significantly different from each other and the protein-stabilized formulas containing acetic acid (MPI, WPI, and CAS) ($P<0.05$, Table A.4.1, Figure 4.1). WPI, CAS, and MPI showed no significant differences in *Z. parabailii* growth for any day during incubation. CON and GLU both had rapid growth of *Z. parabailii* between Day 0 and Day 10, resulting in a 3-log increase in concentration. Both CON and GLU had concentrations above 10^5 CFU/mL after 10 days of storage; concentrations above this threshold lead to noticeable undesirable fermented flavors (Lodovico 2003; Dang 2009). Growth of *Z. parabailii* in CON was also observed in a previous study (Chapter 3). The uninhibited growth of *Z. parabailii* in CON was most likely due to *Z. parabailii* resistance mechanisms. Previous studies have shown that acetic acid can be used as a carbon source for *Z. parabailii* (Suh 2013). If the concentration of acetic acid is sublethal, the rate at which acetate ions are metabolized in tricarboxylic acid cycle increases (Guerreiro 2012). The remaining H^+ ions are expelled from the cell via H^+–ATPase pumps to maintain the cytoplasm pH (Macpherson 2005). As for the rapid growth of *Z. parabailii* in GLU, *Z. parabailii* has shown the ability to assimilate and metabolize d-glucono-1,5-lactone (Suh 2013). D-glucono-1,5-lactone is the cyclic ester of gluconic acid in solution. It is possible that as the gluconic acid dissociated in the cytoplasm of *Z. parabailii*, it was converted into d-glucono-1,5-lactone and used as a food source.

As previously stated, both CON and GLU showed a 3-log increase in *Z. parabailii* during the first 10 days of storage. Growth rates in GLU reached the stationary phase after 10 days of storage and remained in a stationary growth phase for the remainder of the 45-day

storage period. CON also approached the stationary growth phase after 10 days of storage but transitioned into the death phase at approximately Day 15, as determined by the downward trend in concentration.

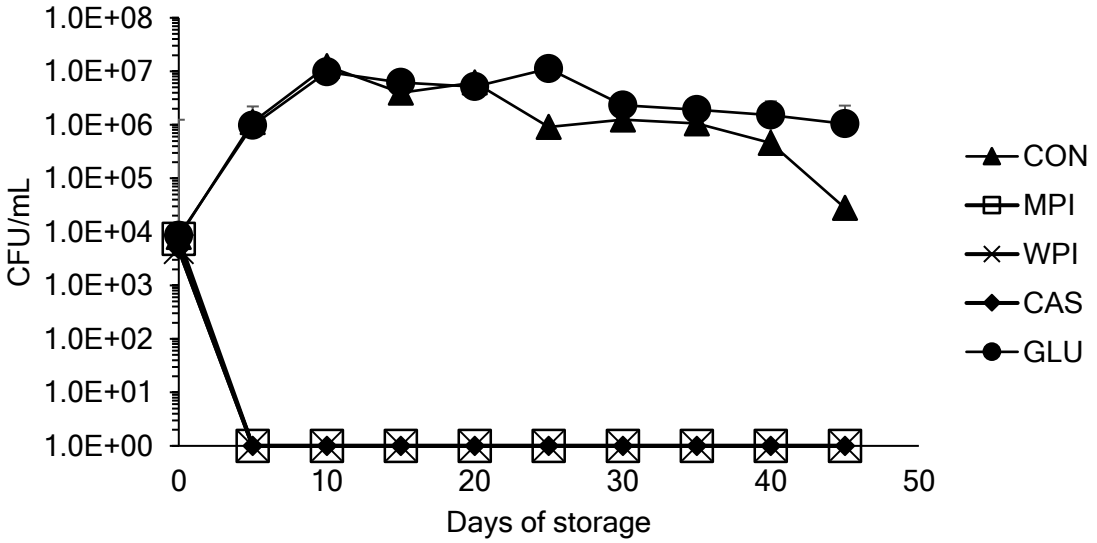

Figure 4.1 Growth of *Z. parabailii* in protein-stabilized dressings stored for 45 days at 25°C

All formulations stored at 25°C had an initially culturable inoculum that was added in before the salad dressings were stored (Figure 4.1). After 5 days of storage, the number of *Z. parabailii* cells in WPI, MPI, and CAS had fallen below the detectable limit for plate counting (25 CFU/mL). The rapid decline of *Z. parabailii* cells indicated that WPI, MPI, and CAS incited cell death. The negligible plate counts for these formulations were attributed to the low availability of fermentable sugars and a high concentration of acetic acid. After the starches, gums, and maltodextrin were removed from the formulation, the major source of carbon was from sucrose. *Z. parabailii* has a limited ability to ferment sucrose as a carbon source (Yang 2003). Limiting metabolites for *Z. parabailii* may have affected the ability of H^+–ATPase

pumps to remove H^+ ions from the cell. Another potential factor contributing to the reduced *Z. parabailii* growth in WPI, MPI, and CAS was the concentration of acetic acid in the sample. The concentration of acetic acid needed to inhibit *Z. parabailii* growth in glucose media ranges between 320–555 mM (Martoell 2007). The concentration of acetic acid in MPI, CAS, and WPI was approximately 550 mM. A higher concentration of acetic acid was needed to reduce the pH of the salad dressings to 4.2 in the protein-stabilized dressings as compared to the polysaccharide-stabilized dressing (CON) due to buffering capacity of milk proteins (Demetriades 1997). The elevated level of acetic acid in MPI, CAS, and WPI may have led to mitochondrial damage, resulting in *Z. parabailii* cell death (Ludovico 2003). *Z. parabailii* cell death in MPI, CAS, and WPI also occurred at both 10°C (Figure 4.2) and 4°C (Figure 4.3) possibly for the same reasons.

Interestingly, GLU at 25°C also required a higher concentration of gluconic acid to reach a pH of 4.2, but gluconic acid did not have the same effect on *Z. parabailii* growth as acetic acid (Figure 4.1). The high concentration of gluconic acid in GLU did not prevent the growth of *Z. parabailii*. Rather, it had the opposite effect. As previously discussed, the gluconic acid or its disassociated form may have been metabolized by *Z. parabailii* as a food source. Gluconic acid will dissociate to D-glucono-1,5-lactone in the cytoplasm to be in equilibrium. If D-glucono-1,5-lactone was metabolized and excess H^+ was removed by the proton pumps, this would both provide a food source and maintain a viable pH in the cytoplasm, allowing *Z. parabailii* to grow at higher acid concentrations. As for CON, the acetic acid concentration of 340 mM, which was at the low end of the inhibitory effect threshold, did not stop the growth of *Z. parabailii*. These results were not surprising because the inhibitory range for *Z. parabailii* is very wide and may vary between *Z. parabailii* strains.

GLU and CON did not show the same rapid growth of *Z. parabailii* when stored at 10°C as when stored at 25°C (Figure 4.2). *Z. parabailii* growth in GLU reached the stationary phase after 25 days of storage at 10°C, which was notably slower than the growth rate of *Z. parabailii* in GLU at 25°C. Similarly, *Z. parabailii*'s growth rate in CON did not reach the stationary phase until

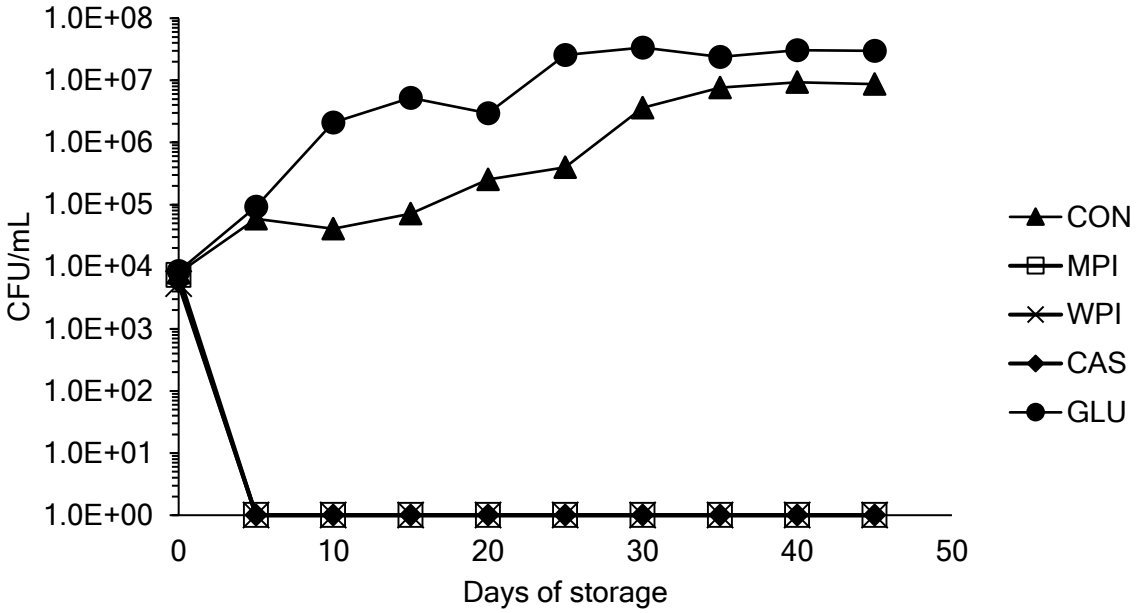

Figure 4.2 *Z. parabailii* growth in protein-stabilized dressings stored for 45 days at 10°C

35 days of storage at 10°C, but took 10 days to reach the stationary phase at 25°C. Although *Z. parabailii* has a slower rate of growth at 10°C compared to 25°C, CON and GLU still exceeded the spoilage threshold of 10^5 CFU/mL before the end of the 45-day storage period (Figure 4.2). GLU and CON reached this threshold between 5-10 days and 15-20 days of storage, respectively.

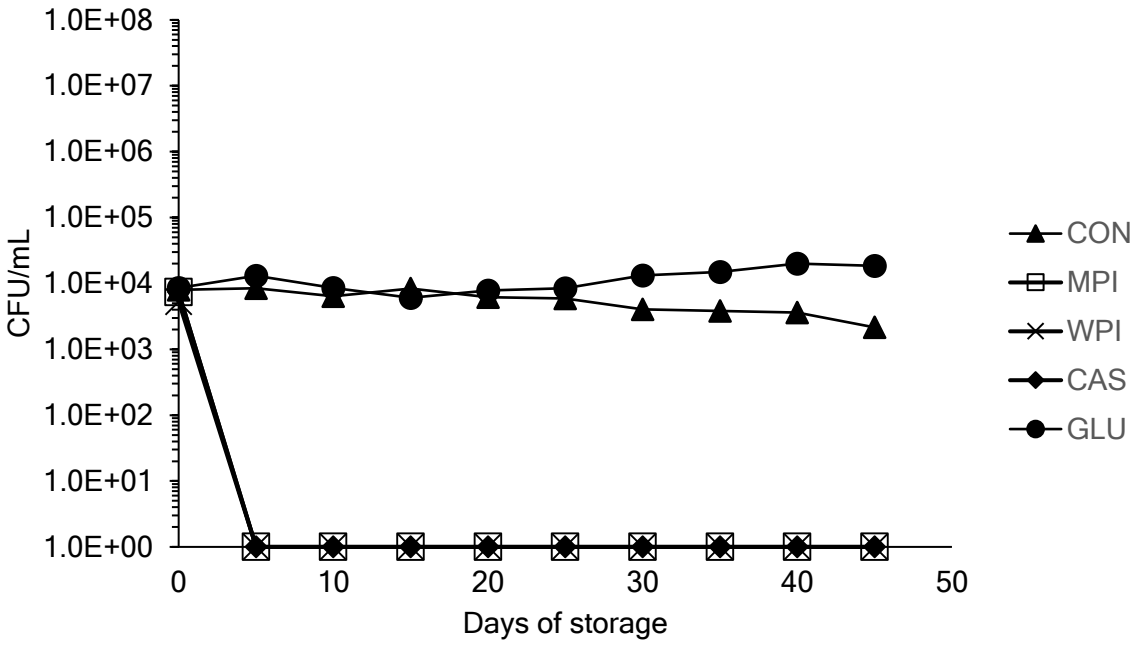

Figure 4.3 *Z. parabailii* growth in protein-stabilized dressings stored for 45 days at 4°C

Z. parabailii in CON and GLU stored at 4°C were in a lag phase of growth for the first 25 days of storage (Figure 4.3). After 30 days, the concentration of *Z. parabailii* in CON showed a downward trend, indicating a death phase, and the *Z. parabailii* concentration in GLU was slowly increasing, possibly transitioning from the lag phase into the log phase. MPI, CAS, and WPI exhibited a similar trend to these formulations stored at higher temperature: there were no detectable cells after five days of storage.

Overall, the factors that had the greatest effect on *Z. parabailii* inhibition were the storage temperature, the type of stabilizer, and the type of acid used in the formulation of the lite salad dressing. *Z. parabailii* stored at refrigeration temperatures (4°C) were more likely to have a lower number of viable *Z. parabailii* cells as compared to the higher storage temperatures. Increasing the storage temperature from 4°C to 10°C was sufficient to allow logarithmic growth of *Z. parabailii.* The substitution of milk protein stabilizers for

polysaccharide stabilizers prevented growth of *Z. parabailii* cells at all storage temperatures but only when acetic acid was used as the acidulant. GLU did not have the same effect on the growth and concentration *Z. parabailii* as MPI, although the two formulations had the same stabilizer. Thus, gluconic acid was insufficient to inhibit *Z. parabailii* growth. This inability to inhibit *Z. parabailii* growth in GLU may be due to how *Z. parabailii* processes gluconic acid. Further study on the metabolism of gluconic acid is needed to provide deeper insight into *Z. parabailii* survival mechanisms in media containing gluconic acid.

Overall, milk protein stabilizers with a 12.5% protein concentration acidified with acetic acid stored at 4°C had the best potential to inhibiting *Z. parabailii* growth in lite salad dressings. A caveat to using a protein-stabilized dressing over a polysaccharide-stabilized dressing may be the differences between the polysaccharides versus proteins stabilization mechanisms in salad dressings. This will be further discussed in the subsequent sections.

4.4.2 Dressing viscosity profiles

Table 4.2 Viscosity profiles for lite salad dressings

	Day 0			Day 45		
Sample	K (Pa.s)	n	σ_0 (Pa)	K (Pa.s)	N	σ_0 (Pa)
CON	7.92	0.584	17.9	13.0	0.522	29.5
MPI	1.56	0.812	0.22	1.15	0.74	0.631
GLU	3.10	0.691	1.98	0.41	0.982	7.26
WPI	5.26	1.00	7.67	3.76	1.00	5.10
CAS	3.46	0.622	1.52	1.42	0.695	0.688

All salad dressing formulations exhibited Herschel–Bulkley behavior except for WPI, which showed Bingham plastic behavior (Table 4.2) (Sun 2006). A Herschel-Bulkley fluid is

a non-Newtonian fluid that shear-dependent behavior with a yield stress. The Herschel-Bulkley model (Eqn. 1) uses three main parameters: the consistency coefficient, K ($Pa.s^n$), yield stress, σ_0 (Pa), and flow behavior index, n (Steffe 1996). Bingham plastic materials have a yield stress but exhibit Newtonian behavior after they begin to flow.

K values, which are strongly correlated to viscosity (Steffe 1996), differed amongst samples based on the type of stabilizer and acidulant used. The protein-stabilized salad dressings all differed in K values, where MPI and WPI showed significant difference from GLU and CAS. K values can be affected by the type (e.g. casein versus serum proteins) and concentration of milk protein used (Dickinson 2001). CON had the highest K value, which was attributed to stronger synergistic effects in the polysaccharide-stabilized formulation. Both polysaccharides and proteins form structures through entanglement, creating resistance to flow. The formation and strengthening of the polysaccharide network over time likely contributed to the increase in K over time for CON. The caseins in CAS can also form network structures. However, the pH of CAS was below the isoelectric point of casein (pI=4.6). When pH<pI, the charge on the casein micelles becomes increasingly positive, resulting in a release of calcium and inorganic phosphorus (Walstra 2006). The release of these minerals lowers the zeta potential of casein, causing them to lose their charge. Thus, these caseins would be unable to create a well-stabilized system. These changes to the casein micelle over time may have caused the decrease in K values for both MPI and CAS.

Interestingly, GLU had a higher K than MPI even though they were stabilized by the same concentration of milk protein isolate. In a previous study (Chapter 3), the acid(s) used in salad dressing formulations affected their rheological behaviors (Table 4.2). Similarly, the different rheological behaviors of MPI and GLU formulations were attributed to the different

acids in their compositions. The differences in rheological behavior may have been related to the pKa of the acids and the ability of the dissociated forms of the acids to interact with milk proteins (Walstra 2006). The charges on the weak acids would neutralize the charges on β-lactoglobulin and casein, which can affect the overall protein structure by changing the electrostatic repulsion among the proteins (Turgeon 1996). A reduction in electrostatic repulsion could have caused the protein network to weaken over time, leading to smaller K values.

K values decreased after 45 days for CAS, MPI, WPI, and GLU (Table 4.2), potentially from colloidal disruption over time. Whey protein solubility plays a key role in emulsion stability (Neirynck 2004). Factors that affect protein solubility include electrostatic interactions, hydrophobic interactions, and reactivity (Walstra 2006). Low pH affects all these factors and may contribute to a decrease in K values for MPI, WPI, and GLU over time due to the weakening of the whey protein-based network. Further, MPI and CAS may have had lower K values after storage due to changes in casein structures. Over time, β-casein undergoes proteolysis into γ-casein and protease peptone by plasmin (Walstra 2006). This proteolysis reduces colloidal phosphate stability. As the unstable phosphate converts to a more stable form, the casein–phosphate interactions are changed, resulting in an unstable casein structure. Instability in the casein structure would result in decreased K values over time, as the structure would be more easily broken down by shear. Furthermore, the alterations to protein structures during storage may have resulted in structures that unable to coat oil droplets as effectively, resulting in destabilization by aggregation and Oswalt ripening. Oswalt ripening occurs when a large oil droplet assimilates smaller surrounding oil

droplets over time. This can lead to a more heterogeneous oil droplet size, which can lower the viscosity (Walstra 2006; De Cássia da Fonseca 2009).

The change in yield stress varied by formulation on Day 0 and Day 45 (Table 4.2). CON had the highest yield stress and MPI had the lowest on Day 0. Yield stresses in CON, MPI, and GLU increased over time, while the yield stresses in WPI and CAS decreased. CON's yield stress likely increased due to increased polysaccharide entanglement over time, resulting in an increased energy requirement to break apart entanglements and initiate flow. The increases in yield stress in MPI and GLU could have been due to the presence of both casein and whey proteins in their formulations. The whey and casein proteins may be interacting with each other to create internal structure through van der Waals interactions, electrostatic interactions, and hydrogen bonding. At low pH, both casein and whey increase in hydrophobicity, promoting hydrophobic interactions.

Both CAS and WPI had a decrease in yield stress during storage due to the changes in the proteins over time that weakened the dressing structure. A weaker structure requires less energy to rupture and flow. The increase in electrostatic interactions at low pH and over time for both casein and whey would cause the proteins to aggregate and become less soluble. The aggregation would result in fewer proteins available to form a network, reducing the resistance to flow provided by the network structures.

Flow behavior index (n) values were dependent on the stabilizers used in the formulations. CAS and GLU showed increased n over time, signifying reduced shear-thinning behavior. The casein structures in CAS can change over time, as previously discussed, leading to the inability for casein to create strong intermolecular networks (Walstra 2006). A weak network reduces shear thinning because the network is quickly broken down at low shear

rates. GLU switched from shear-thinning behavior to Newtonian behavior. In other words, once the yield stress of GLU was surpassed, the internal network created by the proteins was not strong enough to influence the flow behavior. MPI and CON showed decreased n over time, indicating increased shear-thinning behavior. The increase in shear thinning in CON may have come from interaction between the starch and acetic acid in CON. The increase in shear thinning in MPI was likely due to interactions between acetic acid and the casein and whey proteins. WPI had an n of 1 at both 0 and 45 days of storage, indicating Newtonian behavior. This Newtonian behavior may stem from the solubility properties of whey proteins at low pH. Since whey proteins are less soluble at low pH, they are more likely to aggregate than form a network, reducing shear thinning behavior generated by network disruption.

4.4.3 Dressing strain sweep results

Table 4.3. Strain sweep results for salad dressings with different acidulants at 0 and 45 days[a]

Day	Sample	Phase angle (°)	Critical strain (%)	Critical stress (Pa)	G* at critical strain (Pa)
0	CON	11.2^{D}	0.70^{B}	2.42^{AB}	347^{B}
	MPI	20.5^{B}	1.00^{AB}	4.78^{A}	478^{A}
	GLU	24.9^{A}	0.32^{B}	1.10^{B}	345^{B}
	WPI	13.5^{C}	1.60^{A}	1.27^{B}	79.6^{C}
	CAS	20.0^{B}	1.59^{A}	1.71^{B}	108^{C}
45	CON	9.38^{D}	0.517^{C}	2.70^{B}	524^{A}
	MPI	14.7^{B}	0.250^{C}	5.55^{A}	231^{B}
	GLU	21.2^{A}	0.100^{B}	1.05^{CD}	106^{C}
	WPI	14.7^{C}	2.53^{A}	0.12^{D}	30.5^{D}
	CAS	19.3^{AB}	0.818^{B}	1.72^{C}	211^{B}

[a] Letters in each column that are different indicate significant differences ($P<0.05$)

Viscoelasticity is a mechanical property that can be used to predict how a material will behave when exposed to a stress or strain (Franco 1995). Many of these viscoelastic properties can be measured on a rheometer. Changes in viscoelastic properties can indicate stability problems or possible textural changes and help differentiate formulations with similar viscosities but different sensory textures (Chiralt 1992).

All formulations exhibited elastic-dominant behavior at 0 and 45 days of storage based on phase angle values (>45°) (Table 4.3). GLU had the highest phase angle before and after storage; The phase angle was statically different for all samples on Day 0 for all samples except MPI and CAS (Table 4.3). CON had the lowest phase angle before and after storage. The higher elasticity of CON was attributed to the polysaccharide-stabilized internal structure. The protein-stabilized salad dressings would have different internal structures and structural development compared to the polysaccharide-stabilized sample. Because the formulations had a pH of 4.2, milk proteins become less soluble at low pH because of the lack of proper electrostatic repulsion with themselves or other dairy proteins (Dickinson 2008). Over time, the phase angle significantly decreased for CON, MPI, and GLU; WPI and CAS phase angles did not significantly change. A decrease in the phase angle indicates an increase in solid-like behaviors. The starch sample had polysaccharides that entangle themselves over time resulting in a more network-like internal structure which increased elastic behavior. Protein-stabilized samples containing both casein and whey (MPI and GLU) showed differences in phase angle but samples with either casein or whey did not. The interaction between whey and casein over time in a low-pH system likely created a stronger internal structure, resulting in increased solid-like behavior.

Critical strains for all protein-stabilized samples decreased over time, resulting in a smaller LVR. The critical stress increased significantly for CON and MPI and decreased significantly for CAS, WPI, and GLU. The changes in critical values may be related to the degree of destabilization in the protein-stabilized emulsions during storage, as discussed in the previous section.

Critical stress for CON and MPI increased over time. Critical stress is the critical strain multiplied by G*; this value is related to the yield stress. CON and MPI critical stress increased over time and WPI critical stress decreased over time in agreement with the yield stress values from the shear rate sweep data. GLU and CAS showed no statistical difference in critical stress before and after storage, which was not expected. WPI critical stress decreased over time and GLU critical stress had no significant change over time. The WPI and GLU values were not in complete agreement with the yield stress values determined from shear rate sweeps, but that may be due to different manner in which shear is applied in oscillatory versus rotational tests.

4.4.4 Frequency sweeps for protein-stabilized salad dressings

All salad dressing formulations exhibited elastic-dominant behavior over the frequency range tested (Figure 4.4), in agreement with the data at critical strain. All formulations also showed frequency dependence and weak gel behavior. These results were likely due to the gel-like network structure in the protein-stabilized dressings formed through protein entanglement and interactions. These interactions play a role in establishing a weak gel network structure (Diftis 2005).

G' and G" values for all protein-stabilized formulations decreased from Day 0 to Day 45 (Figure 4.4, 4.5). CON had a slight increase in G' and G" from Day 0 to Day 45. The

increase in moduli values for CON may be due to entanglement of polysaccharides over time; a decrease in moduli values for protein samples may have been due to emulsion destabilization influenced by the change in the milk proteins interaction in a low pH environment. While samples were not visibly separated, destabilization at the micron level, caused by changes in the protein charge distribution, could have occurred and caused these results.

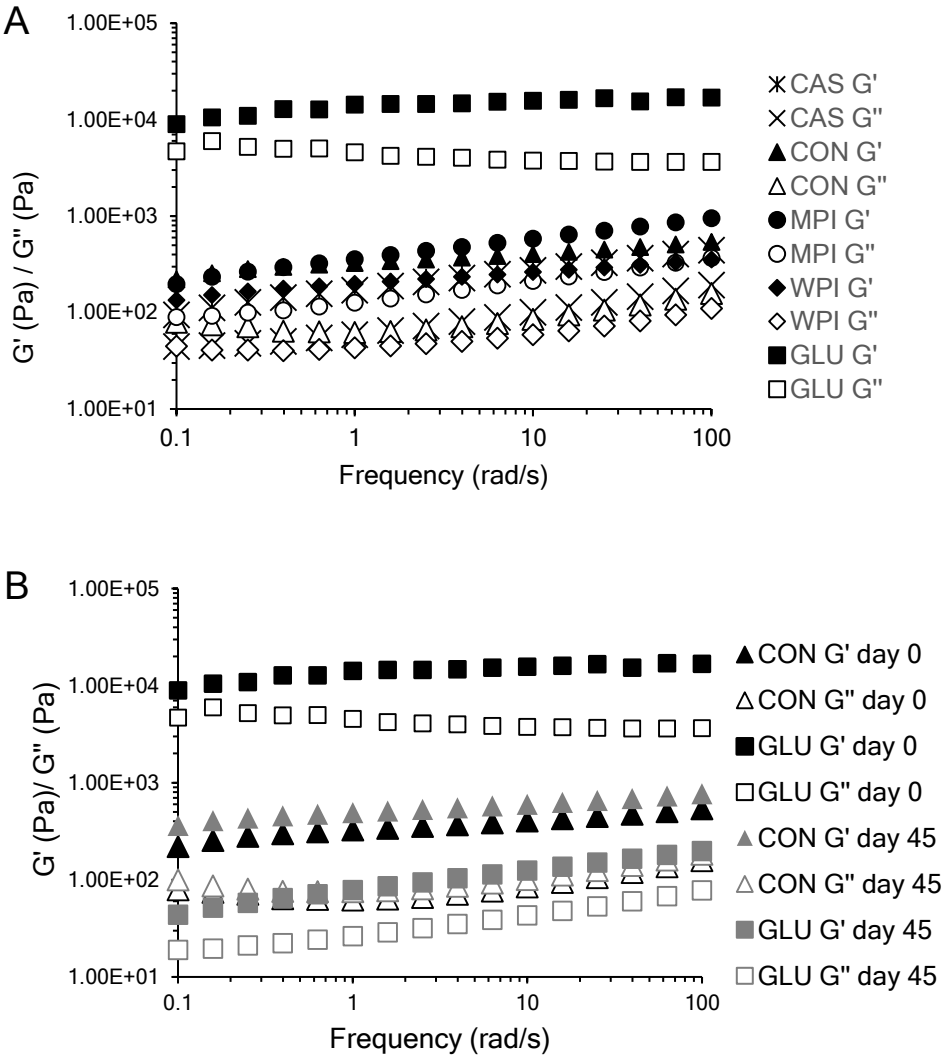

Figure 4.4. A) Frequency sweep for salad dressings formulations with protein based stabilizers before 45-day incubation B) Select comparisons of frequency sweeps for salad dressing formulations for Day 0 versus Day 45 of incubation

All protein-stabilized formulations had a sharp decline in moduli values over time when compared to CON formulations. This suggests that droplet rearrangements were continuously taking place immediately after the emulsion was prepared, leading to a weaker network structure.

4.4.4 Large amplitude oscillatory shear of protein-stabilized salad dressings

Nonlinear behavior was quantified using the LAOS analysis developed by Ewoldt et al. (2008). The amount of nonlinear elastic behavior exhibited by a sample was evaluated through the ratio of G'_3 and G'_1. When $G'_3/G'_1 > 0.01$, samples exhibit nonlinear behavior. As the value of G'_3/G'_1 increases above 0.01, the amount of nonlinear behavior increases. Samples with $G'_3/G'_1 < 0.01$ have linear behavior. G'_3 and G'_1 was used for comparison instead of G''_3/G''_1 because samples exhibited elastic-dominant behaviors. The other parameters used to evaluate the LAOS data was the ratio of the large strain elastic modulus (G_L) to the minimum strain elastic modulus (G_M) and the ratio of the instantaneous viscosities at maximum shear rate (η_L) to minimum shear rate (η_M) . G_L/G_M indicated the type of nonlinear elastic behavior exhibited. $G_L/G_M > 1.1$ indicated strain hardening and $G_L/G_M < 0.9$ indicated strain softening. Similarly, the ratio of η_L/η_M was used to measure viscous-related nonlinear behavior. Values greater than 1.1 indicated shear thickening and values less than 0.090 indicated shear thinning.

LAOS parameters for all samples are shown in Tables 4.4 and 4.5. In general, G_L/G_M and G'_3/G'_1 increased with increased strain. At 1% strain for all frequencies, most protein-stabilized formulations prepared with acetic acid showed linear viscoelastic behavior. On the other hand, GLU and CON showed nonlinear elastic behavior at 1% strain at 0.2 rad/s.

Table 4.4 LAOS elastic table for salad dressings formulations with and without protein stabilization incubated on Day 0[ab]

		CON		GLU		CAS		MPI		WPI	
Frequency rad/s	Strain (%)	G_L /G_M (Pa)	G_3'/G_1' (Pa)	G_L /G_M (Pa)	G_3'/G_1' (Pa)	G_L /G_M (Pa)	G_3'/G_1' (Pa)	G_L /G_M (Pa)	G_3'/G_1' (Pa)	G_L /G_M (Pa)	G_3'/G_1' (Pa)
0.2	1	1.10^{KL}	0.051^{BC}	1.25^{HIJ}	0.081^{BC}	1.00^{KL}	0.002^{C}	1.00^{KL}	0.001^{C}	1.00^{KL}	0.006^{C}
	10	1.28^{GHI}	0.062^{BC}	1.42^{EFG}	0.087^{BC}	1.15^{IJK}	0.017^{C}	0.996^{L}	0.001^{C}	1.09^{JKL}	0.024^{C}
	25	1.45^{EF}	0.107^{BC}	1.46^{EF}	0.129^{BC}	1.67^{CD}	0.059^{BC}	1.04^{KL}	0.0122^{C}	1.1^{JKL}	0.023^{C}
	50	1.55^{DE}	0.115^{BC}	1.65^{CD}	0.133^{BC}	1.73^{C}	0.060^{BC}	1.36^{FGH}	0.27^{B}	1.11^{JKL}	0.025^{C}
	100	1.93^{B}	0.170^{BC}	2.04^{B}	0.181^{BC}	2.28^{A}	0.193^{BC}	1.35^{FGH}	1.82^{A}	1.10^{JKL}	0.024^{C}
2	1	1.00^{K}	0.001^{L}	1.09^{HIJ}	0.0001^{L}	1.00^{K}	0.001^{L}	1.00^{K}	0.001^{L}	1.00^{K}	0.001^{L}
	10	1.17^{FGHI}	0.042^{HI}	1.11^{GHIK}	0.036^{J}	1.17^{FGHI}	0.043^{H}	1.03^{JK}	0.019^{K}	1.05^{JK}	0.0145^{K}
	25	1.32^{C}	0.063^{FG}	1.14^{GHIJ}	0.038^{IJ}	1.30^{CDE}	0.062^{FG}	1.08^{HIJK}	0.015^{K}	1.03^{JK}	0.0146^{K}
	50	1.23^{CDEF}	0.057^{G}	1.27^{CDEF}	0.084^{D}	1.31^{C}	0.077^{E}	1.28^{CDE}	0.064^{F}	1.19^{DEFG}	0.044^{H}
	100	1.31^{CD}	0.083^{ED}	1.48^{B}	0.106^{B}	1.32^{C}	0.096^{C}	1.69^{A}	0.134^{A}	1.18^{EFGH}	0.042^{HI}
20	1	1.00^{HIJ}	0.001^{B}	1.00^{HIJ}	0.001^{B}	0.995^{HIJ}	0.003^{B}	1.00^{HIJ}	0.001^{B}	1.00^{HIJ}	0.003^{B}
	10	1.21^{CDEF}	0.054^{AB}	1.00^{HIJ}	0.001^{B}	1.00^{HIJ}	0.001^{B}	1.01^{HIJ}	0.005^{B}	0.92^{IJ}	0.021^{B}
	25	1.30^{CDE}	0.072^{AB}	1.08^{FGHI}	0.0236^{B}	1.15^{DEF}	0.011^{AB}	1.24^{CDE}	0.114^{AB}	1.04^{HIJ}	0.017^{B}
	50	1.28^{CDEF}	0.054^{AB}	1.42^{ABC}	0.225^{A}	1.10^{DEF}	0.040^{B}	1.53^{AB}	0.052^{AB}	1.04^{HIJ}	0.013^{B}
	100	1.33^{BCD}	0.072^{AB}	1.54^{AB}	0.096^{AB}	1.15^{DEF}	0.042^{B}	1.58^{A}	0.134^{AB}	0.85^{J}	0.028^{B}

[a] Absolute values of G_3'/G_1' and G_L'/G_M' were used

[b]Tukey's letter designations showed statistical differences between samples tested at the same frequency

Table 4.5 LAOS viscous table for salad dressings formulations with and without protein stabilization incubated on Day 0

		CON		GLU		CAS		MPI		WPI	
Frequency rad/s	Strain (%)	η_L/η_M (Pa)	G_3''/G_1'' (Pa)	η_L/η_M (Pa)	G_3''/G_1'' (Pa)	η_L/η_M (Pa)	G_3''/G_1'' (Pa)	η_L/η_M (Pa)	G_3''/G_1'' (Pa)	η_L/η_M (Pa)	G_3''/G_1'' (Pa)
0.2	1	1.00^{C}	0.008^{DEFG}	0.764^{J}	0.116^{B}	1.00^{C}	0.028^{DEFG}	1.00^{C}	0.001^{G}	1.00^{A}	0.009^{G}
	10	1.04^{EF}	0.001^{DEFG}	0.627^{K}	0.136^{B}	0.943^{EF}	0.011^{FG}	1.04^{B}	0.011^{FG}	0.953^{FG}	0.012^{EFG}
	25	0.96^{B}	0.027^{G}	0.802^{I}	0.066^{CDE}	0.944^{EF}	0.014^{FG}	1.12^{A}	0.023^{EF}	0.908^{EFG}	0.027^{EF}
	50	0.98^{C}	0.019^{FG}	0.843^{H}	0.059^{C}	0.960^{DE}	0.017^{FG}	1.09^{A}	0.015^{DC}	0.925^{EFG}	0.023^{E}
	100	1.12^{C}	0.025^{FG}	0.872^{H}	0.051^{CDE}	0.904^{G}	0.041^{DEFG}	0.989^{CD}	0.028^{DC}	0.941^{FG}	0.016^{EF}
2	1	1.00^{DE}	0.001^{H}	1.10^{B}	0.009^{GH}	1.03^{DC}	0.001^{H}	1.00^{DE}	0.001^{H}	1.00^{DE}	0.001^{H}
	10	0.93^{C}	0.030^{EFGH}	0.744^{J}	0.057^{CD}	1.04^{C}	0.030^{EFGH}	1.04^{C}	0.011^{FG}	0.934^{G}	0.016^{EFGH}
	25	0.84^{EF}	0.045^{EFGH}	0.796^{I}	0.041^{CDF}	0.961^{FG}	0.045^{DEFG}	1.12^{B}	0.023^{FG}	0.934^{G}	0.016^{EFGH}
	50	1.30^{EF}	0.056^{EFGH}	0.760^{J}	0.070^{C}	1.32^{A}	0.056^{EFG}	1.09^{B}	0.015^{EF}	0.851^{H}	0.042^{CDE}
	100	1.32^{B}	0.071^{EFGH}	0.765^{JI}	0.408^{B}	1.12^{B}	0.025^{GH}	0.989^{EF}	0.028^{FG}	0.855^{H}	0.039^{CDEF}
20	1	1.00^{C}	0.001^{J}	1.00^{C}	0.002^{J}	1.00^{C}	0.001^{J}	1.00^{C}	0.001^{J}	1.00^{C}	0.001^{J}
	10	1.06^{D}	0.016^{EFGHI}	1.00^{C}	0.009^{IJ}	0.935^{D}	0.001^{J}	1.07^{E}	0.018^{HI}	0.913^{D}	0.023^{GHI}
	25	1.16^{E}	0.037^{CD}	0.989^{C}	0.003^{J}	0.843^{E}	0.027^{GHIJ}	1.07^{E}	0.013^{FG}	0.833^{E}	0.048^{DFG}
	50	1.16^{A}	0.024^{CD}	0.863^{E}	0.038^{EFG}	0.787^{F}	0.15^{B}	0.917^{D}	0.098^{A}	0.784^{F}	0.064^{CE}
	100	0.99^{A}	0.028^{CD}	0.725^{G}	0.087^{C}	0.796^{F}	0.053^{DEFG}	0.785^{F}	0.068^{C}	0.768^{F}	0.061^{FCE}

[a] Absolute values of G_3''/G_1'' and η_L'/η_M' were used

[b]Tukey's letter designations showed statistical differences between samples tested at the same frequency.

The presence of nonlinear viscoelastic behavior at 1% strain was not unexpected because the critical strain of GLU and CON (Table 4.4) was lower than 1%. As frequency increased, all formulations tended to have reduced elastic nonlinear behavior. This may have been due to a shift to viscous-dominant behaviors with increased strain. As strain increased, the amount of nonlinear behavior increased, as expected. Strain hardening was the predominant type of nonlinear elastic behavior at lower frequencies for all samples; shear thinning nonlinear viscous behaviors appeared at higher frequencies for protein-stabilized formulations (Tables 4.4, 4.5).

GLU had both significant nonlinear elastic-dominated behavior and significant viscous-dominated behavior. GLU showed strain hardening behavior for all strains at 0.2 rad/s and for strains ≥10% at 2 and 20 rads/s. GLU also had shear thinning behavior at all strains for 0.2 rad/s, at strains ≥ 10% for 2 rad/s, and at strains ≥ 50% for 20 rad/s. This was indicated by having a $\eta_L/\eta_M < 0.9$. G_M/G_L increased with increased strain where η_L/η_M. generally decreased with increased strain. The amount of nonlinear viscous behavior decreases as frequency increases due to the same reasons stated above. Comparing GLU to MPI, which had the same protein stabilizer, the onset of the nonlinear behaviors in GLU occurred at smaller strains. This was expected because the critical strain of MPI is greater than GLU (Table 4.3). MPI had the same trends in η_L/η_M and G_L/G_M as GLU, which was expected because they both used the same protein stabilizer. Strain hardening behavior for MPI occurred at ≥25% strain at 0.2 and 2 rad/s, and at ≥10% strain at 20 rad/s. MPI also differed from GLU in that MPI did not show shear thinning behavior until 20 rad/s at 100% strain.

CAS had significant nonlinear behavior for both viscous- and elastic- behaviors. CAS showed shear thickening behavior at strains ≥10% at 0.2 rad/s and 2 rad/s, and ≥25% at 20 rad/s. The greatest extent of shear thickening was found in CAS at 0.2 rad/s at 0.2% strain. As strain hardening increases, the amount of stress needed to deform the structure also increases. CAS also showed both shear thinning and thickening behaviors. Shear thickening behaviors were shown at 2 rad/s at strains ≥50%, and shear thinning behaviors were shown at 20 rad/s for strains ≥25%. The increase in shear thinning behavior at higher frequencies may be due to the increase in fluidlike behaviors at higher frequencies and strains.

WPI had significant elastic nonlinear behaviors at 2 rad/s and 20 rad/s. WPI showed strain hardening ≥ 25% strain at 2 rads/s and exhibited strain softening at 100% at 20 rad/s which was completely different from all of the other samples. This may be due to whey proteins being less soluble at pH near their isoelectric point, reducing their ability to create rigid structures. Strain softening in WPI may also be due to a decrease in relaxation time increasing fluidlike behavior. The WPI has shear thinning viscous behaviors ≥ 50% at 2 rad/s and 25% at 20 rad/s.

The variations in nonlinear behaviors among the samples over time can also be viewed in Lissajous plots (Figure 4.5). Lissajous plots are plots of stress versus strain, where the deviation from an elliptical shape indicates nonlinear behavior (Ewoldt 2008). The ellipses on Figure 4.5 show greater distortion at higher strains. This distortion is related to the amount of nonlinear behavior the samples are undergoing. This increase in nonlinear behavior is also shown in Table 4.4. After 45 days, the area encompassed by the curve at 1% strain greatly decreased for MPI and WPI, corresponding to the decrease in G'_3/G'_1 at lower strains. During storage, the protein-stabilized formulations MPI and WPI had the most change in their

nonlinear behaviors because MPI and WPI 1% and 100% strain Lissajous had the largest decreased in size and change shape between Day 0 and Day 45.

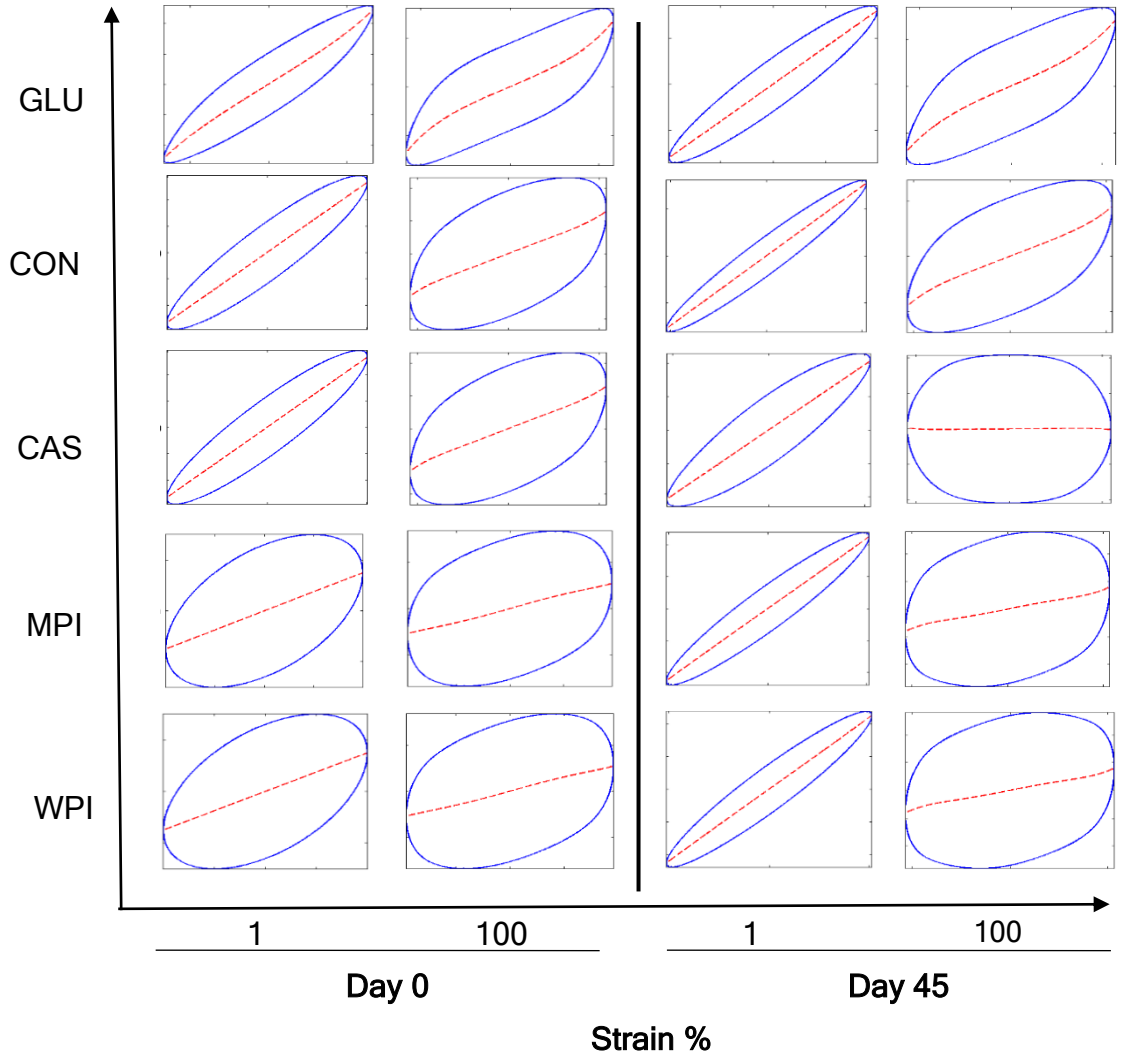

Figure 4.5 Summarized elastic Lissajous plots for LAOS parameters at Day 0 and at Day 45 at 2 rad/s. More detailed plots are included in the appendices (Figures A.4.1-10)

Of the formulations tested, CON had the fewest changes in small- and large-strain rheological behavior over time. MPI had the least amount of change in viscosity behavior over time comparted to the other protein-stabilized dressings. The polysaccharide-based

stabilizer used in CON was able to maintain a stable structure over the 45-day storage period due to polysaccharide self-interactions and interactions among the polysaccharides. However, CON was not able to prevent the growth of *Z. parabailii* at storage temperatures above 4°C. The concentration of *Z. parabailii* in CON at these temperatures was greater than 10^5 CFU/mL, which would cause off-flavors from fermentation. The protein-stabilized formulations can prevent the growth of *Z. parabailii* better than polysaccharide-based stabilizers, but the use of a protein stabilizer in place of the polysaccharide stabilizer may result in different internal structure development. Protein-stabilized dressings acidified with acetic acid prevented *Z. parabailii* growth at all storage temperatures studied. Of the protein-stabilized formulas evaluated, CAS was the closest to the traditional salad dressing formula (CON) on Day 0 in terms of rheological behavior, but the structural changes in CAS during storage resulted in a decrease in many of the viscoelastic properties and increased nonlinear viscoelastic behavior compared to CON. MPI had the most rheological stability over time while maintaining a closer match to the rheological behaviors of CON. Overall, the formulation containing milk protein isolate and acetic acid (MPI) had good inhibition of *Z. parabailii* at all storage temperatures for 45 days and had the least amount of change in rheological behaviors of the protein-stabilized samples. These results indicate that protein stabilizers, particularly MPI, are a viable option for use as a stabilizing system in salad dressing.

4.5 Conclusions

Z. parabailii growth can be difficult to inhibit in salad dressings because of its high acid tolerance. This study showed that the incorporation of milk proteins for stabilizing lite salad dressings can inhibit the growth of *Z. parabailii*. Other factors that were important for *Z.*

parabailii inhibition were the type of acidulant used in conjuncture with the proteins and the storage conditions of the formulations. Refrigeration temperatures (4°C) showed promising results for inhibiting *Z. parabailii*; salad dressings stored at temperatures above 4°C have a risk of *Z. parabailii* spoilage. Salad dressings made with a milk protein stabilizer and acetic acid stopped growth of *Z. parabailii* between 4 and 25°C, showing that the protein-based stabilizers were effective at reducing *Z. parabailii* growth.

Salad dressing formulations with protein stabilizers showed changes in viscosity and viscoelastic properties over time. The formulation prepared with milk protein isolate showed the least amount of change in rheological behaviors over time, indicating that this formulation may be more stable than the other protein stabilized dressings. The protein-stabilized dressings had a higher variability in rheological properties compared to the polysaccharide-stabilized dressing. Because starch stabilizers may be more proficient at preventing colloidal disruption during storage, additional work should be done to determine protein–starch combinations that will prevent *Z. parabailii* growth over time at temperatures above 4°C while maintaining emulsion stability.

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CHAPTER 5: CONCLUSIONS

Z. parabailii is an important spoilage yeast that can grow in low-pH foods such as lite salad dressing. This yeast is known for its resistance to weak acids. The inhibiting abilities of weak acids on *Z. parabailii* growth changed based on the different acid combinations used in the formulations, storage temperature, and stabilizer composition. The information from this study on *Z. parabailii* growth can be used to develop strategies reducing spoilage in lite salad dressings caused by *Z. parabailii*.

In this work, different combinations of acidulants were used to inhibit *Z. parabailii* growth. Of the acids evaluated, acetic acid used alone was the most effective for inhibiting *Z. parabailii* growth in both polyaccharide- and milk protein isolate-stabilized dressings. Unfortunately, none of the acid combinations used in polysaccharide-stabilized salad dressing formulations stored at 10 or 25°C prevented spoilage from *Z. parabailii.* However, storage of these formulations at 4°C inhibited the growth of *Z. parabailii* and reduce *Z. parabailii* concentration over time regardless of acidulant(s) used. These results demonstrated the importance of a low, stable storage temperature when working with *Z. parabailii*, as it will grow at temperatures even slightly elevated from 4°C.

The best inhibitor of *Z. parabailii* was acetic acid combined with the milk proteins used to create a protein-stabilized salad dressing. In these formulations, *Z. parabailii* cells were no longer culturable after 5 days of storage at 4, 10, or 25°C. Based on the results of this work, the optimal way to prevent *Z. parabailii* growth is to use a milk protein stabilizer in the dressing formulation and store the dressing at 4°C.

Changes in formulations of lite salad dressings to inhibit *Z. parabailii* growth in salad dressings may impact their rheological behaviors. The viscoelastic behavior and viscosity of all salad dressing formulations evaluated showed changes over time. The degree of change was affected by the acid and stabilizer used. Of the protein-stabilized formulations, the formulation incorporating milk protein isolate and acetic acid had the least amount of change in rheological behavior over time. For the best reduction of *Z. parabailii* growth and the least impact on rheological behavior, a dressing made with milk protein isolate and acetic acid is recommended.

Possible follow-up studies on this research topic revolve around sensory analyses and use of a combination of proteins and polysaccharides to stabilize salad dressing. Protein ingredients can be expensive and finding an optimal formulation that inhibits *Z. parabailii* growth and includes both polysaccharides and proteins can reduce production costs while maintaining dressing quality and texture. A sensory study comparing 1) a pure protein-stabilized lite salad dressing, 2) a polysaccharide- and protein-stabilized dressing, and 3) a pure polysaccharide-stabilized dressing to determine how specific stabilizers affect human perception of salad dressing texture and flavor attributes. While future studies further explore ways to inhibit *Z. parabailii* growth in salad dressings while maintaining appealing sensory attributes, the results of this study provide the key information for the reduction of *Z. parabailii* growth in lite salad dressings.

APPENDIX A: Supplemental Tables

Table A.3.1 Tukey's grouping for *Z. parabailii* growth in salad dressings formulations stored at 4°C, 10°C, and 25°C[a]

Formulations	Tukey grouping 25°C	Tukey Grouping 10°C	Tukey Grouping 4°C
GDL	A	CB	CD
GDL 2%	ABC	A	D
A	BCD	E	CB
L	ABCD	C	D
GA	AB	ED	CB
GA2	ABCD	E	B
GL	CD	CB	B
GL2	D	B	B
GAL	ABCD	CD	A
AL	AB	C	CB

[a] Different letters in different columns indicate significant differences ($\alpha = 0.05$)

Table A.4.1 Tukey grouping for CFU/ml for Zygosaccharomyces parabailii between formulations stored at 25°C, 10°C and 4°C[a]

Formulations	Tukey grouping 25°C	Tukey Grouping 10°C	Tukey Grouping 4°C
WPI	C	C	C
MPI	C	C	C
GLU	A	A	A
CAS	C	C	C
CON	B	B	B

[a] Different letters in different columns indicate significant differences ($\alpha = 0.05$)

APPENDIX B: Supplemental Figures

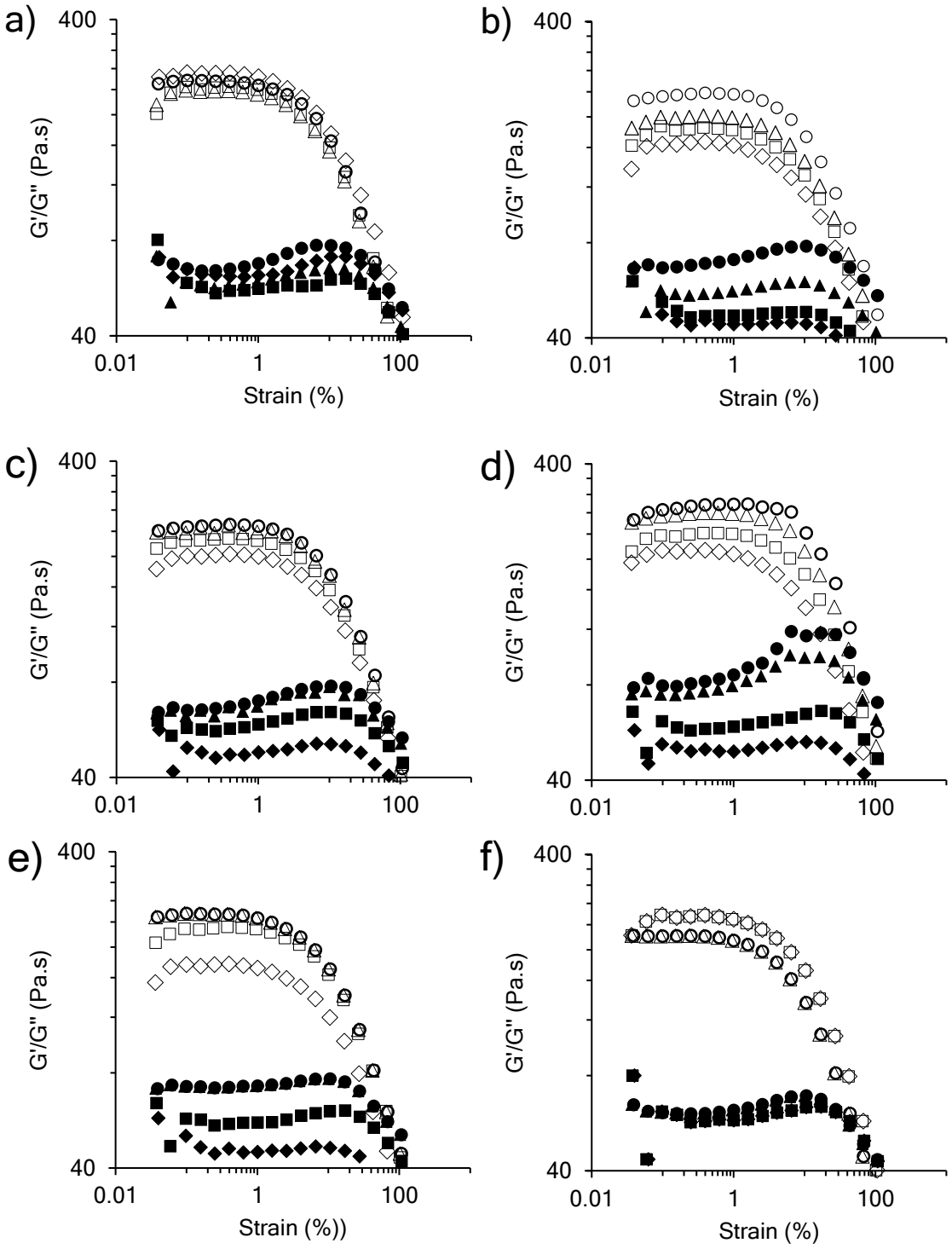

Figure A.3.1 Strain sweep from 0 to 100 rad/s at 0.075% strain where A) GLU, B) AC, C) LA, D) GA, E) GA2, F) GL, G) GL2, H) GAL, and I) AL. Each time point is represented as follows: Day 0 (○) Day 15 (Δ) Day 30 (□) Day 45 (◊). Open symbols represent G', closed symbols represent G"

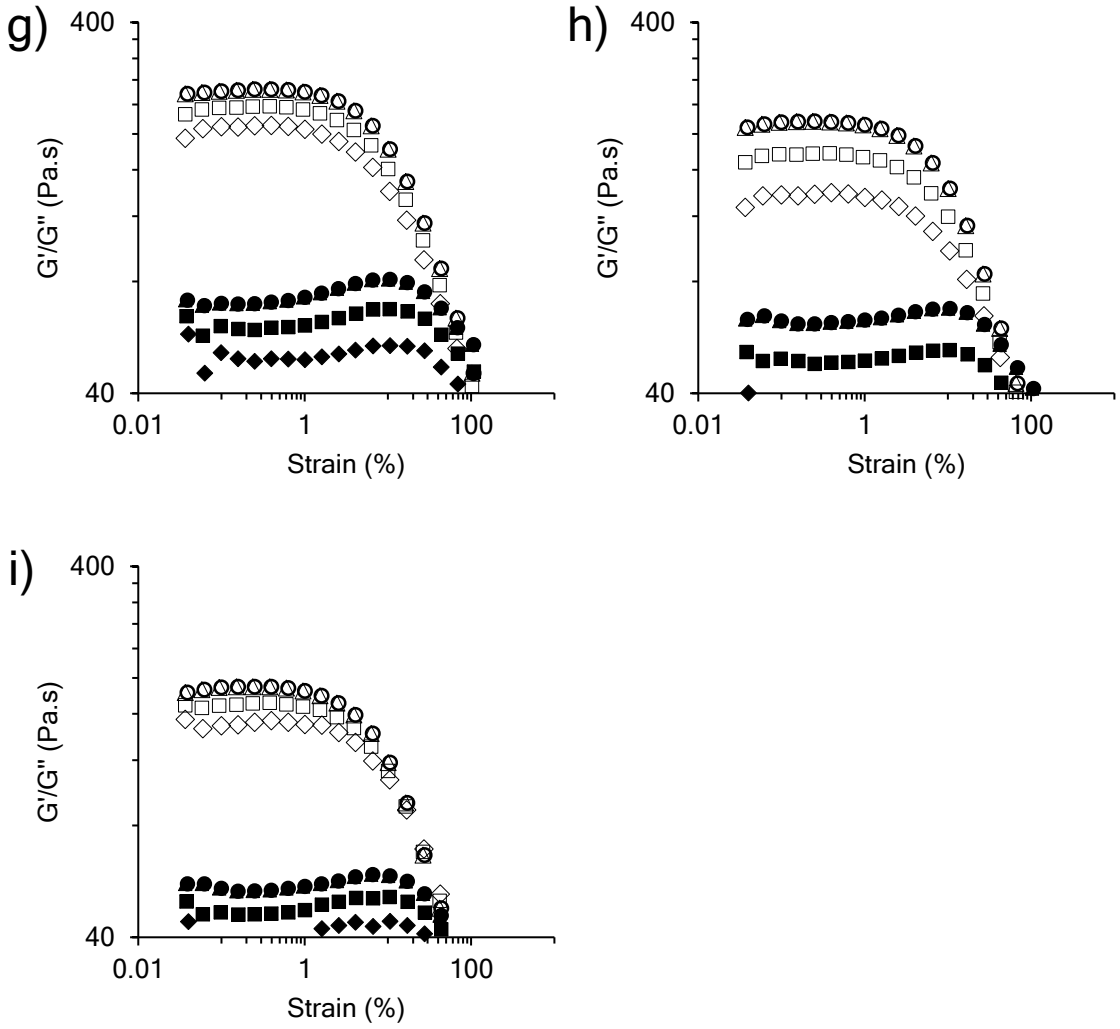

Figure A.3.1 (continued from previous page)

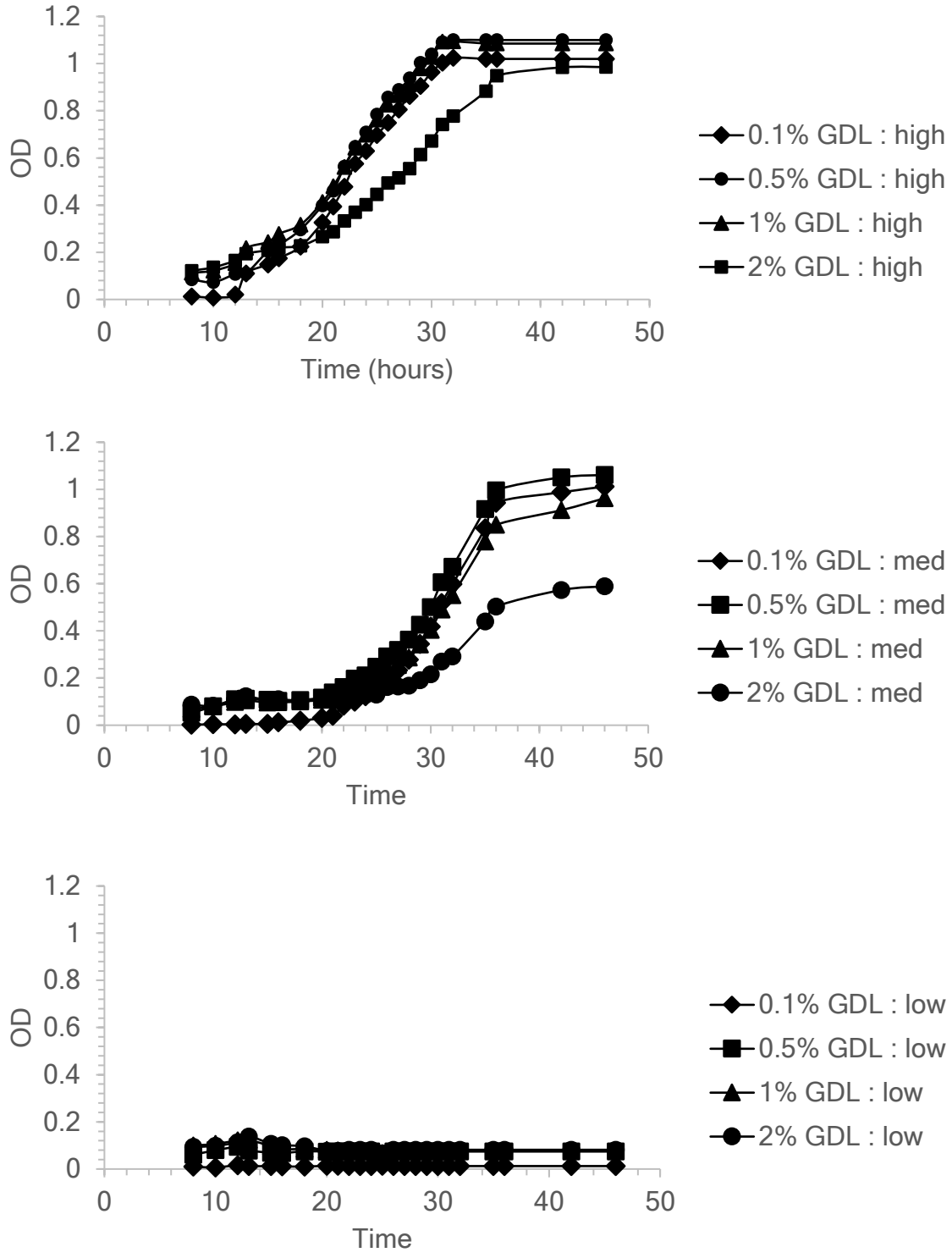

Figure A.3.2 Growth curves of Z. parabailii grown in yeast mold broth with different concentrations of gluconic acid (GDL) at a high inoculum (10^4 CFU/mL), medium inoculum (10^3 CFU/mL), and low inoculum (10^2 CFU/mL). The growth Z. parabailii was observed for 46 hr using a spectotronic 20D

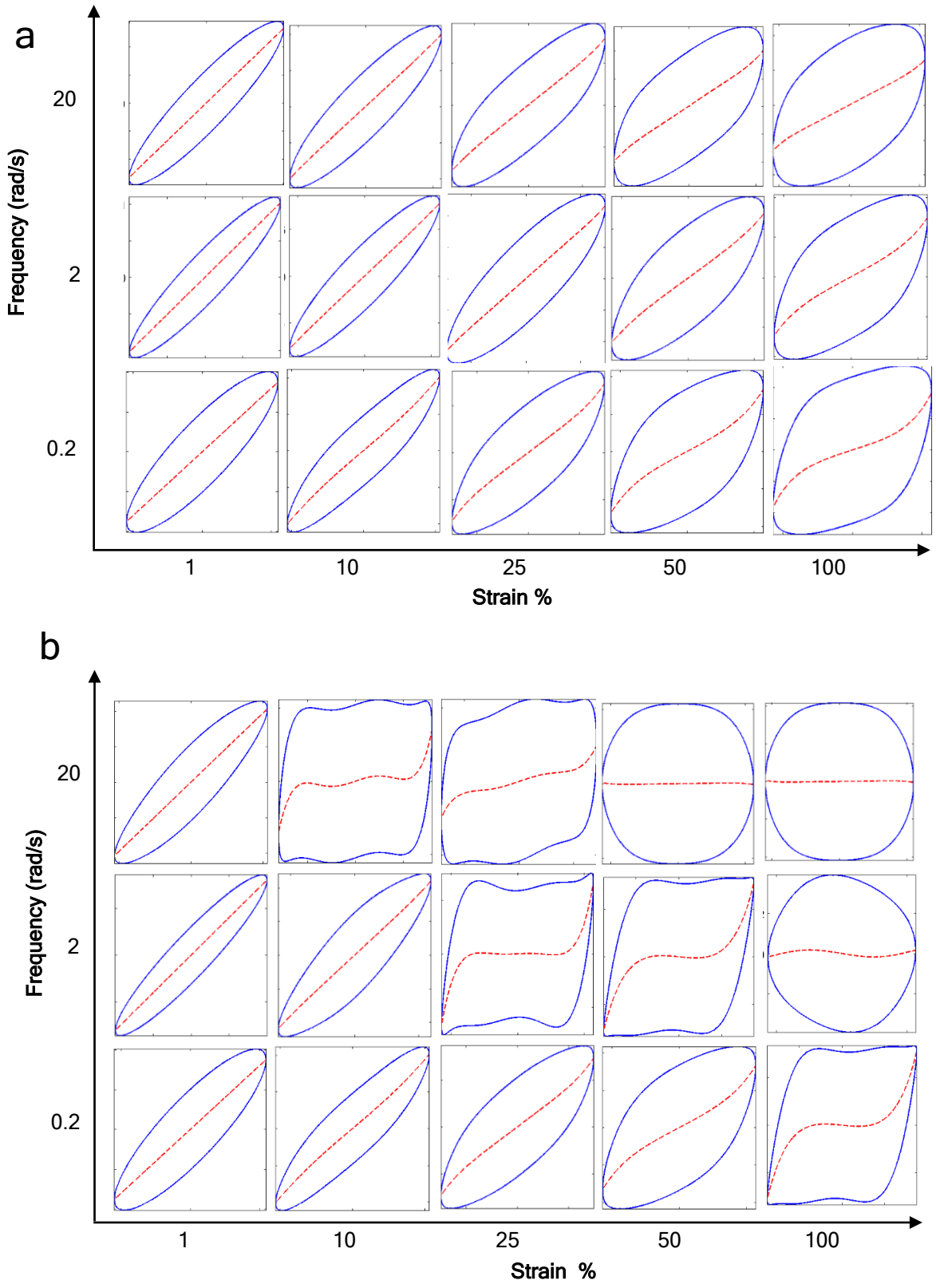

Figure A.4.1 Elastic Lissajous plots for CAS at a) Day 0 and b) Day 45

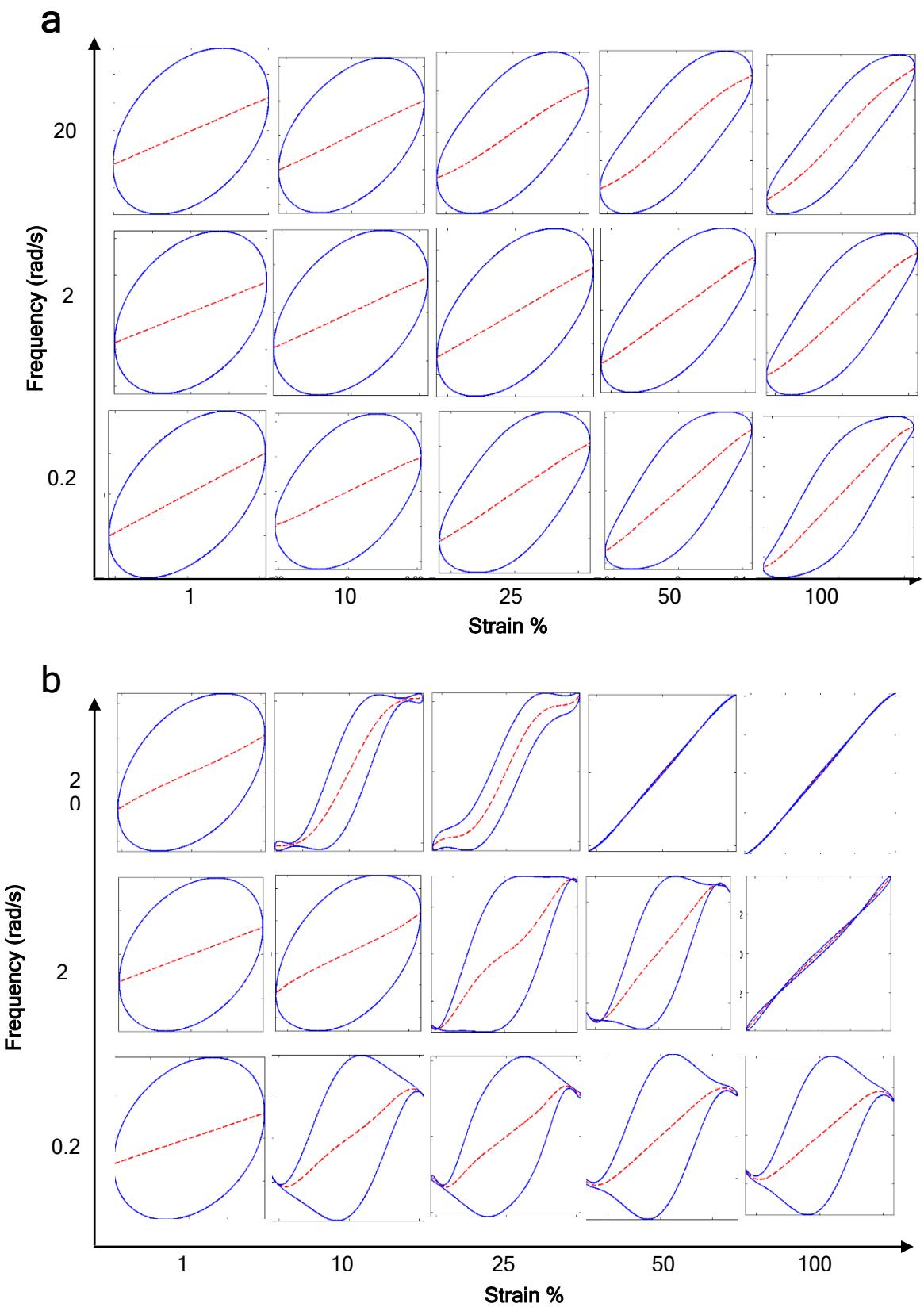

Figure A.4.2 Viscous Lissajous plots for CAS at a) Day 0 and b) Day 45

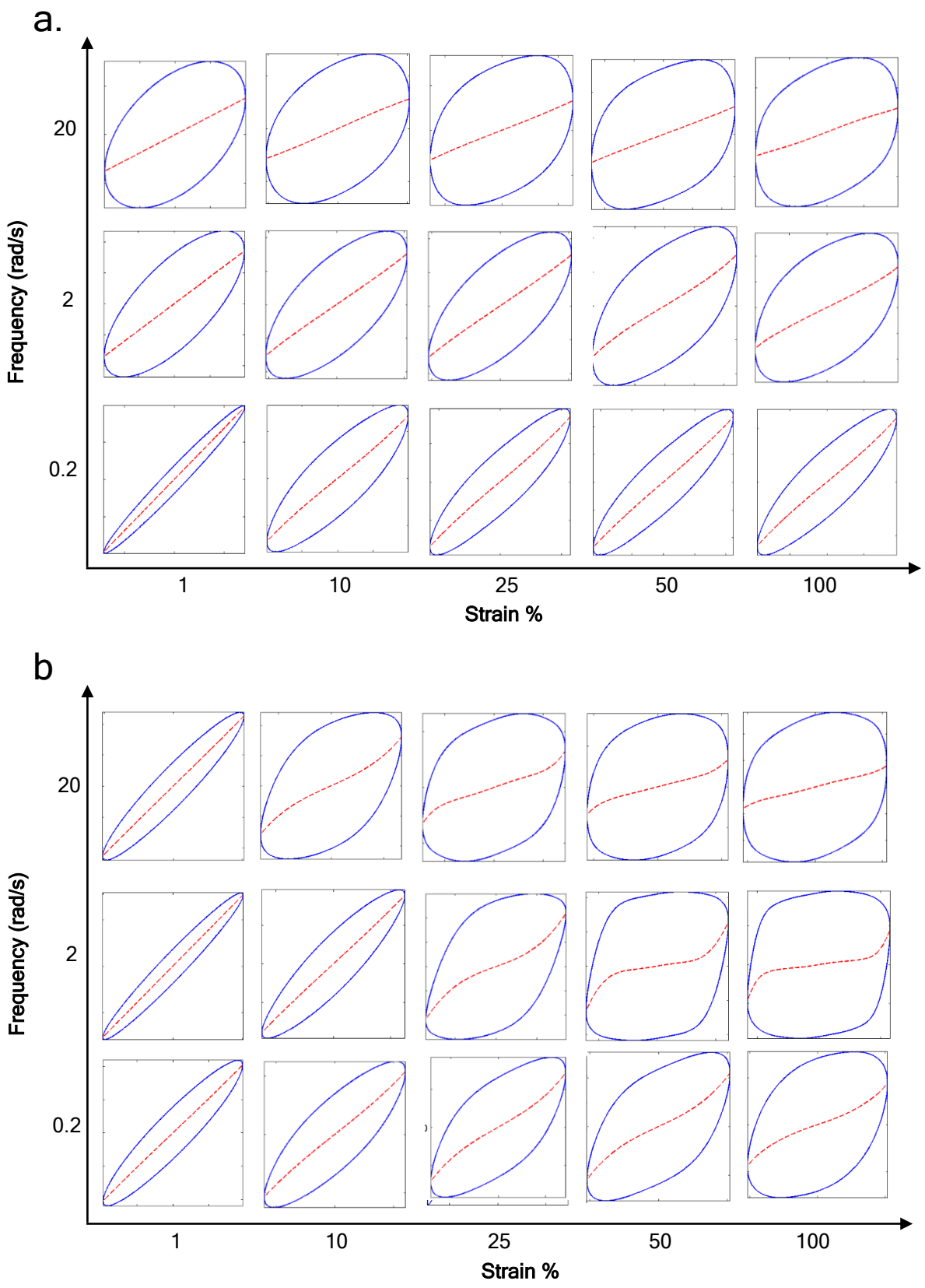

Figure A.4.3 Elastic Lissajous plots for WPI at a) Day 0 and b) Day 45

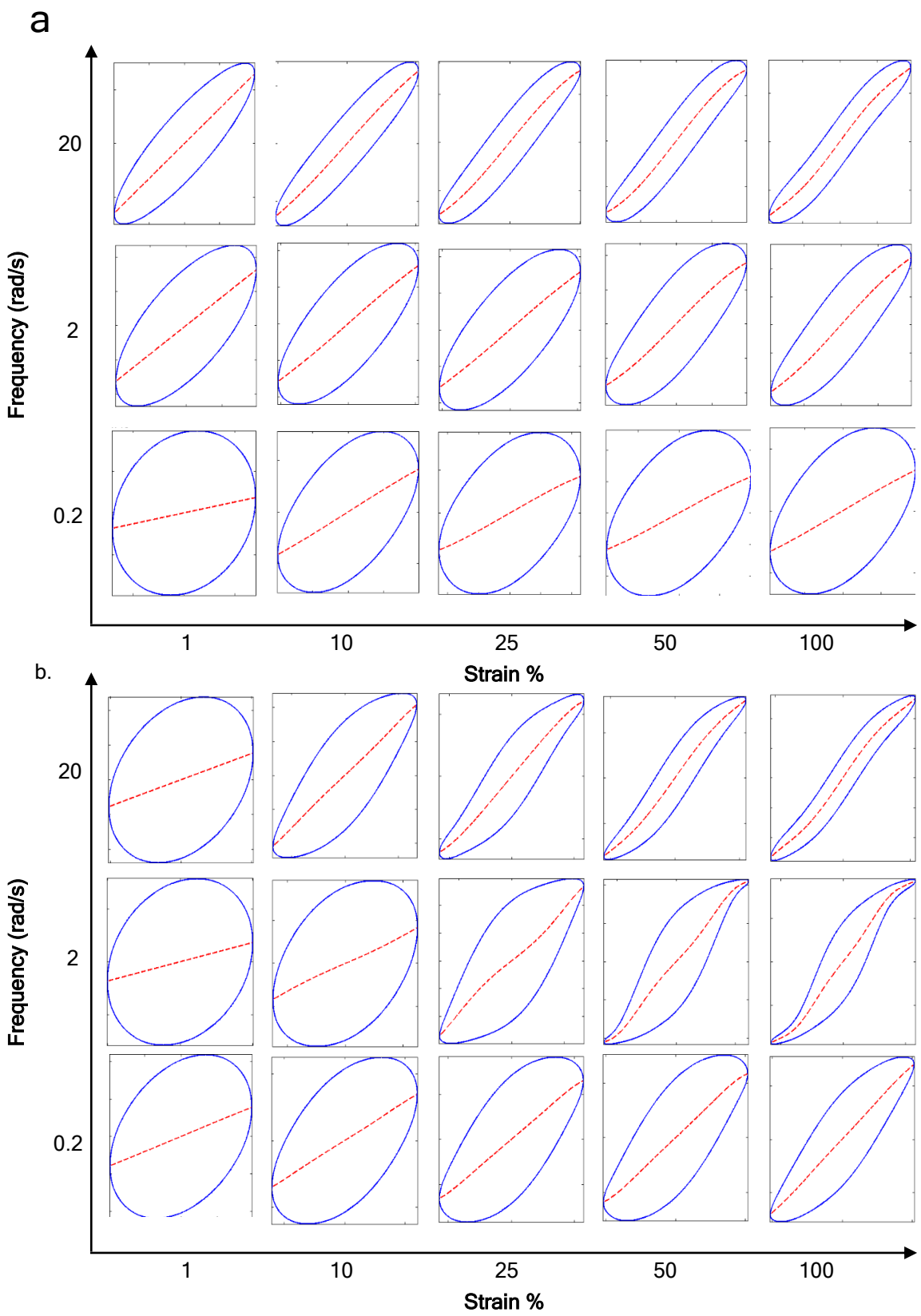

Figure A.4.4 Viscous Lissajous plots for WPI at a) Day 0 and b) Day 45

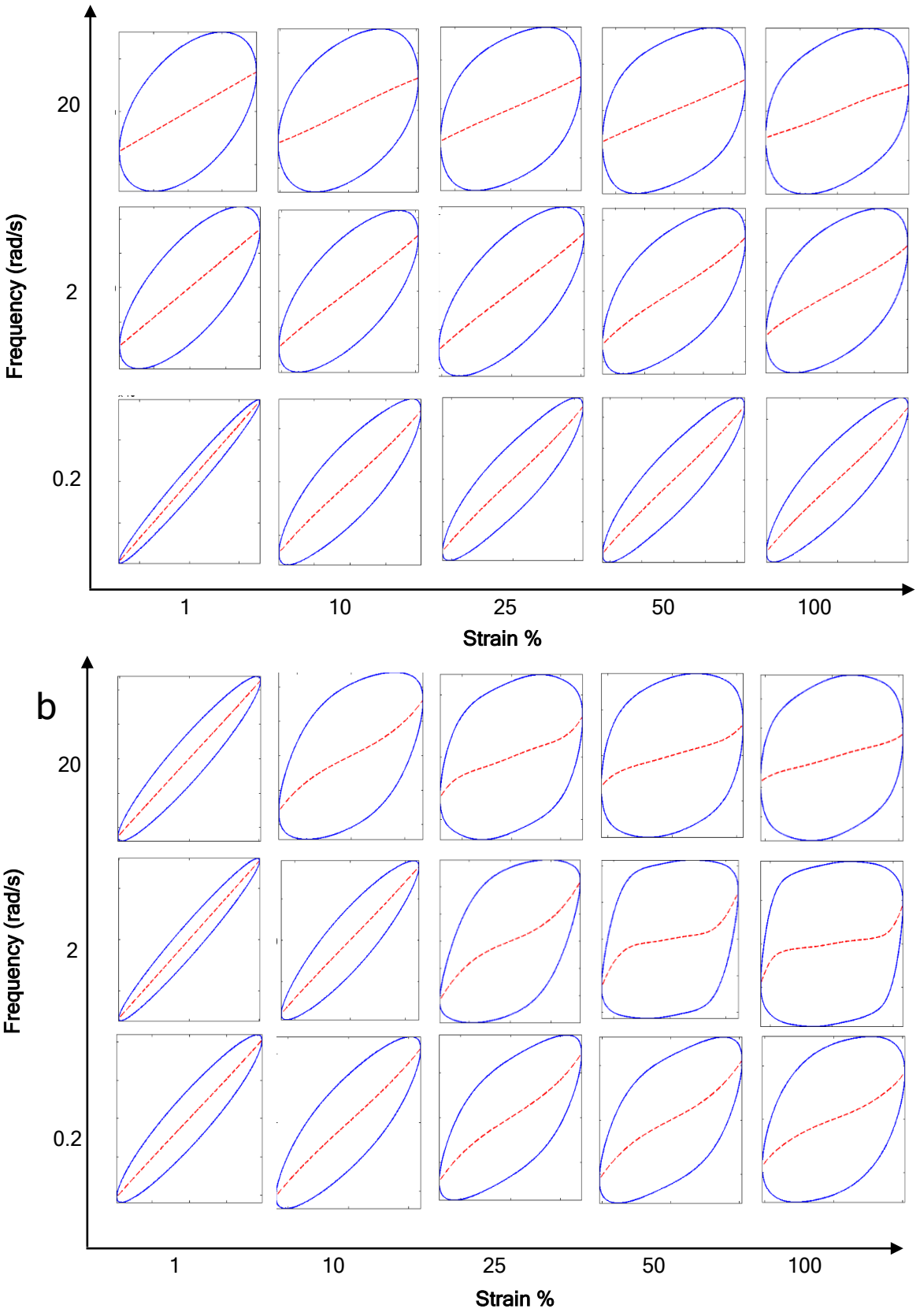

Figure A.4.5 Elastic Lissajous plots for MPI at a) Day 0 and b) Day 45

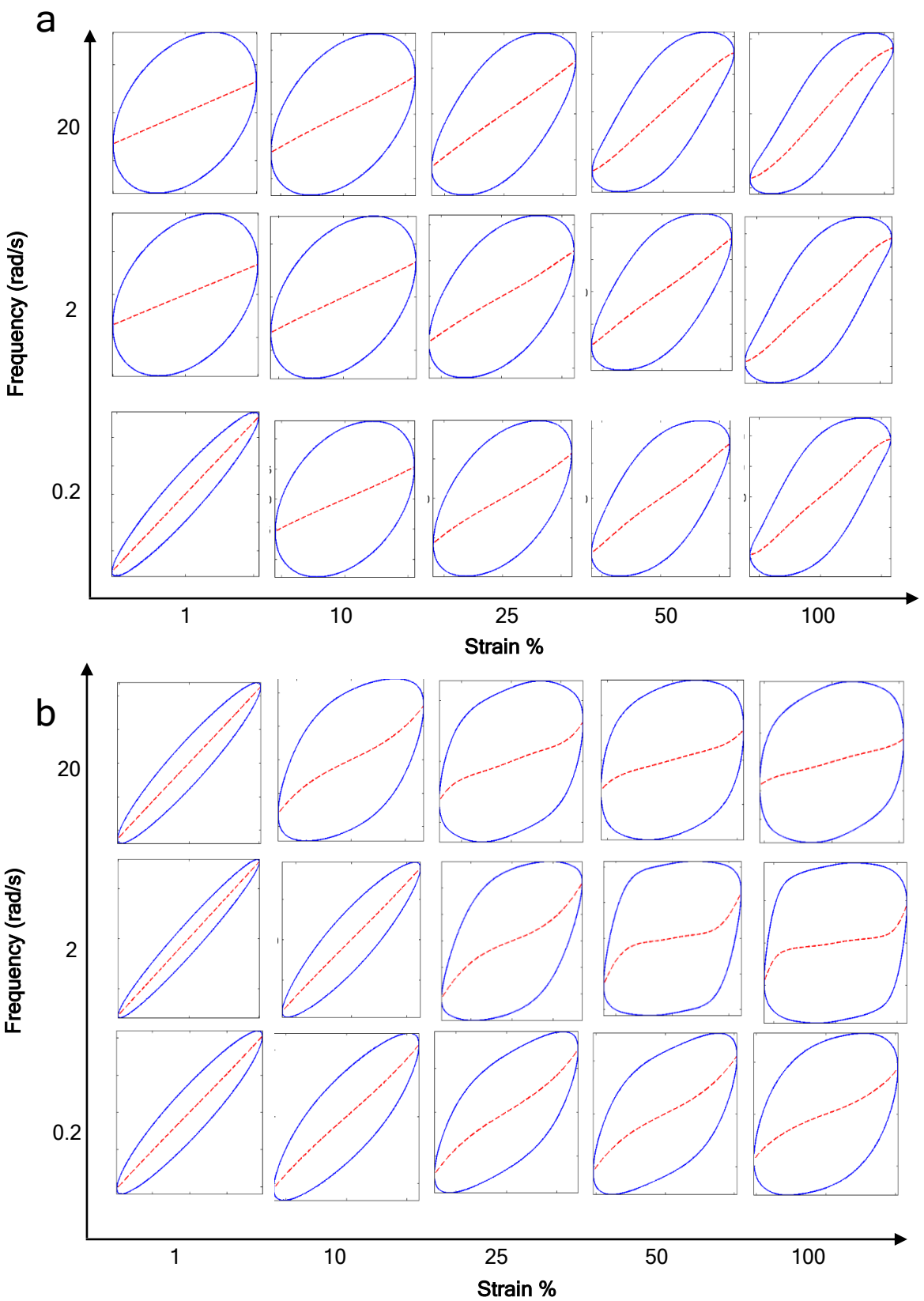

Figure A.4.6 Viscous Lissajous plots for MPI at a) Day 0 and b) Day 45

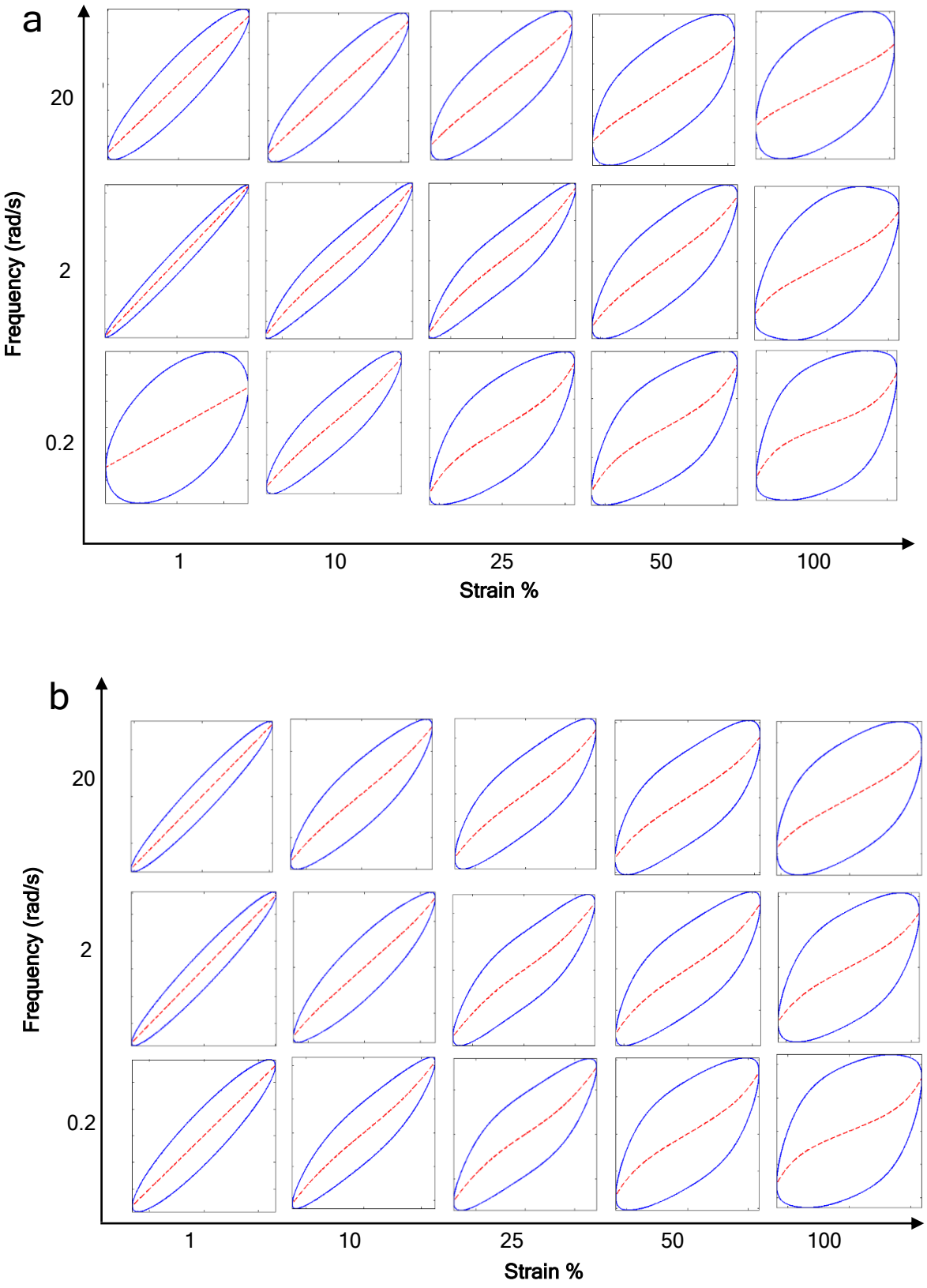

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Figure A.4.7 Elastic Lissajous plots for CON at a) Day 0 and b) Day 45

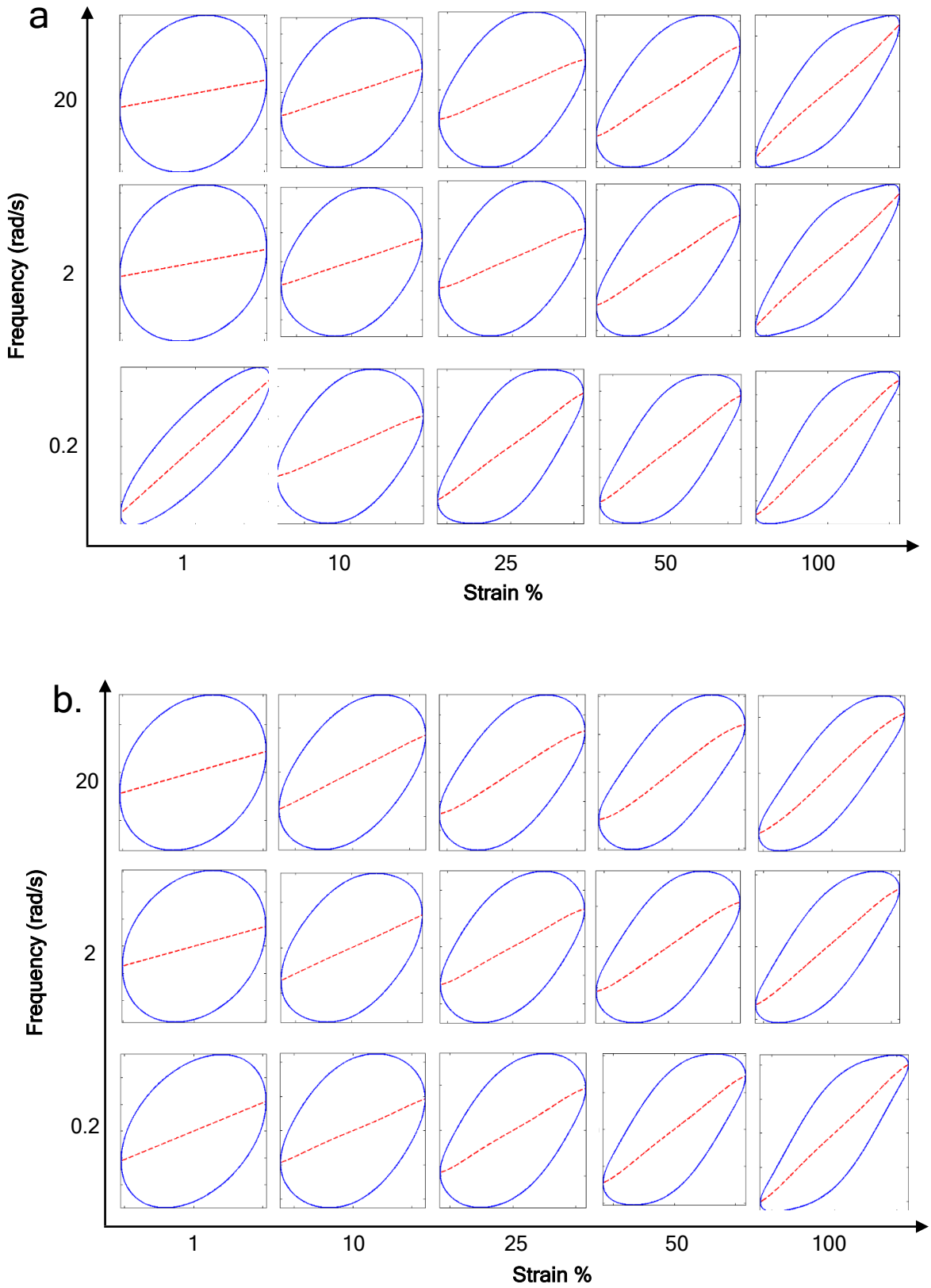

Figure A.4.8 Viscous Lissajous plots for CON at a) Day 0 and b) Day 45

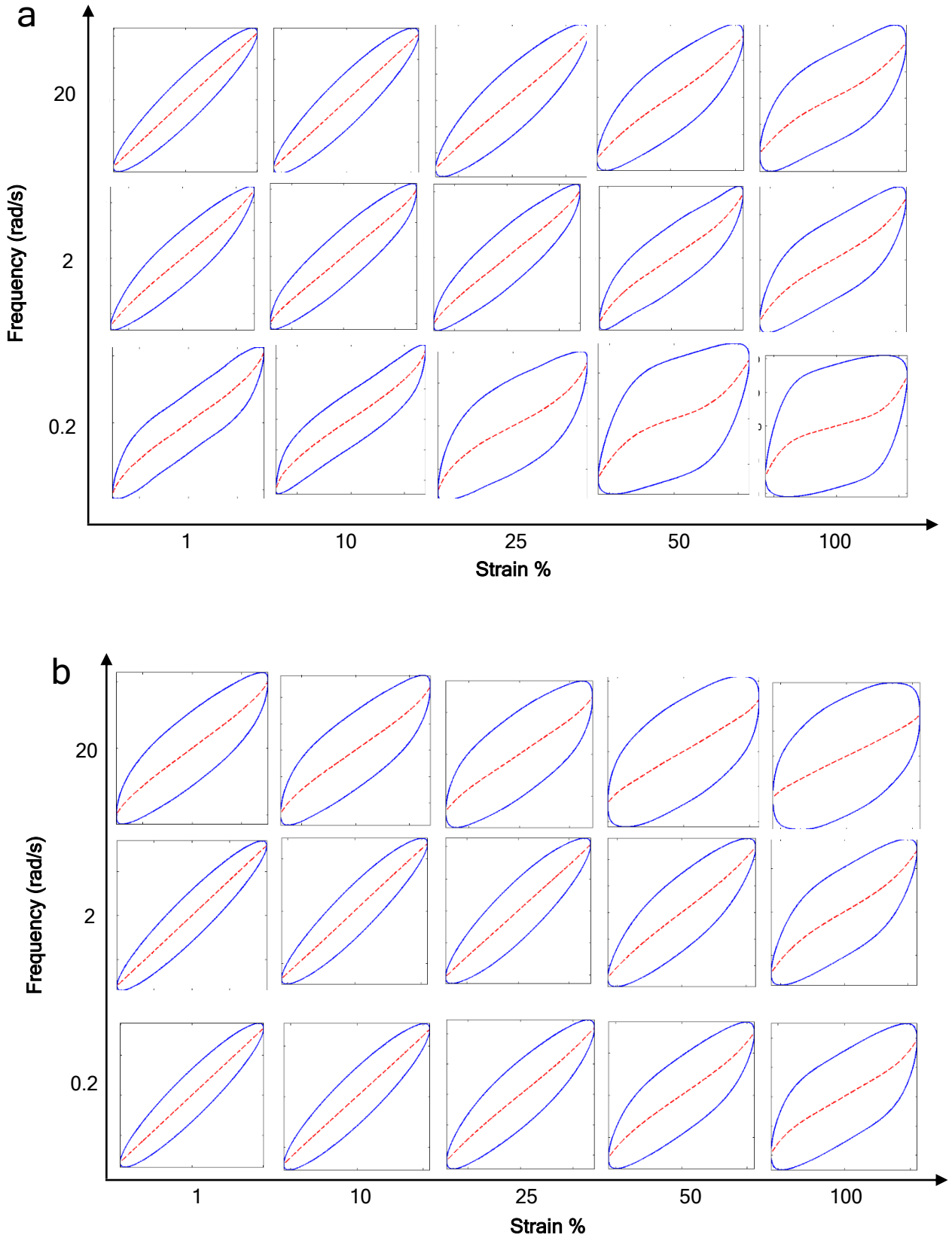

Figure A.4.9 Elastic Lissajous plots for GLU at a) Day 0 and b) Day 45

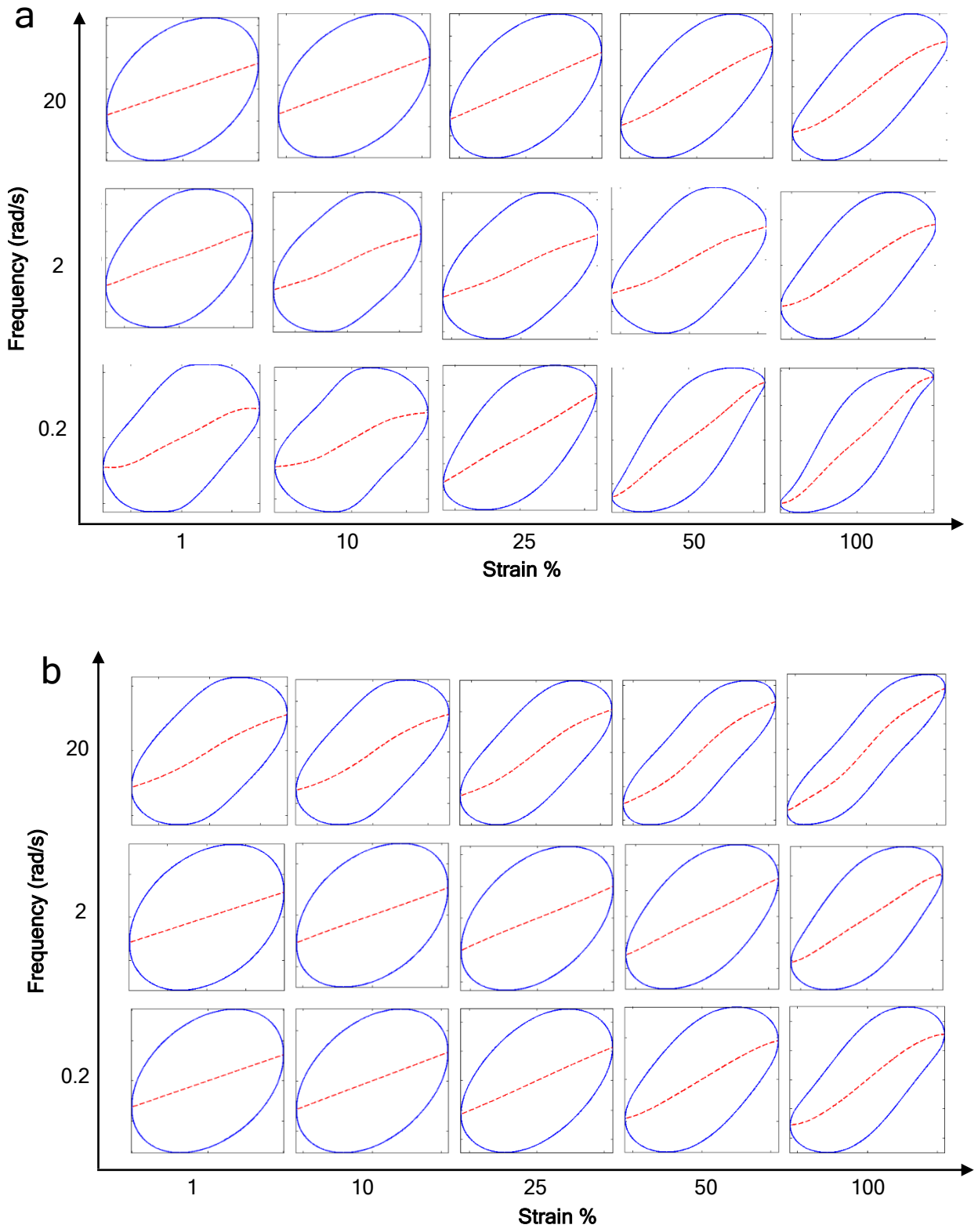

Figure A.4.10 Viscous Lissajous plots for GLU at a) Day 0 and b) Day 45