EFFECT OF LACTIC ACID SOURCE ON PROPERTIES OF SILVER CARP RESTRUCTURED WITH ALGINATE GEL

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by

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The undersigned, appointed by the dean of the Graduate School,

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A candidate for the degree of Master of Science,

And hereby certify that in their opinion it is worthy of acceptance.

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EFFECT OF LACTIC ACID SOURCE ON PROPERTIES OF SILVER CARP RESTRUCTURED WITH ALGINATE GEL

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ABSTRACT

Silver carp, *Hypophthalmichthys molitrix*, is an invasive species in the United States which can be used to make value-added products. The objective of this study is to compare different acidification methods' effect on developing silver carp patties by using alginate as a cold setting binder. There were four treatments: lactic acid bacteria (LAB) fermentation (F), control (C), encapsulated lactic acid (En) and powdered lactic acid (LA). Fish were mixed with the same amount of sodium alginate (3.6%), calcium carbonate (1.2%) and dextrose (3%). The F treatment was inoculated with log5/g fish *Lactobacillus. curvatus* in sterile peptone water, the C treatment had sterile peptone water as a blank control. Then the two treatments were incubated at 37^{o}C for 30 hours. After obtaining the level of different lactic acid amounts (LD) from the F and C treatments, the LD was applied to En and LA and then they underwent fermentation for 30 hours. Bacteria count, pH and lactic acid concentrations were determined at 0h, 6h, 10h, 18h, 25h and 30h for every treatment. After cooking, the puncture test and texture profile analysis were conducted to test the internal bindings of the four treatments. Results showed that F had the lowest pH, highest level of lactic

acid concentration (LAC), highest binding, highest lightness and whiteness ($P<0.05$). En had the second highest binding strength ($P<0.05$), LA and control had the lowest binding strength ($P<0.05$). Although there were strong positive relationships between LAC and binding strength, this did not apply to comparisons of C, LA and En, since C had the lowest LAC but the binding strength was very similar to those of En and LA. This study indicated that organic acid by fermentation can support alginate gel formation in restructured silver carp patties and slow acidification by fermentation resulted in a different product than fast acidification by encapsulated and powdered lactic acid.

CHAPTER 1

INTRODUCTION

Silver carp (*Hypophthalmichthys molitrix*) originated from China and had been spread over 80 countries for various reasons such as aquaculture, capture fisheries enhancement and plankton control (Kolar and others 2007). In 1973, it was introduced to the United States to control algae in lagoons and later it spread into the Mississippi river basin through floods (Kolli 2008). However, because of their excessive feed requirements (Thiel 2007) and temperature range (4-30^{o}C) for growth, they are considered an invasive species (Conover and others 2007; Kolar and others 2007) and many efforts have been made to prevent their proliferation in the Laurentian Great Lakes (Asian Carp Control Strategy Framework 2012).

Although hazardous to the water ecology system, they have nutritional values as human food. Buchtova and Jezek (2011) studied the nutritional value of each part of silver carp and found that the fat lipids were an alternative source of polyunsaturated fat, specifically, the α-linolenic acid C18:3n-3, EPA C20:5n-3, and DHA C22:6n-3. Ashraf and others (2010) studied the nutritional profiles on wild silver carp and the moisture, protein and lipids contents were 78.79%, 15.50%, 2.19%, respectively. According to the USDA nutrient data (2009, 2011), the nutritional contents for fresh pork and beef from various parts were approximately averaged at 70% moisture, 20% protein, and over 6% lipid. Therefore, silver carp could be a potential low fat food. Moreover, Steffens and others (1992) stated that the silver carp could be a dietary food for cardiovascular disease prevention.

However, as a food for human consumption, silver carp has a strong earthy/musty taste and odor and contains a large amount of small bones, which decreased the acceptability and consumption (Xu and others 2010). Though it has the low economic value and low acceptability, it can be made into restructured meat by incorporating binding and flavor ingredients to alter the texture and taste. Traditional restructured meat products employ heat binders such as salt and phosphate, which extract salt-soluble proteins (myofibrillar) that bind meat pieces together during the heating process (Boyer and others 1996). This technology needs a high temperature to induce protein cross-linking. However, silver carp surimi will undergo gel softening during heating (Liu and others 2006), due to the degradation of myosin heavy chain caused by myofibril-bound serine proteinases (Cao and others 1999, 2004; Zhong and others 2012).

The problem caused by heat processing could be solved by using cold-binders. In this way, the texture can be developed in a cold state. Alginate is an FDA approved generally recognized safe ingredient and is widely used in cold set binding technology (Means and others 1987). Means and Schmidt (1986) tested various combinations of alginate, calcium carbonate and sodium erythorbate in restructured beef. The role of organic acid in the alginate system is very important; many studies have concluded that higher amounts of organic acid resulted in higher internal binding (Clarke and others 1988a; Ensor and others 1989; Kolli 2008). Schmidt and Means (1986) explained these as the slow hydrolysis of the acid produced hydrogen ions to stimulate the calcium ions release from calcium salt. However, there is a lack of research about whether lactic acid bacteria fermentation will support alginate gel formation. In fermentation process, many biochemistry changes will occur. Acid production and proteolysis might be the most important factors impacting texture. If

fermentation (whether natural or with starter culture) supported alginate gel formation in meat products, there would be practical application values because it eliminates the addition of an extra acid source, which might be more expensive than fermentation. Therefore, the objectives of the study were:

1. To test whether the acid produced by lactic acid bacteria in the meat block can support the alginate gel-system in silver carp patties.
2. To compare the internal binding caused by different sources of lactic acid: fermentation, encapsulated lactic acid and powdered lactic acid.

CHAPTER 2

LITERATURE REVIEW

2.1 Alginate

2.1.1 Alginate structure and gel formation principle

Alginates are salts of alginic acid, they are natural polysaccharides extracted from brown algae (Yang and others 2012). Alginate consists of two linear linked blockwise copolymers: β-D-mannuronic acid (M) and α-L-guluronic acid (G) (Fischer and Dorfel 1955) (Fig 1.1). The distribution of the blocks (MM, GG, MG and GM, Fig1.2) is dependent upon the origin of the algae species, the extracted part in the algae and the harvest season (Jorgensen and others 2007; Smidsrod and Akjak-Braek 1990).

Figure 1.1: Structure of alginate (mannuronic and guluronic acid) (Draget and others 1997)

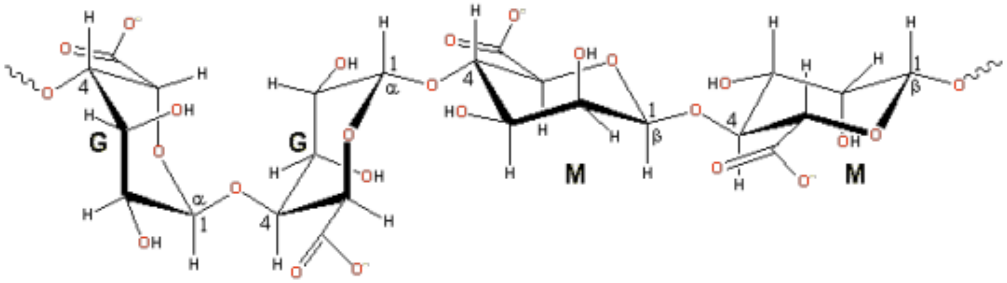
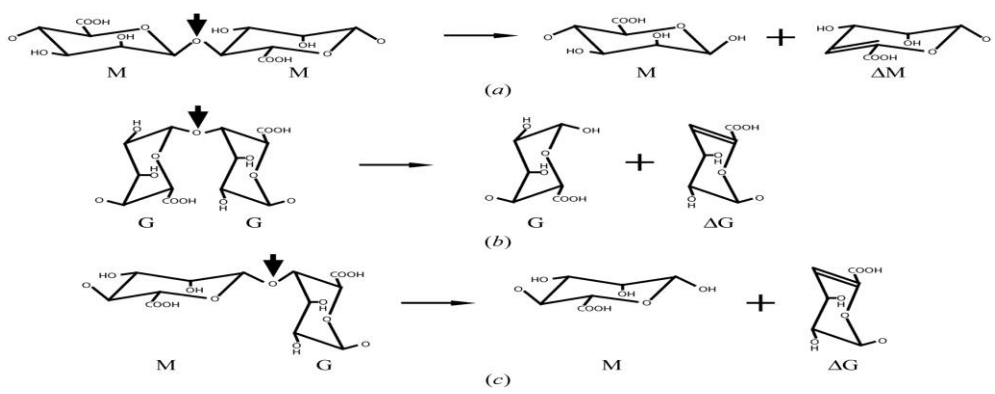
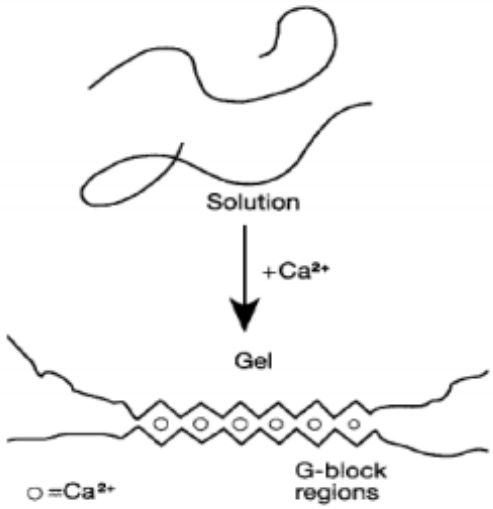


Fig 1.2 Blocks in alginate (Yamasaki and others 2005)



The alginate gel is thermo irreversible; once formed, the characteristic remains throughout the production process (Kolli 2008). An egg-box model was proposed to describe alginate interchain association with Ca^{2+} (Fig 1.3). The formation is very complex and dependent on the type of alginate, calcium form and preparation methods (Moe and others 1995). The zigzag-shaped chain forms by the G molecular fractions creating pocket-like cavities which capture Ca^{2+} (Grant and others 1973). Experiments demonstrated that both G and M contents are important in the gel-forming ability and strength (Draget and others 1997; Liu and others 2003). In the presence of Ca^{2+}, G-rich alginates form strong but brittle gels whereas M-rich alginates form weak but elastic gels (Lzydorczyk and others 2005).

Figure 1.3 Egg-box structure (Anonymous 1998)



2.1.2 Alginate application in food

Cold binding system in restructured meat, compared to conventional sausage production technology which employs salt, phosphate and heating, has some

advantages. First, the meat product can be sold in a raw or refrigerated state (Schmidt and Means 1986), and is able to reduce problems of discoloration and oxidative rancidity (Means and Schmidt 1987; Raharjo and others 1989). Devatkal and Mendiratta (2001) found that the salt-phosphate restructured pork rolls have higher amounts of thiobarbituric acid reactive substances (byproduct of lipid peroxidation) than alginate ones. Second, the demand of low sodium in food is increasing as consumer raise concerns of adverse health effects such as hypertension (Tsao and others 2002) and the cold binding system can reduce the use of salt.

Alginate has been widely used in meat products as a binder (Moreno and others 2008). Sodium alginate gels are formed by intermolecular association with polyvalent cations (mainly calcium) and these gels may have interactions with myofibrillar proteins (Montero and others 2000). However, the exact interaction mechanisms were unknown and studies had controversial conclusions. Some studies reported (Shand and others 1993; Montero and others 2000) the interactions were mainly electrostatic between anionic groups from alginate and positively charged groups from protein, whereas Moreno and others (2008) indicated that electrostatic bonds, hydrogen bonds and covalent bonds were all important and hydrogen bonds dominated; therefore they concluded that the gelation was established by alginate themselves and not dependent on protein-protein or protein-alginate bonds. These contradictions could be attributed to different origins of meat materials or different preparation methods.

The use of a calcium source is important in alginate gel formation. Calcium carbonate was strongly recommended (Draget and others 1991) because other ion sources, such as magnesium, manganese, aluminum, ferrous and ferric ions were not as effective as calcium to form bridges with alginate (Gannadios and others 1997). Sodium chloride is also not acceptable because a higher salt level had a negative

effect on the gel. Hong and Chin (2010) investigated the sodium alginate on cold-set gelation of porcine myofibrillar protein with various salt levels and found that the gel strength decreased greatly when the salt level increased from 0.1 M to 0.2M.

Acidulants such as lactic acid and glucono-δ-lactone have been used to accelerate the reaction with calcium carbonate to control the alginate gel formation (Boles and Shand 1998) and reducing off-flavor caused from insufficient calcium carbonate dissociation (Means and Schmidt 1986). Hambleton and others (2009) stated that sodium alginate forms a gel where the pH is near its pKa in the presence of polyvalent cations. The acids that have been employed in restructured silver cap were acetic acid, citric acid, lactic acid and glucono delta lactone (Kolli 2008). Many studies have indicated that the lower the pH, the stronger the gel would be. Kolli (2008) employed different levels of acid to make restructured whole silver carp and silver carp fillets, and found that the strongest gels were obtained when the pH were the lowest. Hong and others (2012) investigated the use of calcium alginate in pork myofibrillar protein under the pH from 5-6.5 and found that at the pH of 5, the gel strength was the strongest. Clarke and others (1988b) concluded that the increase in lactic acid increased the firmness in restructured beef. Ensor and others (1989) stated that higher level of lactate (30% lactic acid, 20% calcium lactate, 50% hydrogenated vegetable oil) addition to alginate and calcium carbonate complex with meat resulted higher bind in raw and cooked turkey breast patties. Glucono delta lactone was the most frequently used because it provided slow acidification and the calcium ions can be released slowly (Mouquet and others 1997). Fast acidification was not preferred since it generated crumbly texture (Barbut 2005).

Other than used as a binder in meat, alginate were used as coating ingredients and prebiotics. Liu and others (2013) reported that the coating preservative effectively

mitigated fats oxidation and protein degradation in preserving frozen oyster meat. When used as prebiotics, Wang and others (2006) reported that alginate oligosaccharides stimulated greater growth of *Bifidobacterium bidifum* and *Bifidobacterium longum* in *vitro* than fructo-oligosaccharides. In addition, in an *vivo* study, the fecal bifidobacteria of the rats fed with a 2.5% alginate supplement diet increased by 13 fold and 4.7 fold compared with control and with fructo-oligosaccharides, respectively and the lactobacilli increased by 5 fold compared with the control. In contrast, the enterobacteriaceae and entrococci decreased more in alginate diet fed rats fecal (Wang and others 2006). Peso-Echarri and others (2012) used alginate as a prebiotic supplement to feed the reared sea bream and reported that specimen fed with 5% alginate had higher contents of fat and ash than the control diet, and no significant differences in mineral content, fatty acid profiles, cholesterol content, texture parameters and sensory acceptability were observed. This kind of research was valuable because it could reduce or eliminate the use of antimicrobial agents (Peso-Echarri and other 2012).

2.2 Meat fermentation

Fermentation is a biotechnological method to preserve food which can be achieved by fungi, yeasts or bacteria, or a combination of them (Leroy and De Vuyst 2004). The microorganisms convert fermentable carbohydrates to end metabolites such as organic acids, alcohols and carbon dioxide (Leroy and De Vuyst 2004). Lactic acid bacteria (LAB) are the major microorganisms in fermentation food, because they produce lactic acid as a common product (Stiles and Holzapfel 1997). This compound has the function of preventing growth of pathogenic and spoilage microorganisms so that the shelf life can be prolonged (Ravyts and others 2012). LAB that have been generally regarded as safe in food genera are *Carnobacterium, Enterococcus,*

Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Steptococcus, Tetragenococcus and *Weissella* (Wessels and others 2004). There are three groups in LAB according to their metabolism. Obligately homofermentative: LAB metabolise hexoses through glycolysis only and lactic acid is the only product. Obligately heterofermentative: LAB convert hexoses and pentoses to form lactate, ethanol or acetate through phosphoketolase pathway. Facultatively heterofermentative: LAB degrade hexoses through glycolysis and pentoses through phosphoketolase pathway (Ravyts and others 2012).

2.2.1 Spontaneous fermented sausage

Drying, salting and fermentation are the oldest methods to preserve raw meat. Fermented dry sausage is a mixture of comminuted fat and lean meat, salt, nitrate/nitrite, sugar and spices (Hugas and Monfort 1997). The ground meat is stuffed into casings and undergoes fermentation, smoking/moulding and ripening/drying processes (Ravyts and others 2012). During fermentation, pH decreases due to the LAB action, the meat protein coagulates and the texture changes (Ravyts and others 2012). In raw meat under hygienic conditions, the LAB count is 10^2-10^4 (Ravyts and others 2012). The most common LAB in spontaneously fermented dry sausage are *Lactobacillus sakei, Lactobaciilus curvatus* and *Lactobacillus plantarum*, followed by lactobacilli, pediococci (*Pediococcusacidilactici*), Leuconostoc species (*Leuconostoc gelidum*), Lactococci (*Lactococcus lactis*), Weissella species (*Weissella viridescens*), and Enterococci (*Enterococcus faecalis* and *Enterococcus faecium*) (Montel 1999; Leroy and others 2006; Rantsiou and Cocolin 2006). Other than LAB, other microorganisms could be found, such as coagulase-negative staphylococci (CNS), filamentous fungi and yeast (Leroy and others 2006). The non-pathogenic CNS have multiple desirable properties, for example, promoting desirable color by nitrate

reduction, preventing rancidity by decomposing peroxides and generating flavor compounds through lipolysis and proteolysis (Montel and others 1998; Talon and others 1999). *S. exlosus* and *S. saprophyticus* were the most common species presented in naturally fermented products (Ravyts and others 2012). However, these CNS are less competitive in the presence of LAB and they may not exist in the entire fermentation process because of the unfavorable acidic environment, and they are more important in the ripening process (Ravyts and others 2012). In Southern Europe, the filamentous fungi and yeast, grown on the surface of the sausage, are essential because their activities, such as catalase activity, oxygen consumptions and protection against light can influence the flavor development and color stabilization (Bruna and others 2001).

2.2.2 Directed fermented sausage

LAB have been used as starter cultures in fermented dry sausages and commercial starters are mixtures of LAB and CNS (Ammor and Mayo 2007). Starter cultures should have three functions: rapid pH drop of the meat batters to inactivate pathogens; elongate product stability and shelf life by inhibiting spoilage microorganisms or their metabolisms and promote sensory properties (Lucke 2000). Ammor and Mayo (2007) wrote a detailed review about how to choose starter cultures and proposed some important rules, these are: starter cultures should be able to produce abundant organic acid (mainly lactic acid) to drop the pH rapidly; starter cultures should have the capacities to survive at a broad temperature range (2-24^{o}C), strong salt (2%-10%) and pH (4.2-6) tolerance ranges; heterofermentative LAB are not suitable for meat fermentation since gas formation (CO_2) will lead holes in the sausage and acetic acid will cause a pungent flavor (Buckenhuskes 1993); starter cultures that can produce catalase to hydrolyse hydrogen peroxide are preferred because the hydrogen peroxide

will accelerate rancidity and discoloration; LAB that have nitrate and nitrite reductases are desirable, even though their activities are weaker than CNS, this is good to promote nitrosomyoglobin formation; LAB that can produce proteinases, peptidases and amino-peptidases are preferred since it can help to form precursors of flavor compounds (Ammor and others 2005). Some studies have demonstrated that *L. plantarum, L. curvatus* and *L. sakei* were able to hydrolyze sarcoplasmic proteins and decompose peptides into amino acids (Fadda and others 1999a, 1999b; Sanz and others 1999); LAB strains that produce L(+) lactic acid are favored since the counterpart D (-) is not hydrolyzed by human lactate dehydrogenase and may cause health problems (Buckenhuskes 1993). L(+) is more inhibitory than D(-) (Benthin and Villadsen 1995); LAB that can produce bacteriocin are preferred because bacteriocin have an inhibitory effect on gram-positive pathogens such as *Listeria monocytogenes, Staphylococcus aureus, Clostridium perfrigens* and *Bacillus cerues* (Aymerich and others 2000; Enan and others 1996); LAB that do not have amino decarboxylase activity are preferred (Ammor and others 2007), in that amino acid can be transferred to biogenic amines by decarboxylase activity (ten Brink and others 1990).

The strain used in the current research was *Lactobacillus curvatus*. According to the technical data sheet, the strain can suppress the growth the endogenous lactic acid bacteria and *Listeria monocytogenes*. The growth range is 4-40^{o}C, the maximum salt tolerance is 10% salt in water. Also, this strain is homofermentative, which only produces L(+) lactic acid (Chr Hansen 2012).

2.3 Silver carp restructured food

Restructured meat products are meat shaped into a more appealing form (Anon 1983), they can be made by low value meat, such as the processing waste of meat

(Kuraishi and others 1997) and under-utilized meat. Although Xu and others (2010) stated that silver carp has an earthy/musty texture and contains many small bones, Laird and Page (1996) reported silver carp in the United States has a potential market because of their large size, rapid growth and acceptable flavor. Zivkovic and others (2004) also reported that silver carp gels may have a higher water binding ability than the gels from beef and poultry meat. Therefore, to make silver carp into restructured products by reshaping it and incorporating species may eliminate the presence of bones and undesirable flavor.

There are many methods to produce restructured silver carp. Some research used downstream processing methods to alter the texture and some research employed salt, proteins, hydrocolloids and microbial enzymes to develop restructured silver carp. Wang and others (2012) used salt as an ingredient in the formula and processed the chopped silver carp meat via air drying (AD), freeze drying (FD), microwave drying (MVD) and vacuum drying (VD) and reported that drying alleviated the unpleasant flavor and MVD treatment had a crispy texture and good aroma. The degree of likeness was in this order: FD, VD, AD and MVD.

Proteins that were employed in restructured fish were egg white (Yetim and Ockerman 1995), casein and beef plasma-thrombin (Baker and others 2000), and soy protein (Hasanpour and others 2012). Uresti and others (2004) reported that sodium caseinate, whey protein and their combinations with levels of salts were used, and it was found that both of the proteins increased the gel strength, but sodium caseinate resulted in a stronger gel than whey protein. The combination of salt and protein had the strongest binding strength.

Hydrocolloids that were employed in restructured fish were xanthan, guar, kappa and iota carrageenan and pectins (Ramirez and others 2002a, 2002b; Perez-Mateos and Montero 2002; Perez-Mateos and others 2002; Uresti and others 2003). Uresti and others (2003) found that in fish paste, the firmness and consistency had the lowest value when the pectin content was 1% and they increased later on as the pectin content went up; however, the gel strength was the highest at the 1% pectin content and was decreasing as the pectin content went up. Some researchers (Montero and others 2001; Perez-Mateos and others 2002) used carrageenans, alginate, ionic and non-ionic pectin in fish mince under different combinations of pressure and temperature, and they found that under atmospheric pressure gels were more adhesive and less cohesive, and under a lower pressure and temperature, gel were more cohesive and less adhesive.

Microbial transglutaminase (MTG), an enzyme produced from *Streptoverticillium ladakanum* and *Streptoverticillium mobaraense* (Jiang and others 1998), was also used in restructured silver carp. Uresti and others (2004) and Ramirez and others (2002b) studied the use of MTG and combined it with different levels of salt in silver carp. The enzyme worked best under the condition of at 40^{o}C for 60 min and then 90^{o}C for 20 min. MTG resulted in higher binding and the addition of salt increased the binding.

CHAPTER 3

MATERIALS AND METHODS

The following materials and methods were used to prepare restructured silver carp and conduct various tests. All processing and tests were done in the product development research lab, microbiology teaching lab and food engineering lab of W.C. Stringer Wing at the University of Missouri.

3.1 Materials

Sodium alginate (Lot#612508), calcium carbonate (Lot#4-320-13), and encapsulated lactic acid (Lot#0409001647) were provided by Danisco USA Inc., St. Joseph, MO. Sodium nitrite (6.25%, Lot#A37220) was purchased from Excalibur Seasonings & Marinades, Perkin, IL. Powdered lactic acid (60%, Lot#1206000510) was donated by Purac, Lincolnshire, IL. *Lactobacillus curvatus* (Material#690607) was donated by Chr. Hansen Inc., Milwaukee, WI. Dextrose (Lot#12713), acetonitrile (Lot#112539), sulfuric acid (98% ACS grade, Catalog#A300-500) were purchased from Fisher Scientific Company, Fair Lawn, NJ. Man Rogosa Sharpe (MRS) broth (Lot#2249190), MRS agar (Lot#2354215) and peptone water (Lot#8252722 Bacto^{TM} peptone) were purchased from Becton, Dickinson and Company, Sparks, MD.

3.2 Preparation of silver carp patties

Twenty two silver carp (*Hypophthalmichthys molitrix*) were caught in the Missouri River by personnel from the Columbia Environmental Research Center (CERC), United States Geological Services (USGS). After the capture, each silver carp was eviscerated, rinsed and transported on ice in insulated coolers to the walk-in cooler

(2^{o}C) in the meat lab of Food Science Department, University of Missouri. Within the next 48 hours, all the silver carp were deboned, the deboned fish from each silver carp was placed in a Ziploc bag, and stored in the walk-in freezer. Before the experiments, all 22 deboned fish were mixed and divided into multiple Ziploc bags, with every bag weighed approximately 930g. When needed, bags were transferred to a home-style refrigerator, thawed for 48 hours, then the bags were placed in a bowl filled with cold tap for 2 hours. In each replicate, the fermentation treatment (F) and control treatment (C) were conducted first and those fish were from the same Ziploc bag. After calculating the different levels of lactic acid concentrations (LD) from the two groups, the LD was applied to the encapsulated (En) and powdered lactic acid treatments (LA). The fish of En and LA were from another Ziploc bag. When the F and C treatments were tested, the fish of En and LA group were kept in freezer.

After blending the fish with other ingredients as assigned for two minutes (Cuisinart DLC-2009CHB Prep 9-Cup Food Processor, Brushed Stainless, Cuisinart, Windsor NJ), they were placed in sterile petri dishes, with 70 g in each one. Then the fish were covered with plastic clear wrap and lids, upon pushing the lids on the samples, they were evenly distributed in the plates. The plates were placed in the incubator at 37^{o}C for 30 hours. At 0h, 6h, 10h, 18h, 25h, 30h, plates were taken out to obtain samples, after which they were put back in the incubator. At 30 h, the fish from the plates were taken out, cooked in the water bath until the inner temperature reached 72^{o}C (USDA 2011). After cooling down to room temperature, they were ready for the puncture test and texture profile test. The four treatments are shown in Table 3.1.

Table 3.1 Treatments of restructured silver carp

Ingredients[b]	Treatment 1 (F)	Treatment 2 (C)	Treatment 3 (En)	Treatment 4 (LA)
Fish	450	450	450	450
Sodium alginate (3.6%)	16.2	16.2	16.2	16.2
Calcium carbonate (1.2%)	5.4	5.4	5.4	5.4
Dextrose (3%)	13.5	13.5	13.5	13.5
Sodium Nitrite (0.0156%)	1.12	1.12	1.12	1.12
Lactic acid bacteria	Lg5/g fish	-	-	-
Encapsulated lactic acid	-	-	5.46g[c]/8.12g[d]	-
Powdered lactic acid	-	-	-	3.55g[c]/5.28g[d]
Peptone water (10%)	45mL	45mL	45mL	45mL

[a]All ingredients were weighed in grams

[b]Values in parentheses were percentages of the fish weight

[c]The first replicate lactic acid concentration difference (from treatment 1 and treatment 2). The lactic acid concentration difference was originally 4.73mg/g fish, the purities of encapsulated lactic acid and powdered lactic acid were 39%, 60%, respectively. Therefore the amount for En and LA were $4.73/0.39\times450\times10^{-3}$=5.46g and $4.73/0.6\times450\times10^{-3}$=3.55g.

[d]The second replicate lactic acid concentration difference (from treatment 1 and treatment 2) was originally 7.04mg/g. Therefore the amount for En and LA were $7.04/0.39\times450\times10^{-3}$=8.12g and $7.04/0.6\times450\times10^{-3}$=5.28g.

3.3 Preparation of starter culture.

The starter culture preparation method was following Yin and others (2002) with slight modifications. *Lactobacillus curvatus* was donated from Chr-Hansen Inc. Approximately 0.013g frozen powder was enriched in deMan Rogosa Sharpe MRS broth at 37^{o}C for 24 hours. Finally, cell pellets were harvested by centrifuging at 13500 rpm for 2 min and they were suspended in peptone water. Lastly, the bacteria numbers were adjusted to reach 0.1-0.2 (viable cell count log6/mL) by using a spectrophotometer (50 Bio UV-visible spectrophotometer, Varian-Agilent technologies, Santa Clara, CA) at the wavelength of 600 nm (Yin and others 2002).

3.4 Determination of purity of encapsulated lactic acid powder

The encapsulated lactic acid was placed in distilled water and heated on a hot plate until the liquid became clear. The lactic acid concentrations were measured by HPLC method as described later in section 3.7. Four samples were measured and the purity was 39%±0.83.

3.5 Determination of pH

The pH measurement was conducted using Wang's method with modifications (Wang 2000). Five grams of fish were homogenized with 50 mL distilled water, and the pH was measured by a pH meter (Model 230A, Fisher Scientific Company, Pittsburgh, PA). Before measuring, the electrode was calibrated by pH 7 and pH 4 buffer solutions. The pH values were measured in triplicate for each treatment at 0h, 6h, 10h, 18h, 25h and 30h.

3.6 Determination of moisture content

Moisture content determination was done following AOAC (1997) method (950.46 moisture in meat) with modifications. Three grams of the fish sample was placed in an aluminum tray and placed in vacuum oven at 80^{o}C for 24 hours. Three samples were measure for each treatment. Moisture was calculated by the formula:

$$1 - \frac{\text{dried sample weight}}{\text{sample weight before drying}} \qquad \text{Eq.1}$$

3.7 Determination of lactic acid concentration

Lactic acid concentrations for each treatment were measured at 0h, 6h, 10h, 18h, 25h and 30h using high performance liquid chromatography (HPLC) following Castellari and others (2000) method with slight modifications. The system consisted a pump (Series LC410, Perkin-Elmer, Norwalk, CT), an organic acid column (Aminex HPX-87H Ion Exclusion Column, 300 mm×7.8 mm, Catalog#125-0140, Bio-Rad Laboratories Inc, Hercules, CA), an autosampler (Model 410, Varian-Agilent Technologies, Santa Clara, CA) for auto injection, UV detector (LC90 UV spectrophotometric detector, Perkin-Elmer, Norwalk, CT) and a Galaxie Chromatography data system (Varian-Agilent technologies, Santa Clara, CA) calculating the quantity. The mobile phase consisted of acetonitrile (6%) and 0.045N H_2SO_4, with a flow rate of 0.5 ml/min, the UV detector wavelength was 220nm and temperature was 55^{o}C. Five grams of fish was homogenized with distilled water, after centrifugation at 3900 rpm for 20 min at 4^{o}C. Suspensions were obtained, which were diluted 10 times and were filtered and ready for the auto injections. Three samples from each treatment were measured at 0h, 6h, 10h, 18h, 25h and 30h. The lactic acid concentrations were calculated by the following equation:

Lactic acid concentration (mg/g fish) =

$$\frac{(5 \times \text{moisture content} + 50) \times \text{result obtained from the HPLC program}}{5} \quad \text{Eq.2}$$

3.8 Microbiology analysis

Lactic acid bacteria growth determination method as described by Zhang and others (2013) with slight modifications was followed. Five grams of fish was homogenized with 45 mL sterile peptone water in a sterile stomach bag, blended in for 2 min and decimal dilutions were prepared. Aliquots of 1ml of proper dilutions were pour plated with Man Rogosa Sharpe agar (MRS) in petri dishes in duplicate.

3.9 Colorimeter

Lightness (L*), redness/greenness (a*) and yellowness/blueness (b*) were measured using colorimeter (Chroma Meter CR-410, Konica Minolta, Ramsey, NJ). Whiteness was also calculated by the following formula (Lanier and others 1991):

$$\text{Whiteness} = 100 - [(100\text{-}L^*)^2+(a^*)^2+(b^*)^2]^{1/2} \quad \text{Eq.3}$$

Three patties were measured in each treatment.

3.10 Puncture test

The puncture test was following Clarke and others (1988a) method. A Stevens-LFRA Texture Analyzer (Texture Technologies Corp, Scarsdale, NY) was used to penetrate the approximate 2-cm diameter fish patty disks. The diameter of the spherical probe was 0.635 cm, the penetrating speed was 2.00mm/sec. The highest values throughout the puncturing process were recorded. Five to six samples were measured for each treatment.

3.11 Texture profile analysis (TPA)

Texture profile analysis was conducted as described by Bourne (1978) using a texture analyzer (TA-HDi, Texture Technologies Corp, Scarsdale, NY). Five or six samples (1 cm thick and 2 cm diameter) were compressed by the probe (diameter 50mm, thickness 20mm) twice to 50% of their heights. Other test conditions were: test speed 1 mm/g, pre-test speed 5 mm/s, 50 kg load cell, and 5 s between the two compression cycles. All the compression procedures were carried out at room temperature (25^{o}C).

3.12 Statistical analysis

One-way ANOVA was used to compare means in four treatments. Repeated measure ANOVA with a covariate was used to analyze the impacts of time, treatment and the interactions on pH values, microbiology and lactic acid concentrations during fermentation. Co-variance (ANCOVA) was used to analyze the impacts of treatment on texture properties. Pearson correlation coefficients were also calculated between attributes in texture profile analysis and their relationships with pH values and lactic acid concentrations. All analyses were done using the SPSS program (IBM SPSS, Version 20) using 0.05 as a significant level. Tukey's multiple range test was used to determine significant differences among treatments.

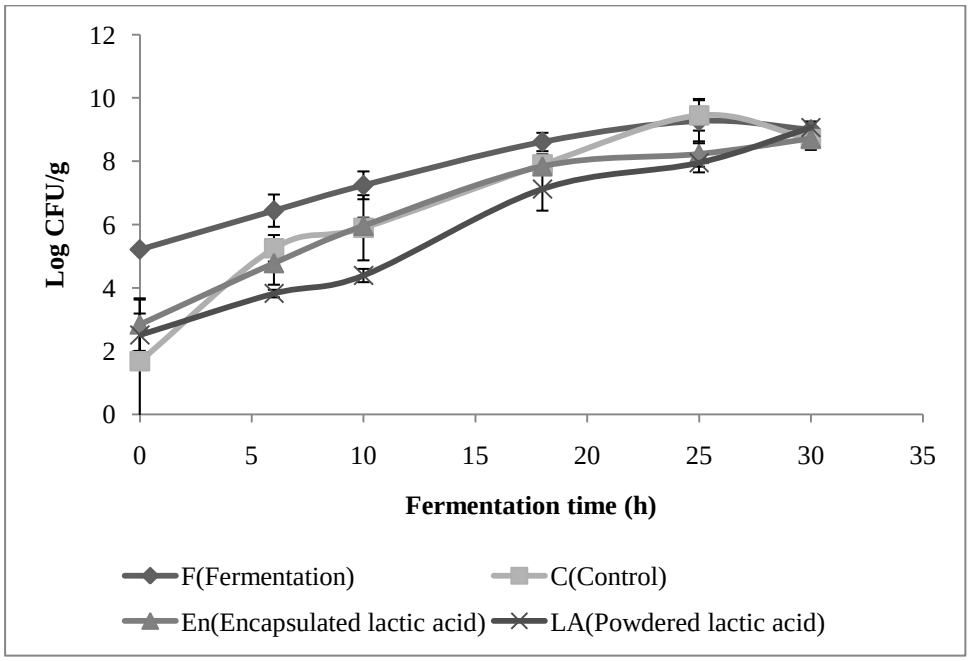
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Microbiology analysis

Figure 4.1 shows the lactic acid bacteria growth during the fermentation process in four treatments. The four treatments underwent a similar trend with the bacteria gradually increasing and the counts in each treatment were different at different time points ($P<0.05$, time's effect, Appendix B). Whereas, their changes varied differently as the interaction of time and treatment effect ($P<0.05$): the fermentation treatment at the initial point had the highest bacteria count because the inoculation level was log5/g fish, the rate of growth was stable until 25 h and stopped afterwards. The C and En treatments had a similar bacteria count over time and they reached the similar level with the fermentation treatment at the 30 h. The C had the highest growth rate from 18h to 25h among all four treatments and then started to decrease, while En had a stable rate throughout the fermentation process. The powdered lactic acid treatment had the lowest bacteria count until 25h and finally reached the similar level as other treatments. The lactic acid differences between F and C treatments (LD) applied to En and LA treatment were also important factors influencing bacteria growth ($P<0.05$), especially in LA, in that it was possible that the direct and fast addition of powdered lactic acid delayed the bacteria growth but the slow acidification method (En) did not.

Figure 4.1: Lactic acid bacteria growth during fermentation in silver carp patties



It was noticeable that MRS agar is not very selective to lactic acid bacteria at the last two time points (25h and 30h) in all four treatments, because different morphologies of colonies were seen. Those colonies included the spread white ones with ragged edges and unsmooth surface which were grown on the agar surface; colonies that were round or oval were grown inside the agar, etc. These were unlike the first four time points where similar morphologies were observed (round, oval ones inside the agar). Our preliminary research also indicated that *Staphylococcus* (Gram stain positive cocci, isolated from mannitol salt agar) was able to form colonies in the MRS agar. These indicated that as the fermentation proceeded, other bacteria that can stand acidic environment started to grow.

The lactic acid bacteria in F and C treatments were increasing, which were consistent with other studies where lactic acid bacteria were employed to ferment silver carp or other fish species (Yin and others 2002; Hu and others 2007; Liu and others 2009). However, at the 25 h, the C treatment reached the same level as the F,

while in those studies, the lactic acid bacteria remained nearly 1-2 log lower in C group than F treatment throughout the fermentation process. The reason might be that in their research, different types or levels of anti-microbial ingredients were added, such as salt (Yin and others 2002; Hu and others 2007, 2008), ginger and garlic juice, pepper flour (Liu and others 2009) so that the endogenous lactic acid bacteria did not grow rapidly. Also, the reason could be the different starter cultures, the origins of the fish, and the endogenous bacteria species presented in the fish. Hagi and others (2004) performed a yearlong research project to investigate the seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish and found out in October (when the fish were deboned), the major one in common carp was *Lactococcus lactis.* For LA treatment, bacteria counts were very low. The reason could be that the endogenous bacteria were inhibited by the direct high amount of an acid addition thus the lag phase of growth was delayed. In contrast, the bacteria count in En group was similar with C, indicating the slow release of acid did not influence the endogenous bacteria growth.

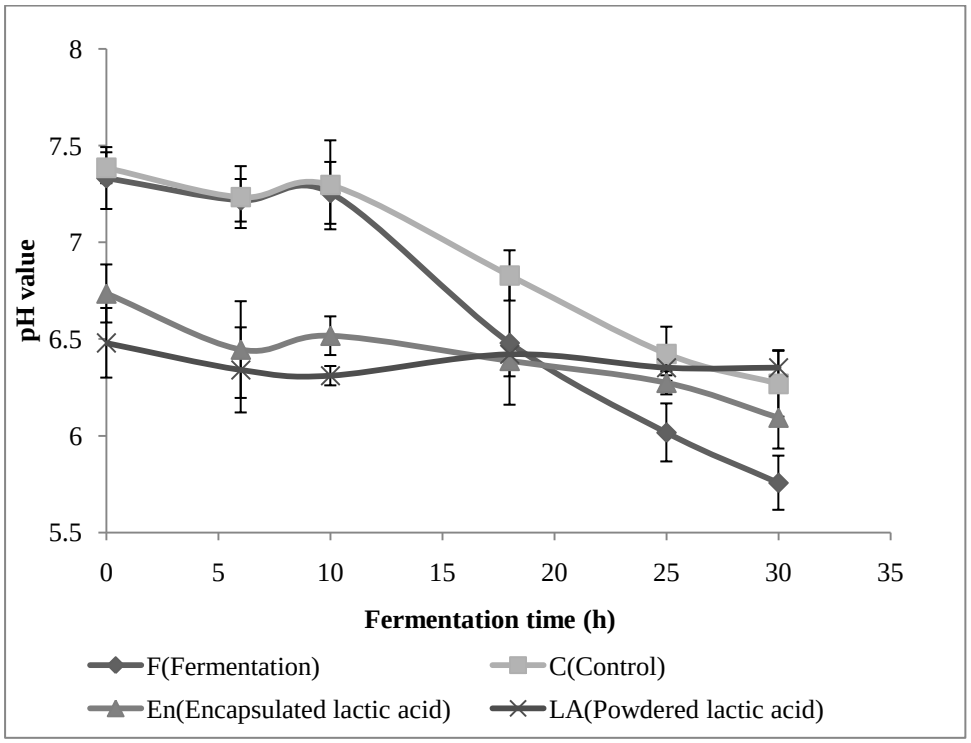
In this study, sodium nitrite was used to inhibit endogenous bacteria, as referred by Korkeala and others (1992), who found that the addition of 200 mg/L nitrite to MRS broth had a slight inhibition effect on lactic acid bacteria, such as homofermentative lactobacilli, Leuconostocs, *Lactobacillus curvatus* and *Lactobacillus sake.* The addition of 156 ppm nitrite in this study was the maximum amount that can be added to comminuted meat products required by USDA (1995).

4.2 pH values and lactic acid concentrations during fermentation

Figure 4.2 shows the pH changes during fermentation. The pH values of F and C treatments dropped drastically after 10h; however, the F treatment had approximately

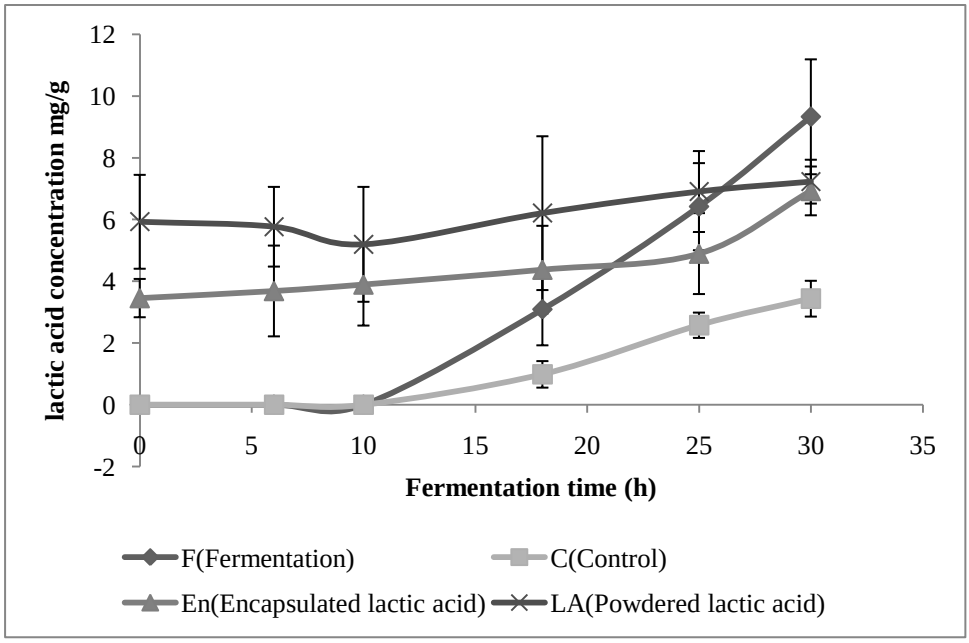
two units drop and C only dropped about 1 unit at the end of fermentation. The En group had a much lower pH value at the starting point due to direct acidification and it underwent a slow decreasing rate. The LA group had the lowest pH value at 0 h and the pH remained stable around 6.5.

Figure 4.2 Changes in pH during fermentation in silver carp patties



The lactic acid concentration (Fig 4.3) at 0 h, 6 h, 10 h for F and C treatments were so small that they were under the detection limit, then they increased until 30 h. En and LA treatments initially had a higher amount than F and C ($P<0.5$), while En was lower than LA. This was because the acid was encapsulated in the En treatment, thus they had a lower initial amount than that in LA. The acid in the LA treatment remained stable but En increased slowly.

Figure 4.3 Changes in lactic acid concentration during fermentation in silver carp patties



The pH and lactic acid concentration (LAC) trends can be explained by the lactic acid bacteria growth. The decrease in pH and increase in lactic acid concentration in F treatment were due to the higher LAB population in the fish, which secreted lactic acid and thus dropped the pH the most. The C treatment had slower rates in pH and LAC because of the initial low LAB load and other bacteria competition. The slow or unchanged tendencies in En and LA treatments indicate there was not much lactic acid produced by microorganisms, and may also imply that the microorganism observed on MRS agar were not lactic acid bacteria, or only part of them were. These cultures also grew at the later fermentation hours (25 h and 30 h) in F and C treatments, and those in En and LA treatments could be acid-resistant cultures, such as *Staphylococcus*, yeast or molds. In research investigating other fish products or antimicrobial microorganisms, Walters and Levin (1994) isolated a fermentative film-forming yeast, which was notably resistant to propionic acid at the concentration of

2%; *Acinetobacter*, which can be isolated from freshwater and foodstudff (Towner 1996), are indicators of antimicrobial resistance, Guardabassi and others (2000) found that some of the *Acinetobacter* genus have oxolinic acid resistance.

The repeated ANOVA indicated that time, treatment and level of lactic acid difference (LD) and their interactions were important factors predicting pH, LAC and microbiology analysis (Appendix B. a, b, c). In the first replicate where the LD was 4.73 mg/g fish, the LAC of En and LA treatments kept increasing and reached similar levels of F. In the second replicate where the LD was 7.03 mg/g fish, the LAC remained stable or had very low increasing rate and did not reach the same level of F. The reason was that a lower amount of LD did not inhibit lactic acid bacteria grow while a higher dose of LD inhibited more LAB.

The pH of F and C did not show any differences until after 10 h, after which both of them started to decrease with a higher dropping rate in F. The dropping trend in control treatment was different from those in other studies. Hu and others (2008) inoculated mixed starter cultures in silver carp, the experimental pH changes were similar to our study, but the control group without any starter culture remained at about 7.5 for 48 hours. In the researchers' another study (Hu and others 2007), the pH in control groups where no starter culture added started at 6.5, had a slight decrease at 12 h and increased afterwards. Both of the studies had the same level of sugar (3%) as in our study but they had additional 3% NaCl. Liu and others (2009) employed salts, ginger and garlic juice and pepper flour, the control group showed a decrease until 16 h and then started to increase. The differences in pH changes in our study and others might be their stronger antimicrobial ingredients effects that inhibit more endogenous lactic acid bacteria while our 156 ppm nitrite were not able to. These are consistent

with the microbiology analysis where their bacteria counts on MRS agar were less than ours.

4.3 Color observation

Table 4.1 shows the color observations of the four treatments. The F and C treatments had the highest L* and whiteness values ($P<0.05$), which were desirable in fish products. The LA and En had the highest a* value ($P<0.05$), indicating they had a higher redness than the other two treatments, these were caused by fast acidifications. The higher lightness and whiteness indicate that using fermentation (F) or natural fermentation (C) have a positive effect in terms of appearance.

Table 4.1 Color observations after 30h fermentation in silver carp patties

Treatment	L*	a*	b*	Whiteness
F	77.74^{a}	1.67^{c}	9.93^{ab}	75.56^{a}
C	76.98^{ab}	1.32^{bc}	11.01^{a}	74.37^{ab}
En	75.21^{bc}	2.64^{ab}	9.17^{bc}	73.43^{bc}
LA	73.54^{c}	3.84^{a}	8.66^{c}	71.89^{c}

F (Fermentation), C (Control), En (Encapsulated lactic acid), LA (Powdered lactic acid). ^{abc}The different letters in each column indicate significant difference ($P<0.05$).

The F and C treatments had better appearances in terms of lightness and whiteness, which indicated that fermentation, whether by endogenous lactic acid bacteria, or inoculants, had positive effects. These were consistent with two studies (Yin and others 2002, Hu and others 2008), where the fermentation treatments had higher values in L* and whiteness. Yin and others (2002) also tested the color parameters before and after 72h fermentation and found the fermentation process increased the L* and whiteness values. They also stated the reasons could be that the proteolysis and partially pH-induced muscle and pigment proteins aggregation. The SDS-PAGE

(Sodium dodecyl sulphat-polyacrylamide gel electrophoresis) tests from previous research indicated that there were less high molecular weight bands in salt-soluble and water-soluble protein of fermentation treatments than those in unfermented treatments (Xu and others 2010; Liu and others 2009), and the high molecular weight bands gradually disappeared over time during the fermentation process (Yin and others 2002). Hu and others (2008) studied the amino acids profile and found there were more amino acids in fermentation groups than in the control group. Therefore, the proteolysis could be an important reason leading to the increased lightness and whiteness.

The redness indicator (a*) was not different between F and C treatments, but in their studies, the control group had higher values. These could due to the magnitude of fermentation that our C treatment had stronger fermentation than theirs because less anti-microbial agents were employed. It is noticeable that the faster acidification method had a huge impact on the redness of the products, that the En and LA products looked more reddish than slow acidification ones, which meant the acid changed the protein conformations so that the light reflection also changed. There were not any differences in b* values in F and C, which were consistent with Yin and others (2002) conclusion, but Hu and others (2008) reported that b* in fermented groups had higher values than control groups.

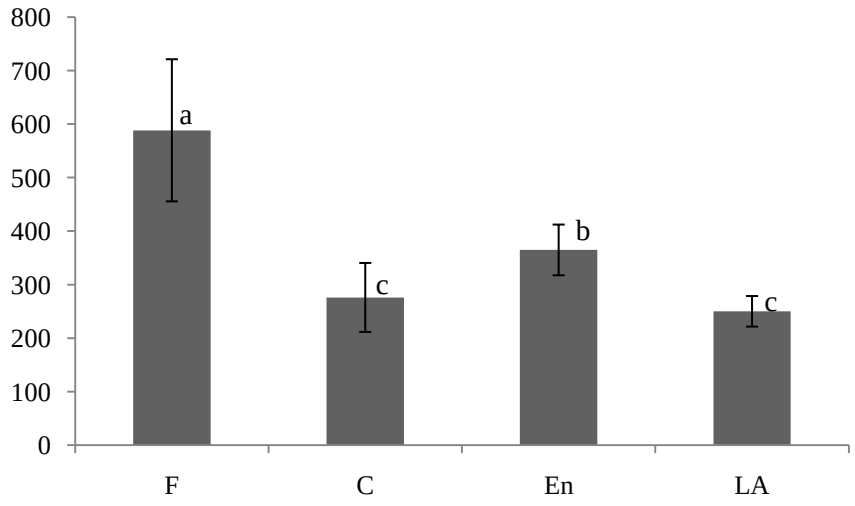
The lightness and whiteness of silver carp products are depending on the processing methods to a large degree. Wang and others (2012) using various drying methods to process restructured silver carp patties and found that fresh ones had the highest lightness (79.32, $P<0.05$), the rehydrated samples had the second highest values (73.00-78.00) and the dried ones had the lowest values (60.00-70.00). Wang and others (2011) tested the color parameters for silver carp fillets that underwent

different convective drying temperatures (40^{o}C, 50^{o}C, 60^{o}C, 70^{o}C) and found fresh samples had the highest value in lightness (approximately 55) whereas the values decreased almost 10 units in all dried samples. Taskaya and others (2009) extracted silver carp proteins under acidic (2.0-3.0) and basic pH (11.5-12.5) conditions and tested the protein paste color parameters and reported that protein paste precipitated under acidic environment had lower whiteness (58-65) than those in basic condition (68-70), but incorporation with functional ingredients (potato starch, beef plasma protein, transglutaminase, polyphosphate and titanium dioxide) increased the whiteness (approximate 75). Therefore, the lightness or whiteness varied from different origins of the silver carp and the preparation methods.

4.4 Puncture test and texture profile analysis (TPA)

The F treatment had the highest puncture value ($P<0.05$), indicating they had the strongest binding strength. The En treatment had the second highest value ($P<0.05$), whereas there was no difference between C and LA treatments.

Figure 4.4 Puncture test after 30h fermentation of silver carp patties



F (Fermentation), C (Control), En (Encapsulated lactic acid), LA (Powdered lactic acid). ^{abc}The different letters on different bars indicate significant difference ($P<0.05$)

As shown in Table 4.2, F had the highest hardness, cohesiveness, gumminess, chewiness and springiness ($P<0.05$), indicating that the internal binding and elasticity were stronger than other treatments. En and LA showed a weaker binding property in terms of hardness, cohesiveness, gumminess and chewiness ($P<0.05$). C had the lowest hardness but there were not differences with En and LA treatments with respect of cohesiveness gumminess and chewiness, indicating C and LA had very similar binding properties. For adhesiveness, En and LA treatments had the lowest values ($P<0.05$), indicating upon eating, they would be more sticky to mouth palate. For springiness, LA had the least property of elasticity.

Table 4.2 Texture profile analysis after 30h fermentation in silver carp patties

Treatment	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness
F	7067.65^{a}	-44.26^{b}	0.87^{a}	0.49^{a}	3633.44^{a}	3147.71^{a}
C	2644.09^{c}	-47.66^{b}	0.76^{b}	0.29^{b}	772.26^{b}	603.91^{b}
En	3984.05^{b}	-98.53^{a}	0.87^{a}	0.30^{b}	1189.3^{b}	1036.41^{b}
LA	2859.81^{bc}	-106.00^{a}	0.56^{c}	0.32^{b}	912.23^{b}	512.01^{b}

F (Fermentation), C (Control), En (Encapsulated lactic acid), LA (Powdered lactic acid). ^{abc}The difference letters in each column indicate significant difference ($P<0.05$)

The puncture test determines the force to push a probe into food samples (Bourne 2002a). In TPA, hardness is the force required to break food samples into pieces during the first bite (Guraya and Toledo 1996), which is associated with soft, firm or hard (Szczesniak 1963). Cohesiveness is defined as the strength of the internal bonds of the products (Szczesniak 1963). Chewiness is resistant to breaking down on mastication (Jowitt 1974), or defined as the energy needed to masticate a solid food product (Szczesniak 1963); Bourne (2002b) defined chewiness as the length of time required to masticate a sample to reduce it to constant sizes for swallowing; the attribute is related to tender, chewy or tough in human sensory analysis (Szczesniak

1963). Gumminess is energy required to disintegrate a semisolid food to a state that are suitable for swallowing (Szczesniak 1963), which measures the denseness throughout the mastication process (Bourne 2002b). However, gumminess is usually used to describe semi-solid food; therefore, it is unnecessary to use this descriptor to assess fish patties quality (Texture Technologies Corp 2012). Szczesniak (1963) also stated that since chewiness and gumminess are similar in definitions, in TPA reporting, it is recommended that only one of the two attributes would be reported. Chewiness and gumminess are the secondary parameters of cohesiveness (Szczesniak 1963). In the TPA program, the gumminess and chewiness are derived from calculations: gumminess = hardness × cohesivenss, chewiness = gumminess×springiness (Bourne 2002a). Springiness is measured by the force in which the sample returns to the original size after compression, which reflects the elasticity of the product (Munoz 1986). Adhesiveness is the force required to remove the sample that adheres to the mouth palate (Bourne 2002b).

Some properties had high correlations (Table 4.3), which are consistent to their definitions. Hardness, cohesiveness, gumminess and chewiness have strong correlations (Pearson correlation coefficients > 0.70) with each other, which indicated they are similar properties in showing internal binding, although in sensory testing, they have different perceptions.

Table 4.3 Pearson's correlation coefficients of the texture profile attributes in silver carp patties

	Hardness	Adhesiveness	Cohesiveness	Gumminess	Springiness	Chewiness
Hardness	1	0.166	0.886**	0.974	0.483**	0.979**
Adhesiveness	0.166	1	0.238	0.193	0.217	0.213
Cohesiveness	0.886**	0.238	1	0.946**	0.301*	0.938
Gumminess	0.974**	0.193	0.946**	1	0.377**	0.995
Springiness	0.483**	0.217	0.301*	0.377**	1	0.361
Chewiness	0.979**	0.213	0.938**	0.995**	0.461**	1

** Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed)

4.5 Effect of fermentation on puncture test and texture profile analysis

Kolli (2008) concluded that more organic acids resulted in stronger binding strength of restructured whole silver carp and silver carp fillets. The strength was negatively correlated to pH (-0.77). These were because more organic acid lowered the pH and made more calcium ions available to calcium ions.

This study and the studies stated above had consistency (Appendix B. g). The correlation of pH and puncture test was -0.888, the lactic acid concentration and puncture test correlation was 0.742, which also indicated there was a strong relationship between the acidity and puncture values. As for the hardness, the coefficients with pH and lactic acid concentration were -0.86 and 0.797, respectively, which demonstrated that the hardness and puncture test were similar in testing the binding of meat products. The correlation of pH and lactic concentrations with chewiness, cohesiveness were very strong, too.

The F treatment had the highest binding strength. One of the reasons may be the highest acid production by LAB. Also, it is highly possible that the proteolysis

resulted in smaller peptides and amino acids that make the gel easier to bind them or the protein may coagulate induced by acid that generates the harder texture. The study of Xu and others (2010) showed that the fermentation treatment had a higher hardness, Barbut (2005) also reported that also showed that the fermentation group had higher modulus of rigidity than other direct acidification treatments. However, these two studies did not employ any binders; therefore, fermentation alone can increase the internal binding of meat products.

The co-variate ANOVA analysis for puncture and TPA are shown in appendix B e and f. The treatments, levels of different amounts of lactic acid applied to En and LA groups, and their interactions were important factors influencing all the texture properties. The interactions were the most important, which means that the levels of different lactic acid concentrations (4.73 mg/g fish and 7.03 mg/g fish) had different impacts on the texture properties in four groups. For example, in the first replicate (LD = 4.73 mg/g), the ranking (from high to low) of puncture test and hardness was F (1st), En (2nd), LA and C (3rd); in the second replicate (LD =7.03 mg/g), the ranking of puncture test and hardness was F (1st), C, En and LA (2nd). The En had different ranks in the two replicates because of the LD dosages that a higher amount would result in higher binding compared to C and LA. It is worthy to address that in both replicates, although C had the lowest lactic acid concentration at 30h than En and LA ($P<0.05$), the binding strength were similar with those two treatments, indicating that slow acidification by natural fermentation was more effective in increasing the binding. For LA, fast and direct adding acid resulted in loose texture of the meat so it was harder to bind them in the next 30 hours. Barbut (2005) stated that the liquid lactic acid addition resulted in breaking down of proteins so the meat pieces were

crumbled. Therefore, although higher acid may result in higher binding, acidification methods were important. Slow acidification was better than fast acidification.

CHAPTER 5

CONCLUSION AND FUTURE WORK

This study confirms fermentation, whether with starter cultures or by endogenous LAB, can support alginate gel formation and result in a superior quality of silver carp patties, in terms of appearance and internal binding. Also, the statistics analysis also confirm Kolli 's (2008) study that a lower pH resulted in higher binding. Moreover, this study demonstrated slow acidification method by fermentation is better than other direct addition acid methods, such as adding encapsulated lactic and powdered lactic acid. Last but not least, the Pearson's correlation coefficient can also demonstrate that the puncture test, hardness, cohesiveness, gumminess and chewiness of TPA are good indicators of internal binding of meat products.

However, there is possible future work to do to find out the reason why the fermentation treatment was more effective or to develop a mature silver carp products. To find out the changes in the meat, proteolysis detection by electrophoresis, microstructure observations and amino acid profiles can be tested. To develop a product, more confirming steps should be conducted since this study only incorporated two replicates and the LD were different. Therefore, future studies can either focus on controlling the LD at a very similar level by knowing the pattern of starter culture growing, to confirm the current study; or do more replicates to have more levels of LD to test if fermentation had superior quality over a broad range of inoculants, which is more practical in applying fermentation in industry. In addition, future study can incorporate some spices and perform preference and descriptive sensory test in order to know how consumers think about the products.

Appendix

A. Experiments results

a. Data for microbiology analysis (Unit: log10 CFU/g fish)

		0h	6h	10h	18h	25h	30h
First Rep	F	5.16	6.91	6.91	8.41	8.72	8.81
	F	5.26	6.80	6.80	8.32	8.62	8.79
	C	3.34	5.03	5.03	8.14	9.09	9.03
	C	3.40	4.99	4.99	8.17	8.99	8.95
	En	2.11	4.13	5.67	7.89	7.88	8.78
	En	2.15	4.26	5.80	7.48	7.88	-*
	LA	1.78	3.80	4.51	7.68	8.14	9.26
	LA	2.11	3.79	4.56	7.74	8.24	9.20
Second Rep	F	-*	6.27	7.57	8.88	9.92	9.23
	F	-	5.81	7.67	8.86	9.83	9.19
	C	-	5.88	6.79	7.81	9.86	8.36
	C	-	5.11	6.80	7.49	9.88	8.45
	En	3.58	5.35	6.16	7.95	8.57	8.82
	En	3.56	5.39	6.19	8.04	8.59	8.53
	LA	3.26	3.99	4.41	6.63	7.85	8.87
	LA	2.90	3.70	4.08	6.43	7.57	8.92

*Colonies were not observed, the reason could be over diluted or human error.

b. Data for pH

	Treatment	0h	6h	10h	18h	25h	30h
First Rep	F	7.16	7.13	7.13	6.80	6.14	6
	F	7.18	7.1	7.1	6.80	6.17	5.77
	F	7.23	7.11	7.11	6.71	6.15	5.82
	C	7.31	7.08	7.08	6.91	6.38	6.5
	C	7.28	7.09	7.09	6.95	6.63	6.37
	C	7.38	7.09	7.11	6.95	6.52	6.38
	En	6.84	6.63	6.65	6.32	6.21	5.97
	En	6.9	6.65	6.55	6.37	6.24	5.94
	En	6.87	6.72	6.47	6.27	6.27	5.94
	LA	6.65	6.49	6.35	6.36	6.31	6.23
	LA	6.64	6.54	6.32	6.38	6.33	6.33
	LA	6.64	6.59	6.38	6.38	6.32	6.31
Second Rep	F	7.4	7.34	7.38	6.22	5.88	5.62
	F	7.52	7.3	7.35	6.19	5.84	5.67
	F	7.5	7.32	7.46	6.16	5.92	5.66
	C	7.38	7.33	7.58	6.62	6.22	6.08
	C	7.5	7.41	7.46	6.75	6.42	6.15
	C	7.46	7.4	7.46	6.79	6.37	6.13
	En	6.63	6.16	6.53	6.47	6.38	6.23
	En	6.59	6.26	6.55	6.45	6.25	6.24
	En	6.58	6.25	6.35	6.44	6.29	6.24
	LA	6.31	6.12	6.28	6.44	6.37	6.42
	LA	6.3	6.12	6.26	6.49	6.38	6.47
	LA	6.34	6.18	6.27	6.48	6.4	-

c. Data for lactic acid concentration (Unit: mg/g fish)

	Treatment	0h	6h	10h	18h	25h	30h
First Rep	F	-*	-	-	4.408	5.594	6.987
	F	-	-	-	2.256	5.166	8.173
	F	-	-	-	2.257	4.734	7.853
	C	-	-	-	0.537	3.012	2.902
	C	-	-	-	0.752	1.935	2.903
	C	-	-	-	0.752	2.903	3.010
	En	2.793	2.471	2.683	3.002	3.114	7.521
	En	2.578	2.468	2.683	3.325	4.081	7.737
	En	3.544	2.300	2.681	3.113	4.187	7.199
	LA	4.724	4.403	3.644	3.647	5.262	6.754
	LA	4.295	4.515	3.540	3.326	6.014	6.871
	LA	4.723	4.941	3.971		6.012	6.765
Second Rep	F	-	-	-	2.370	7.753	11.029
	F	-	-	-	2.477	7.532	10.981
	F	-	-	-	4.741	7.755	10.984
	C	-	-	-	0.754	2.585	3.988
	C	-	-	-	1.400	2.801	3.658
	C	-	-	-	1.508	2.262	4.208
	En	4.082	4.185	5.045	4.940	6.122	6.448
	En	3.866	5.473	5.256	6.225	6.446	5.586
	En	3.867	5.266	5.041	5.686	5.477	7.095
	LA	7.635	7.309	5.037	8.057	8.064	7.849
	LA	7.526	6.770	7.518	7.727	7.731	8.388
	LA	6.665	6.665	7.500	8.271	8.390	6.778

*Lactic acid concentrations were too low to detect. Undetectable.

d. Date for colorimeter

	Treatment	L	a	b	Whiteness
First Rep	F	77.85	2.22	9.85	75.66
	F	77.11	2.05	10.23	74.84
	F	78.27	2.17	9.59	76.15
	C	76.58	2.65	9.76	74.49
	C	75.18	2.53	9.97	73.13
	C	73.53	2.51	10.99	71.23
	En	76.01	2.61	8.78	74.32
	En	74.47	3.04	10.36	72.28
	En	76.63	2.84	8.55	74.95
	LA	74.88	4.07	8.14	73.28
	LA	73.63	3.98	9.16	71.80
	LA	72.85	4.27	8.29	71.29
Second Rep	F	77.6	1.45	9.61	75.58
	F	77.55	1.41	9.69	75.51
	F	78.07	0.73	10.58	75.64
	C	79.1	0.04	12.42	75.69
	C	78.49	0.08	10.52	76.06
	C	79.01	0.1	12.4	75.62
	En	74.34	2.34	8.82	72.77
	En	75.06	2.13	9.32	73.29
	En	74.77	2.86	9.19	73.00
	LA	73.55	3.43	8.73	71.94
	LA	72.52	3.88	8.78	70.89
	LA	73.79	3.43	8.87	72.12

e. Date for puncture test (Unit: gram)

	Treatment	Puncture test		Treatment	Puncture test
First Rep	F	560	Second Rep	F	540
	F	521		F	632
	F	400		F	546
	F	419		F	708
	F	580		F	740
	F	545		F	870
	C	210		C	346
	C	212		C	348
	C	226		C	332
	C	190		C	347
	C	285		C	349
	C	240		C	227
	En	398		En	425
	En	305		En	398
	En	450		En	360
	En	351		En	356
	En	324		En	337
	LA	267		En	309
	LA	256		LA	290
	LA	234		LA	247
	LA	183		LA	265
	LA	251		LA	257
				LA	226
				LA	275

f. Date for texture profile analysis

		Hardness	Adhesiveness	Springiness
First Rep	F	5173.747	-61.295	0.920
	F	5914.483	-51.420	0.876
	F	4042.027	-17.199	0.876
	F	5475.549	-76.939	0.908
	F	4675.903	-7.126	0.842
	F	6465.329	-14.652	0.853
	C	2429.444	-83.549	0.779
	C	2317.524	-15.077	0.572
	C	2252.065	-7.501	0.662
	C	1810.652	-71.897	0.678
	C	2169.097	-98.576	0.661
	C	2413.033	-71.388	0.732
	En	3952.149	-86.636	0.910
	En	3281.859	-88.758	0.890
	En	2720.802	-81.963	0.863
	En	4340.818	-88.186	0.855
	En	3994.736	-90.845	0.844
	En	4536.008	-93.921	0.903
	LA	3137.261	-98.414	0.566
	LA	2668.321	-96.826	0.552
	LA	3031.574	-122.421	0.490
	LA	3188.978	-168.138	0.555
	LA	2542.604	-146.474	0.689
	LA	3068.347	-114.733	0.427
Second Rep	F	7714.146	-77.053	0.894
	F	9759.245	-70.994	0.85
	F	6647.527	-16.726	0.878
	F	8728.585	-35.261	0.882
	F	10708.99	-84.097	0.835
	F	9506.27	-18.4	0.847
	C	3232.534	-68.168	0.891
	C	2692.739	-6.961	0.798
	C	3521.711	-11.152	0.857
	C	2946.487	-8.959	0.788
	C	3534.427	-54.248	0.852
	C	2409.379	-74.501	0.851
	En	3750.374	-64.346	0.826
	En	4445.384	-105.221	0.937
	En	4624.077	-73.574	0.855
	En	3625.273	-80.468	0.839

En	4225.634	-134.364	0.895
En	4311.434	-194.035	0.848
LA	2873.23	-136.412	0.550
LA	2728.869	-29.474	0.554
LA	2383.052	-18.487	0.531
LA	3075.69	-126.191	0.641
LA	2759.946	-108.473	0.591

	Cohesiveness	Gumminess	Chewiness
First Rep	0.392	2027.459	1866.235
	0.446	2634.985	2309.064
	0.328	1325.784	1161.751
	0.383	2096.657	1903.450
	0.372	1738.717	1464.183
	0.396	2559.755	2182.624
	0.236	574.247	447.453
	0.258	596.968	341.384
	0.276	622.180	411.939
	0.257	464.627	315.013
	0.275	595.845	393.730
	0.240	580.066	424.405
	0.250	986.438	897.585
	0.287	943.185	839.801
	0.258	701.824	605.545
	0.308	1338.566	1144.423
	0.289	1152.981	973.229
	0.307	1391.609	1257.011
	0.303	949.503	537.784
	0.274	731.179	403.695
	0.288	874.404	428.538
	0.281	895.416	496.648
	0.325	827.586	570.568
	0.288	882.385	376.994
Second Rep	0.613	4727.289	4225.247
	0.644	6282.357	5340.95
	0.582	3870.817	3399.707
	0.522	4559.64	4019.638
	0.595	6369.059	5320.635
	0.569	5408.707	4578.985
	0.312	1007.972	897.663
	0.299	803.836	641.713
	0.358	1261.172	1080.621

0.307	905.92	713.993
0.307	1085.825	924.935
0.319	768.455	654.076
0.334	1253.089	1035.161
0.287	1276.183	1196.421
0.366	1692.239	1446.591
0.298	1080.904	906.565
0.266	1123.726	1005.439
0.309	1330.826	1129.186
0.340	975.727	536.650
0.294	803.205	444.852
0.368	876.475	465.628
0.390	1200.798	769.261
0.369	1017.905	601.489

B. Date analysis

a. Repeated ANOVA measurement for microbiology analysis

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Time	Pillai's Trace	.996	301.928[b]	4.000	5.000	.000
	Wilks' Lambda	.004	301.928[b]	4.000	5.000	.000
	Hotelling's Trace	241.542	301.928[b]	4.000	5.000	.000
	Roy's Largest Root	241.542	301.928[b]	4.000	5.000	.000
Time * treatment	Pillai's Trace	2.644	12.989	12.000	21.000	.000
	Wilks' Lambda	.000	25.549	12.000	13.520	.000
	Hotelling's Trace	109.868	33.571	12.000	11.000	.000
	Roy's Largest Root	96.521	168.912[c]	4.000	7.000	.000
Time * diff[d]	Pillai's Trace	.970	41.108[b]	4.000	5.000	.001
	Wilks' Lambda	.030	41.108[b]	4.000	5.000	.001
	Hotelling's Trace	32.886	41.108[b]	4.000	5.000	.001
	Roy's Largest Root	32.886	41.108[b]	4.000	5.000	.001
Time * treatment * diff	Pillai's Trace	2.694	15.431	12.000	21.000	.000
	Wilks' Lambda	.000	24.072	12.000	13.520	.000
	Hotelling's Trace	66.624	20.357	12.000	11.000	.000
	Roy's Largest Root	46.213	80.873[c]	4.000	7.000	.000

a. Design: Intercept + group + diff + group * diff

Within Subjects Design: Time

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

d. level of difference of lactic acid applied to En and LA

b. Repeated ANOVA measurement for pH analysis

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
time	Pillai's Trace	.966	63.407[b]	5.000	11.000	.000
	Wilks' Lambda	.034	63.407[b]	5.000	11.000	.000
	Hotelling's Trace	28.821	63.407[b]	5.000	11.000	.000
	Roy's Largest Root	28.821	63.407[b]	5.000	11.000	.000
time *treatment	Pillai's Trace	1.957	4.875	15.000	39.000	.000
	Wilks' Lambda	.002	16.137	15.000	30.768	.000
	Hotelling's Trace	51.626	33.270	15.000	29.000	.000
	Roy's Largest Root	44.305	115.194[c]	5.000	13.000	.000
time * diff	Pillai's Trace	.829	10.668[b]	5.000	11.000	.001
	Wilks' Lambda	.171	10.668[b]	5.000	11.000	.001
	Hotelling's Trace	4.849	10.668[b]	5.000	11.000	.001
	Roy's Largest Root	4.849	10.668[b]	5.000	11.000	.001
time * treatment * diff	Pillai's Trace	1.897	4.469	15.000	39.000	.000
	Wilks' Lambda	.002	18.919	15.000	30.768	.000
	Hotelling's Trace	93.370	60.172	15.000	29.000	.000
	Roy's Largest Root	87.841	228.386[c]	5.000	13.000	.000

a. Design: Intercept + group + diff + group * diff

Within Subjects Design: time

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Repeated ANOVA measurement for lactic acid concentration analysis

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Time	Pillai's Trace	.996	301.928[b]	4.000	5.000	.000
	Wilks' Lambda	.004	301.928[b]	4.000	5.000	.000
	Hotelling's Trace	241.542	301.928[b]	4.000	5.000	.000
	Roy's Largest Root	241.542	301.928[b]	4.000	5.000	.000
Time * treatment	Pillai's Trace	2.644	12.989	12.000	21.000	.000
	Wilks' Lambda	.000	25.549	12.000	13.520	.000
	Hotelling's Trace	109.868	33.571	12.000	11.000	.000
	Roy's Largest Root	96.521	168.912[c]	4.000	7.000	.000
Time * diff	Pillai's Trace	.970	41.108[b]	4.000	5.000	.001
	Wilks' Lambda	.030	41.108[b]	4.000	5.000	.001
	Hotelling's Trace	32.886	41.108[b]	4.000	5.000	.001
	Roy's Largest Root	32.886	41.108[b]	4.000	5.000	.001
Time * treatment * diff	Pillai's Trace	2.694	15.431	12.000	21.000	.000
	Wilks' Lambda	.000	24.072	12.000	13.520	.000
	Hotelling's Trace	66.624	20.357	12.000	11.000	.000
	Roy's Largest Root	46.213	80.873[c]	4.000	7.000	.000

a. Design: Intercept + group + diff + group * diff

Within Subjects Design: Time

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

d. Covariate ANOVA for colorimeter test

Dependent Variable: L*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	86.822[a]	7	12.403	17.291	.000
Intercept	4895.261	1	4895.261	6824.408	.000
treatment	15.960	3	5.320	7.417	.002
difference	1.961	1	1.961	2.734	.118
treatment * difference	21.174	3	7.058	9.840	.001
Error	11.477	16	.717		
Total	138242.396	24			
Corrected Total	98.300	23			
a. R Squared = .883 (Adjusted R Squared = .832)					

Dependent Variable: a*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	34.307[a]	7	4.901	86.153	.000
Intercept	23.450	1	23.450	412.213	.000
treatment	2.177	3	.726	12.758	.000
difference	7.107	1	7.107	124.928	.000
treatment * difference	4.187	3	1.396	24.536	.000
Error	.910	16	.057		
Total	169.739	24			
Corrected Total	35.217	23			
a. R Squared = .974 (Adjusted R Squared = .963)					

Dependent Variable: sqrt b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.554[a]	7	.079	7.663	.000
Intercept	7.621	1	7.621	738.467	.000
Treatment	.024	3	.008	.785	.520
difference	.027	1	.027	2.647	.123
treatment * difference	.056	3	.019	1.819	.184
Error	.165	16	.010		
Total	232.600	24			
Corrected Total	.719	23			
a. R Squared = .770 (Adjusted R Squared = .670)					

Dependent Variable: whiteness

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	56.814[a]	7	8.116	9.690	.000
Intercept	4688.878	1	4688.878	5597.852	.000
treatment	10.461	3	3.487	4.163	.023
difference	.904	1	.904	1.079	.314
treatment * difference	12.558	3	4.186	4.997	.012
Error	13.402	16	.838		
Total	130833.211	24			
Corrected Total	70.216	23			
a. R Squared = .809 (Adjusted R Squared = .726)					

e. Covariate ANOVA for puncture test

Dependent Variable: sqrth puncture

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	565.133[a]	7	80.733	37.091	.000
Intercept	937.724	1	937.724	430.820	.000
treatment	62.330	3	20.777	9.545	.000
difference	35.744	1	35.744	16.422	.000
treatment * difference	24.182	3	8.061	3.703	.020
Error	82.711	38	2.177		
Total	17137.000	46			
Corrected Total	647.844	45			
a. R Squared = .872 (Adjusted R Squared = .849)					

f. Covariate ANOVA for texture profile analysis

Dependent Variable: sqrt hardiness

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9134.330[a]	7	1304.904	57.598	.000
Intercept	3238.613	1	3238.613	142.950	.000
treatment	308.418	3	102.806	4.538	.008
difference	683.251	1	683.251	30.158	.000
treatment * difference	852.871	3	284.290	12.548	.000
Error	883.566	39	22.656		
Total	195807.306	47			
Corrected Total	10017.896	46			
a. R Squared = .912 (Adjusted R Squared = .896)					

Dependent Variable: Adhesiveness

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	44890.896[a]	7	6412.985	5.075	.000
Intercept	14748.897	1	14748.897	11.673	.001
treatment	9328.141	3	3109.380	2.461	.077
difference	605.232	1	605.232	.479	.493
treatment * difference	7004.883	3	2334.961	1.848	.154
Error	49278.730	39	1263.557		
Total	347634.437	47			
Corrected Total	94169.625	46			
a. R Squared = .477 (Adjusted R Squared = .383)					

Dependent Variable: Springiness

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.820[a]	7	.117	46.635	.000
Intercept	.767	1	.767	305.197	.000
treatment	.106	3	.035	14.075	.000
difference	.019	1	.019	7.459	.009
treatment * difference	.060	3	.020	7.904	.000
Error	.098	39	.003		
Total	28.789	47			
Corrected Total	.918	46			
a. R Squared = .893 (Adjusted R Squared = .874)					

Dependent Variable: sqrt cohesiveness

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.278[a]	7	.040	62.875	.000
Intercept	.289	1	.289	457.968	.000
treatment	.006	3	.002	3.221	.033
difference	.056	1	.056	88.999	.000
treatment * difference	.025	3	.008	13.080	.000
Error	.025	39	.001		
Total	16.365	47			
Corrected Total	.303	46			
a. R Squared = .919 (Adjusted R Squared = .904)					

Dependent Variable: log10(gumminess)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.540[a]	7	.506	78.480	.000
Intercept	11.867	1	11.867	1841.670	.000
treatment	.100	3	.033	5.159	.004
difference	.429	1	.429	66.570	.000
treatment * difference	.231	3	.077	11.927	.000
Error	.251	39	.006		
Total	456.143	47			
Corrected Total	3.791	46			
a. R Squared = .934 (Adjusted R Squared = .922)					

Dependent Variable: log10 (chewiness)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.951[a]	7	.707	91.345	.000
Intercept	10.196	1	10.196	1316.875	.000
treatment	.176	3	.059	7.567	.000
difference	.553	1	.553	71.475	.000
treatment * difference	.243	3	.081	10.457	.000
Error	.302	39	.008		
Total	422.805	47			
Corrected Total	5.253	46			
a. R Squared = .943 (Adjusted R Squared = .932)					

g. Pearson correlation coefficients in Texture profile analysis pH and lactic acid concentration

		Hardness	Adhesiveness	Springiness	Cohesiveness
Hardness	Pearson Correlation	1	0.166	.483**	.886**
	Sig. (2-tailed)		0.265	0.001	0
	N	47	47	47	47
Adhesiveness	Pearson Correlation	0.166	1	0.217	0.238
	Sig. (2-tailed)	0.265		0.143	0.108
	N	47	47	47	47
Springiness	Pearson Correlation	.483**	0.217	1	.301*
	Sig. (2-tailed)	0.001	0.143		0.04
	N	47	47	47	47
Cohesiveness	Pearson Correlation	.886**	0.238	.301*	1
	Sig. (2-tailed)	0	0.108	0.04	
	N	47	47	47	47
Gumminess	Pearson Correlation	.974**	0.193	.377**	.946**
	Sig. (2-tailed)	0	0.193	0.009	0
	N	47	47	47	47
Chewiness	Pearson Correlation	.979**	0.213	.461**	.938**
	Sig. (2-tailed)	0	0.15	0.001	0
	N	47	47	47	47
LAcon	Pearson Correlation	.797**	0.028	0.226	.776**
	Sig. (2-tailed)	0	0.853	0.127	0
Puncture	Pearson Correlation	-	-	-	-
	Sig. (2-tailed)	-	-	-	-
	N	-	-	-	-
pH	Pearson Correlation	-.860**	-.289*	-.692**	-.759**
	Sig. (2-tailed)	0	0.049	0	0
	N	47	47	47	47

		Gumminess	Chewiness	LAcon	Puncture
	Pearson Correlation	0.974	0.979	.797**	-
	Sig. (2-tailed)	0	0	0	-
Hardness	N	47	47	47	-
	Pearson Correlation	0.193	0.213	0.028	-
	Sig. (2-tailed)	0.193	0.15	0.853	-
Adhesiveness	N	47	47	47	-
	Pearson Correlation	.377**	0.461	.226*	-
	Sig. (2-tailed)	0.009	0.001	0.127	-
Springiness	N	47	47	47	-
	Pearson Correlation	.946**	0.938	0.776	-
	Sig. (2-tailed)	0	0	0	-
Cohesiveness	N	47	47	47	-
	Pearson Correlation	1**	0.995	.795**	-
	Sig. (2-tailed)		0	0	-
Gumminess	N	47	47	47	-
	Pearson Correlation	.995**	1	.784**	-
	Sig. (2-tailed)	0		0	-
Chewiness	N	47	47	47	-
	Pearson Correlation	.795**	0.784	1**	.829**
LAcon	Sig. (2-tailed)	0	0		0
	Pearson Correlation	-	-	.829**	1
	Sig. (2-tailed)	-	-	0	0
Puncture	N	-	-	45	45
	Pearson Correlation	-.806**	-.841*	-.817**	-.888**
	Sig. (2-tailed)	0	0	0	0
pH	N	47	47	47	45

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

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