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Effect of Sourdough Fermentation Parameters on Bread Properties

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EFFECT OF SOURDOUGH FERMENTATION PARAMETERS ON BREAD PROPERTIES

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ABSTRACT

Sourdough is the oldest form of leavening which many believe was invented by the Egyptians. Bread leavened with a sourdough culture relies on the metabolism of naturally occurring lactic acid bacteria and wild yeasts. Historically there were many ties between beer brewing and bread baking. In the fourteen and fifteen hundreds, it was discovered that brewers yeast could also be used to leaven bread. Up until the invention of commercial yeast in the nineteenth century, sourdough cultures and brewers yeast where the only bread leavening methods. By 1910, traditional sourdough was much less common because bread made with commercial yeast was much faster and easier, and produced a more consistent product.[1] The positive qualities of sourdough bread were unfortunately overlooked because of the convenience that commercial yeast offered.

Phytic acid makes up about 1% of wheat and rye flours, and reduces the bioavailability of calcium, magnesium, and iron by forming complexes with the divalent cations. Phytic acid also inhibits enzymes in the digestive system needed to breakdown starch and protein.[2] This explains why some people experience discomfort from eating whole grain wheat products. Sourdough bacteria breakdown phytic acid and "predigest" the grain during the proofing process which releases easy to digest micronutrients.[3] Specific sourdough lactic acid bacteria breaks down sucrose to form exopolysaccharides that contributes to bread volume, texture, and dietary fiber content. This increase in fiber slows the digestion of the sourdough bread and does not cause rapid blood sugar spikes like a commercial white bread often does.[3]

The objective of this study was to gain a better understanding of how fermentation time and temperature affect sourdough production and give insight to why it is sometimes more acceptable than non-fermented breads to the human digestive system. Three identical batches of sourdough bread, 9 samples per batch, were produced and analyzed. Samples 26-2, 26-4, 26-8, and 26-12 were fermented at 26°C and samples 4-14, 4-26, 4-38, and 4-50 were fermented at 4°C to observe the affect of temperature on fermentation. Bread samples were analyzed for moisture, loaf height, and protein content, and parallel dough samples where analyzed for volatiles. This experiment shows evidence of protein hydrolysis with data indicating an increase in alcohol extractable protein as fermentation time increases. It was observed that fermentation temperature, environment (presence or lack of O_2), and time/duration all effect the bread qualities.

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CHAPTER ONE

REVIEW RELATING SOURDOUGH CULTURES AND GLUTEN DISORDERS

INTRODUCTION

In recent years, the identification of individuals with celiac disease has been increasingly more prevalent. Celiac disease is defined as an autoimmune disease of the small intestine that is triggered by the ingestions of gluten proteins from wheat, barley, and rye. When celiac patients ingest gluten proteins their immune cells, T and B, produce antibodies that attack the villi in the small intestine and cause inflammation and damage. This causes inability of the villi to absorb nutrients properly (Darewicz et al., 2008). Some of the common symptoms of this disease include abdominal pain, diarrhea, fatigue, headaches, and irritability (Silvester et al., 2016). Today the only proven cure for this disease is to simply avoid foods containing gluten. If celiac patients continue to consume gluten they are at risk of serious health problems like anemia (iron deficiency), early onset osteoporosis or osteopenia, infertility, lactose intolerance, vitamin and mineral deficiencies, central and peripheral nervous system disorders, pancreatic insufficiency, gall bladder malfunction, and neurological manifestations (celiac Disease Foundation). Some celiac disease individuals have discovered they have no negative reactions after consuming traditional wheat sourdough bread (Cagno et al., 2008) but very few studies have looked into the reason behind this tolerance. Initially, the assumptions focused on the gluten protein reduction occurring in the culture by microbial and enzymatic reactions, but preliminary studies suggest that active probiotics may also be assisting in

the ease of digestion (Cagno et al., 2008)(Caputo et al., 2010). This chapter will review the effects of sourdough production on bread quality and gluten sensitivities.

HISTORY OF SOURDOUGH

Sourdough cultures are the oldest form of leavening, aging back to more than 5,000 years ago. Initially, sourdough batters were a simple mixture of flour and water that were fermented, and were then used as leavening to make bread rise. It was observed that this process could be expedited by keeping the starter culture alive by continuously "feeding it," adding flour and water, and only taking a portion of it when it was time to make bread. The starter, also known as a levain, is a mixture of flour, water, and naturally occurring bacteria and yeast. The starter may be kept indefinitely if it is properly stored and fed. Only a small portion of the starter is used to make bread by mixing with a large portion of flour and a little bit of water. Before the mechanism was understood, the unknown gas producers were called "seeds". In the mid-1800's Louis Pasteur discovered the process was a result of living microorganisms feeding off the slurry. This discovery led to the invention of baker's yeast (Darewicz et al., 2008).

SOURDOUGH CULTURES & BREAD BAKING

Sourdough culture production requires only two ingredients: flour and water. Cultures are not limited to wheat flour, as other types of flours used include rice, rye, spelt, barley, and amaranth (Cagno et al., 2008). Part of the culture includes a third component of wild yeasts and lactic acid bacteria (LAB). These microorganisms are included in the culture

from the environment and different species of yeast and bacteria are introduced in the culture depending on what region of the world it is produced and what types of flours it contains. Every region contains its own unique cocktail of bacteria so no two sourdough cultures are the same. This results in regional flavor profiles (Gadsby, 2003). Traditional San Francisco Sourdough is famous for its unique bacterial and flavor profile. Specific *Lactobacillus* species, like *Lb. sanfranciscensis*, are characteristic of a San Francisco sourdough. These species use co-fermentation to metabolize fructose and maltose or glucose, or citrate and maltose or glucose. Lb. sanfranciscensis prefer to metabolize maltose which is to their advantage because of the lack of competition with yeast for glucose (Salim-ur-Rehman et al., 2006). A sensory study done by the Swiss Society of Food Science and Technology found that sourdough bread made with heterofermentative *Lb. sanfranciscensis* had a pleasant, mild, sour taste and odor, whereas homofermentative *Lb. plantarum* fermented bread had an unpleasant metallic sour taste (Katina et al., 2006). Flavor volatiles that have been correlated to with pleasant flavors of wheat bread crumb include 2-methylpropanoic acid, 3-methyl-butanoic acid, 2/3-methyl-1-butanol, acetaldehyde, 2-nonenal, 2-phenylethanol, benzylethanol, 2,3-butandione, dimethyl sulphide, and 2-furfural (Salim-ur-Rehman et al., 2006).

In the artisanal baking world, sourdough cultures are kept alive for decades and passed down through generations like a family memento. One of the oldest sourdough cultures on record is 126 years old, owned by Lucille Clarke Dumbrill of Newcastle, Wyoming. She got the starter from her mother, who got it from one of her husband's students at the

University of Wyoming. The culture was traced back to a sheepherder's wagon near Kaycee, Wyoming in1889 (Matray, 2011). Like many owners of sourdough culture, Lucille stores her culture in the refrigerator where is remains in a dormant state. To make a batch of sourdough bread, a portion of the culture is removed from the fridge and continually fed at room temperature until it is fully active.

Food for the bacteria and yeasts consists of an even mixture, by weight, of flour and water that is incorporated into the culture. To keep a sourdough culture active at room temperature, the microorganisms must be fed regularly every 12 or 24 hours depending on the flour type and maturity of the culture. When making a new wheat culture for example, it is suggested that you feed the culture every 12 hours for at least the first three days. When activating a dormant culture, the process could take anywhere from 1 to 3 days. Often a portion of the fed culture is discarded after every couple feedings because otherwise there would be an excessive amount of culture. Often cultures are produced in large mason jars covered lightly with a mesh-like fabric or cheesecloth when it is being activated at room temperature. This unsealed environment allows the microorganisms to respire.

Once the culture is active and mature, at least two weeks old, it is ready to produce sourdough bread. This requires about three parts flour, two parts sourdough culture, and one part water by volume. After mixing and kneading, the dough is allowed to proof anywhere from four to twenty-four hours. A double proofing is sometimes done by

punching down after four to twelve hours and then the dough is allowed to proof a second time. The dough is then baked at around 400°F until the internal temperature reaches 190°F to 210°F (Cultures for Health, 2014).

LACTIC ACID BACTERIA & WILD YEASTS

Lactic acid bacteria (LAB) are the primary bacteria utilized to produce sourdough bread. Other types of bacteria may be present in the bread but are not critical to the process. Wild yeast works synergistically with LAB because yeast do not metabolize maltose and compete with the bacteria like baker's yeast would. Many untraditional sourdough breads on the market today utilize baker's yeast to expedite the proofing process. When the artificial yeast is added they compete with the LAB for nutrients and some nutritional benefits of a traditional sourdough are diminished (Lhomme et al., 2014). Lactic acid bacteria found in sourdough cultures include *Lactobacillus sanfranciscensis*, *Lactobacillus rossiae, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus pentosus*, and *Pediococcus pentosaceus*. These species of bacteria are responsible for the production of acid and therefore are classified as obligately heterofermentative, facultatively heterofermentative, or obligately homofermentative (Settanni et al., 2013). Homofermentative LAB primarily produce lactic acid as a by-product of glucose fermentation, while heterofermentative LAB produce lactic acid, carbon dioxide, and ethanol/acetic acid from the fermentation of glucose. Facultatively heterofermentative bacteria use the glycolitic pathway to produce lactic acid, but are able to use the heterolactic fermentation process when there is limited glucose (Dairy Foods Science

Notes, Cornell University). Common wild yeast often captured in the culture include *Saccharomyces cerevisiae, Kazachstania exigua,* and/or *Candida humilis* (Cagno et al., 2008). A study done by the Department of Agricultural and Forest Science at the Università degli Studi di Palermo in Italy looked at the affects of individual lactic acid bacteria on the bread quality and characteristics. When produced with non-sterile flour, bread made with *Lb. sanfranciscensis* showed the greatest loaf height. In the experiment with sterile flour, *Ln. citreum and W. cibaria* consistently produced bread with higher loaf height (Settanni et al., 2013).

ENZYMATIC & MICROBIAL ACTIVITY

The fermentation process in sourdough leads to the activation of naturally occurring grain enzymes. Studies have shown this activity increases nutrient bioavailability that may also facilitate the ease of digestion of the bread. Starch degradation is the main source of fermentable carbohydrates and reducing sugars. Hydrolysis by amylases liberates maltodextrins, maltose, and glucose during fermentation. Maltose accumulation happens in the early stages of fermentation. Once the pH is reduced to 4.5 or below, the maltogenic amylases are inhibited, but glucose from starch and maltodextrins are still released by glucoamylase activity. A specific sourdough lactic acid bacteria breaks down sucrose to form exopolysaccharides that contribute to bread volume, texture, and dietary fiber content (Gänzle, 2014).

Proteolysis is the breakdown of proteins to peptides. In sourdough, this process is dependent on metabolic activity of bacteria that lowers the pH, which leads to the activation of endogenous proteases, the primary source of protein metabolism. The acidic pH of the culture activates proteinases, which are responsible for depolymerisation of proteins and enzymatic degradation of gluten. When the pH of the culture drops due to fermentation there is an accumulation of low molecular weight thiols, which increase the solubility of gluten proteins by decomposing their intermolecular disulfide bonds, making them more prone to breakdown. Lactic acid bacteria help to increase the amount of free amino acids by activating the strain-specific intracellular peptidases (Figure 1)(Gänzle, 2014).

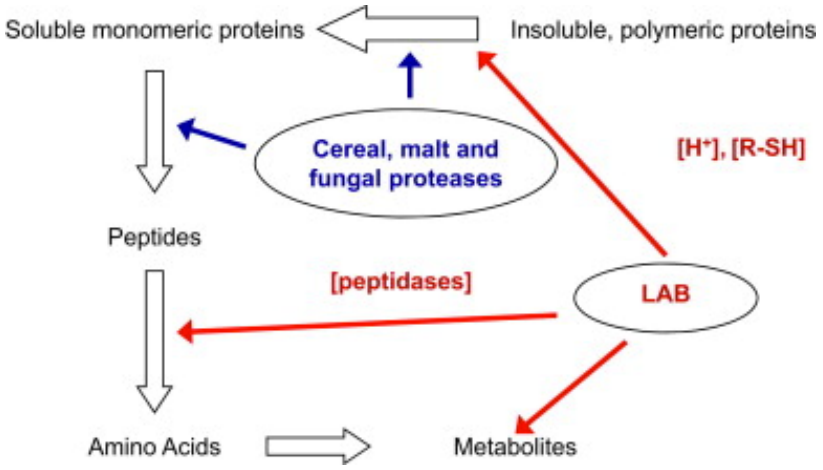

Figure 1. Overview of proteolysis and amino acid metabolism in wheat soughdough
*red arrows indicate conversion by microbial enzymes
*blue arrows indicate conversion by cereal enzymes (Gänzle, 2014)

Lipid oxidation begins when active endogenous lipoxygenase consume oxygen during mixing of the dough. This enzyme oxidizes linoleic acid to form hydroxyperoxy acid.

Flavor active aldehydes are produced by both enzymatic and non-enzymatic degradation of fatty acid hydroperoxydes (Gänzle, 2014)(Hansen and Schieberle, 2005).

Soaking and sprouting grains has been an increasingly popular preparation method because of the nutritional advantages. Much of the increased nutrient value is associated with the enzymatic degradation of phytate. Phytate makes up 1% of the wheat grain and it forms complexes with divalent cations of calcium, magnesium, and iron. The complexes with these nutrients make them unavailable to human digestion. Through enzymatic hydrolysis by phytases, phytate is reduced and nutrient bioavailability is increased (Gänzle, 2014). Vinegar is often added to the soaking liquid to lower the pH and expedite the activation of phytases. Sourdough cultures have the same effect when the fermentation creates an acidic environment resulting in phytase activation (Gänzle, 2014).

GLUTEN & CELIAC DISEASE

Gluten is 75% protein based on dry weight, with the remainder being mostly starch and lipid. The protein portion is responsible for the autoimmune response by celiac disease patients. Majority of the proteins are prolamins, classified by their solubility in alcohol and characterized by their high glutamine and proline content. Gliadins are monomeric prolamins, and glutenins are polymeric prolamins (Shewry et al., 2002). Previously, gliadins were believed to cause the autoimmune response in celiac patients, but more recent research has concluded that glutenins also contribute to the autoimmune response.

The fermentation by bacteria and yeasts cause acidification and production of alcohols including thiols. Cereal grain enzymes that participate in gluten degradation depend on both the presence of thiols and the decreased oxidation-reduction potential caused by fermentation. Sourdough is unique because of this interdependent relationship between the culture microbes and cereal grain enzymes. Thiols increase efficiency of enzymatic degradation of the alcohol soluble gluten proteins. LAB is responsible for the conversion of peptides to amino acids and amino acids to metabolites while the cereal grain enzymes are important to convert gliadins to peptides. In sourdough cultures the rate of gliadin hydrolysis is greater than glutenin hydrolysis mainly due to a more complex glutenin structure (Gänzle, 2014)(Hansen and Schieberle, 2005).

WHEAT PROTEIN ANALYSIS

A study supported by The Research Association of the German Food Industry (FEI) analyzed the individual protein fractions and their extent of degradation during sourdough fermentation. The samples were extracted stepwise by first removing albumins and globulins with NaCl and $HKNaPO_4$, then extracting the gliadin subunits with 60% (v/v) ethanol, and finally the glutenin fractions with a mixture of 1-propanol containing urea, DTT, and Tris-HCl. Data indicated that sourdough fermentation caused the greater decrease in glutenin fractions compared to other breadmaking methods. This leads to an increase in alcohol soluble oligomeric proteins, which reside in the gliadin fraction. This research also discovered that different (homo- or hetero-) fermentative microbial strains caused various degrees of proteolysis (Wieser et al., 2008).

VOLATILE ANALYSIS

Sourdough can be characterized by its unique aroma and flavor profile produced during fermentation. These aromas are dependant on volatile compounds produced from the interaction between the flour and microorganisms present in the culture. These aroma compounds can be identified and quantified by gas chromatography/mass spectrometry (GC-MS). This analysis has given deeper insight into the microbial and enzymatic processes that occur in sourdough fermentation. A study published by the American Association of Cereal Chemists analyzed the proteolysis and liberation of amino acids by cereal and microbial enzymes in sourdough fermentation. This study proved that specific bacteria species cause an accumulation of certain free amino acids that convert to recognizable volitiles (Thiele et al., 2002). Therefore, specific bacteria and yeasts can often be identified knowing that certain volatiles are specific byproducts of that species metabolic process. A study published in the Journal of Cereal Science analyzed the relationship between bacteria species in sourdough and volatiles produced and reported that *L. sanfranciscensis* fermented dough yielded less (E)-2-nonenal, that corresponded to an increase in (E)-2-nonenol (Vermeulen et al., 2007). Sourdough bread flavor is greatly influenced by the lactic and acetic acid content. Homofermentative lactic acid bacteria (LAB) convert over 85% of hexoses into lactic acid, but heterofermentative LAB use hexoses to produce lactic acid, acetic acid/ethanol, and CO_2. Lactic and acetic acid ratios have also been shown to be affected by environmental temperature which would in turn affect flavor (Vaintraub and Bulmaga, 1991).

CONCLUSION

One objective of this research was to analyze the extent of protein hydrolysis within the different sourdough fermentation time periods. Gluten protein breakdown in sourdough cultures is of great interest because of the potential positive impact for celiac and gluten sensitive patients. Some bacteria have shown to break down gluten content more efficiently than others (Caputo et al., 2010)(Hansen and Schieberle, 2005).

With the increasing prevalence and recognition of gluten related disorders, research on reducing this autoimmune response is of great interest. This research will provide an additional understanding of the rate at which sourdough cultures degrade protein. The hope is that these findings will contribute to future discovery of the digestion mechanism and tolerance of celiac patients for sourdough breads.

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CHAPTER TWO

SOURDOUGH FERMENTATION EFFECTS ON BREAD QUALITY

ABSTRACT

The effects of fermentation duration at two different temperatures on the quality and gluten content of sourdough bread were analyzed. A sourdough culture was used to produce 3 separate batches of bread, of which there was 1 Control (0) and 8 fermentation treatments. The Control was baked at time 0:00, not allowed fermentation time. Samples 26-2, 26-4, 26-8, and 26-12 were fermented at 26°C for 2, 4, 8, & 12 hours, respectively and samples 4-14, 4-26, 4-38, and 4-50 were fermented at 4°C for 12, 24, 36, & 48 hours, respectively. Loaf volume, bread moisture, protein content, and volatiles were analyzed for each sample. Bread moisture increased as fermentation time increased for both the 26- and 4- samples. A significant change ($p<0.05$) in moisture was observed between samples 4-14 and 4-50. Ethanol extractable protein consistently increased with fermentation time when comparing the Control (0) with sample 26-12 or 4-14 with 4-50 within batches. This increase was at a significant ($p<0.05$) level when comparing the mean of the Control and 26-12. Analysis on dough samples with a gas chromatograph mass selective system (GC-MS) showed evidence of fermentation progression with an increase in alcohol. An increase in 1-hexanol was observed from the Control-d to samples 26-4d and 26-12d. Control-d, 26-4d, and 26-12d also showed a decrease in hexanal as fermentation time progressed. In the refrigerated temperature (4°C) treated dough samples, 1-hexanol increased but not as significantly as the room temperature samples.

1. Introduction

Sourdough is the oldest and most traditional form of leavened bread (Kiple and Ornelas, 2000). Before the invention of Bakers Yeast, sourdough was the only option to create leavened bread. Because sourdough is a natural process that relies on the metabolism of microorganisms, it requires numerous days of preparation and proofing time. Commercial bakers yeast became available in the United States in the 1860s then quickly became the primary leavening method due to its convenience and consistency (Smith, 2004). It appears that the health benefits and higher bread quality associated with sourdough cultures has just begun to be recognized within the past 30 years.

Although sourdough is more time-consuming than other bread-making methods, there are a number of beneficial reactions that occur during the process. The lactic acid bacteria and yeasts along with the endogenous enzymes are responsible for the microbial metabolism and enzymatic hydrolysis of carbohydrates, phenolic compounds, lipids, and proteins. The metabolism of carbohydrates contributes to the texture, water binding ability, shelf life, nutritional factors, and overall taste of the bread. The production of oligosaccharides, indigestible carbohydrates, is particularly advantageous due to their dietary fiber and prebiotic affects (Gänzle, 2014). In the small intestine the oligosaccharides cannot be broken down therefore acting as a dietary fiber, but in the large intestine these carbohydrates are metabolized by bacteria, acting as prebiotics (Mudgil and Barak, 2013). Exopolysaccharides are carbohydrate polymers produced by

heterofermentative lactobacilli. These bacteria produce exopolysaccharides to form biofilms for protection against environmental factor, but in bread these polymers are beneficial for there water binding capacity which helps prevent bread staling (Gänzle, 2014). The bacteria break down these carbohydrates into short chain fatty acids (SCFA) that can then be absorbed as nutrients in the colon (Gibson et al., 1996). The transformation of phenolic compounds adds both a nutritional and flavor benefit to the bread. Without being broken down, phenolic compounds are considered an antinutrient, they interfere with the absorption of starch and protein, and they also impart a bitter taste. Once the phenols are metabolized by lactic acid bacteria and cereal enzymes the nutrient bioavailability, ratio of absorbed nutrients, is increased and the bitter taste is eliminated. Wheat flour contains about 1% phytate, a saturated cyclic acid that reduces the bioavailability of calcium, magnesium, and iron by forming a complex with divalent cations. Phytate hydrolysis, by phytases, is dependent on a low pH, between 3.5-5, which is achieved in sourdough bread fermentation. The complex with divalent cations is soluble in an acidic pH, 5 or below (Leenhardt et al., 2005). The complexes between phytate and vitamins or minerals are weakened as sourdough ferments, making the nutrients available for digestion. Soaking and sprouting grains is a process in which similar enzymatic reactions occur. The purpose is often to increase availability of nutrients and aid in digestion of the grain by activating cereal enzymes. The metabolism of lipids contributes antioxidant activity, antifungal properties, and some flavor compounds. Lipid oxidation begins in sourdough bread making during the mixing process when oxygen is consumed by endogenous lipoxygenase activity where linoleic

acid is oxidized to hydroxyperoxy acids. Hydroperoxydes are degraded into flavor active aldehydes by enzymatic or non-enzymatic reactions. Coriolic acid, a hydroxy-fatty acid derived from peroxides, has potent anti-fungal property and can increase shelf life over twofold (Czerny and Schieberle, 2002).

Proteolysis in sourdough is caused by the acidification and accumulation of low molecular weight thiols. Here, the pH shifts to the optimum level for aspartic proteases, the main proteinase in the wheat grain. These factors increase both the solubility of gluten proteins and the susceptibility to enzymatic break down (Gänzle et al., 2008). This degradation leads to an accumulation of peptides and amino acid metabolites which in recent years have been recognized for there antioxidant, anti-cancer, or antihypertensive activities (Gänzle, 2014). The protein transformations that occur in sourdough have gained a great deal of attention over the past few decades due to reduced toxicity to some patients with gluten related disorders. It is estimated that about 1% of the worlds population has celiacs disease. In the United State only about 5% of the population affected by celiacs disease have been diagnosed (Celiac Disease Center). It is important to gain a deeper understanding of the disease and the tolerance of sourdough bread so that these individuals have fewer restrictions in there diet.

The objective of this research was to contribute insight on the protein transformations and quality changes that occur resulting from sourdough fermentation, specifically, due to fermentation time and temperature.

2. Materials and Bread Preparation

2.1. Sourdough Culture

Unbleached wheat bread flour (King Author Flour, Norwich, Vermont) was used to make the sourdough samples because it is the most common flour used in sourdough bread. The sourdough culture was donated from Chef Cicely Austin, Executive Pastry Chef, of Aramark Food Service at Clemson University. The culture was stored in a covered container in a refrigerator at 4°C (40°F). For the duration of the experiment, 25 weeks, the culture was feed once a week with 100 mL of spring water (Poland Spring, Poland, Maine) and 100 g of bread flour and then mixed.

In addition, a traditional San Francisco Sourdough Culture was acquired from Sourdoughs International, Inc (Cascade, Idaho). This culture arrived in a lyophilized form and was fully activated by numerous feedings and close temperature monitoring.

2.2. Preparation of Active Sourdough Culture

The entire sourdough sample preparation process, parts 2.2 and 2.3, was done 3 separate times for the production of 3 sourdough batches (Figure 2 & Figure 3).

Thirty-six hours prior to using the culture to make dough, the culture was fully activated by removing 40 g of the culture from the refrigerator and mixing with 40 mL of spring water (Poland Spring, Poland, Maine) and 40 g of bread flour. During this 36-hour

activation process the mixture was covered loosely and kept at room temperature, 21°C (70°F), between each feeding. After 12 hours the mixture was fed for a second time with 120 mL of spring water and 120 g of bread flour. The third feeding was 8 hours later with 360 ml of water and 360 g of bread flour, and the fourth feeding was 8 hours after the third and required 1080 mL of water and 1080 g of bread flour. At this point the culture was active and doubled in size in less than 8 hours (Table 1 & Figure 2).

2.3. Preparation of Dough and Baking Procedure

The bread dough was prepared 8 hours after the forth feeding with 3000 g of the active starter mixed with 2400 g of bread flour, 670 mL of spring water and 40 g of fine sea salt. Ingredients were mixed by hand and then kneaded in a planetary mixer (Model PM10, Berkel Company, South Bend, Indiana) with a Spiral Dough Hook attachment for 10 minutes. The dough was then portioned into 205 g loaves that were formed into oblong balls and placed into loaf pan (5.75" L x 3.25" W x 2.25" H). Each pan was prepared with nonstick cooking spray (Pam Original, ConAgra Foods, Omaha, Nebraska) and 4 g of cornmeal (Quaker, Chicago, Illinois). Nine dough samples, 25 g each, were made in parallel with the 9 bread samples following all the steps except for the baking process. The dough samples (d) were each stored in 2-oz plastic portion cups with lids. Instead of being baked at the time of the loaf sample, this dough sample was frozen in order to terminate further fermentation. These frozen dough samples were used for volatile analysis to observe the progression of fermentation. Loaf 0 (control) was baked immediately (time 0:00) in an Electrolux Icon Dual-Fuel Range (Model#: E36DF76GPS,

Charlotte, North Carolina). Samples 26-2, 26-4, 26-8, and 26-12 were held in a proofing system, rolling rack covered with a large plastic bag, at 26°C and each taken out to be baked at their designated times (2, 4, 8, & 12 hours, respectively). Temperatures of the loaf samples were monitored with probe thermometers. Samples 4-14, 4-26, 4-38, and 4-50 were wrapped in plastic film to hold in moisture and refrigerated at 4°C (40°F) for their allotted time (12, 24, 36, & 48 hours, respectively). Before baking the 4- samples, they were placed in the proofing system, at 26°C for 2 hours prior to baking (Table 2 & Figure 3). A probe thermometer indicated that the 4- samples had an internal temperature of 4°C when they were removed from the refrigerator and an internal temperature of 26°C after their 2 hours in the proofing system. All samples were baked at 204°C (400°F) until the internal temperature reached 93°C (200°F). After each sample was baked, it was taken out of the loaf pan and placed on a drying rack for 2 hours before being tightly wrapped in plastic film, labeled, and stored in the freezer at -18 to -15°C (0-4°F).

2.4. Sample Preparation and Moisture Analysis

Bread samples were removed from the freezer. The outer crust was removed and only the inner portions were used for analysis. The samples were thinly sliced and broken into small pieces not greater than ½ inch. Each sample was weighed (approximately 20 g) and placed in a labeled tin tray. The samples were then placed in a Mechanical Convection Oven (Blue M Electric Company, Blue Island, Illinois) for 24 hours at 100°C. Once removed from the oven, the samples were weighed again for moisture analysis (Table 1).

Samples were then individually pulverized in a blender (Oster Osterizer with 8 oz jar, John Oster MFG. CO, Milwaukee, Wisconsin) and stored in 50 mL centrifuge tubes at 21°C (70°F) until further use.

3. Analysis Methods

3.1. Loaf Height

The maximum height, highest point, of each bread loaf sample was measured in centimeters to the nearest tenth with a standard ruler. Measurements were recorded for further analysis to determine possible correlations between loaf height and extent of fermentation.

3.2. Moisture Analysis

The weight of each sample was taken before and after being dried in the Mechanical Convection Oven (Blue M Electric Company, Blue Island, Illinois) for 24 hours at 100°C. The difference in the original and dried weight was divided by the original weight and multiplied by 100 to get the percent moisture.

3.3. Gluten-Tec® ELISA

Guten-Tec ELISA (EuroProxima, Arnhem, The Netherlands) is an competitive enzyme immunoassay kit that quantitatively detects gliadin and gliadin fragments and was used for gliadin detection for this research. The gliadin antibody used is 100% specific for a T cell stimulatory peptide on the gliadin molecule of wheat, hordein in barley, and secalin

in rye. These epitopes are known to play a major role in eliciting symptoms of celiac disease. The Gluten-Tec ELISA kit is sensitive enough to detect levels a low as 10 parts per million (ppm) (Gluten-Tec® ELISA, 2011).

The Gluten-Tec kit is an indirect ELISA method, meaning that the more gliadin detected will result in less color. This test uses a substrate/chromogen solution that contains horseradish peroxidase (HRP), an enzyme that catalyzes the oxidation of the substrate when hydrogen peroxide is present (Thermofisher Scientific, 2016) and TetraMethylBenzidine (TMB), which makes the HRP visual with color. The HRP binds to the unoccupied sights (where gliadin binds when present) so more color will seen when there are more open binding sites for HRP or less gliadin (Gluten-Tec® ELISA, 2011).

3.3.1. Preparation of Buffers & Samples

Buffer A was made by adding 154 mg of DL-Dithiothreitol (Sigma D0632) and 303 mg of Trisma base (Sigma T1503, SIGMA-ALDRICH Co., St. Louis, Missouri) to a 50 ml tube and dissolved with 10 ml of distilled water. Thirty milliliters of 60% ethanol solution was then added and the mixture was carefully blended. The pH of the solution was adjusted to 8.0 with 1 M HCl using a pH meter (Orion model 420A and Orion 9157BN Triode Refillable pH, Orion Research Inc., Boston, Massachusetts). This solution was then placed in a volumetric flask and filled to 50 ml with distilled water.

Buffer B was made by dissolving 1017 mg of iodoacetamide (Sigma ref. 16125-259, SIGMA-ALDRICH Co., St. Louis, Missouri) and 303 mg Trisma base (Sigma T1503) in 40 ml of distilled water. The pH of this solution was then adjusted to 8.0 with 1 M HCl. This mixture was then placed in a volumetric flask and filled to 50 ml with distilled water.

Each pulverized bread sample was weighed out, 0.25 g, into a 15 ml screw cap Greiner. Four point seven five mL of Buffer A was added to each and vortexed for 1 minute and then incubated in a water bath for 30 minutes at 60°C. Samples were then vortexed for another minute before being centrifuged for 10 minutes at 10,000 x g at room temperature (70°F). Two hundred and fifty µl of the clear supernatant was transferred to a tube and neutralized with 250 µl of Buffer B and vortexed for 1 minute. The neutralized samples were then incubated at room temperature in the dark for 30 minutes before being diluted 1:500 with the Sample Dilution Buffer (ready-to-use from kit) (Gluten-Tec® ELISA, 2011).

3.3.2. Preparation of Reagents

Reagents were prepared and used within the same day. Prior to use, reagents were brought to room temperature (1 hr). Microtiter and plate strips were also brought to room temperature before each use. The unneeded strips were kept in refrigerator in the resealable bag until further use. The Gluten-Tec kit Rinsing Buffer was diluted by a factor of 20 by mixing 2 ml of the concentrated rinsing buffer and 38 ml of distilled

water. The dilution buffers, both the conjugate and antibody dilution buffers, were supplied in a bottle ready-to-use in the kit (Gluten-Tec® ELISA, 2011). To prepare the conjugate solution the vial of conjugate was spun down in the centrifuge for 1 minute at 1000 × g (RPM) before 20 µl was mixed with 2 ml of conjugate dilution buffer. The antibody, biotinylated anti-alpha-20, vial was spun down with a short centrifuge step for 1 minute at 1000 × g, then 10 µl of the antibody is added to 1 ml of the antibody dilution buffer. The substrate solution was delivered ready-to-use. It was mixed well and brought to room temperature before each use. The ethanol solution, 60%, was prepared by mixing 300 ml of ethanol and 200 ml of distilled water (Gluten-Tec® ELISA, 2011).

3.3.3. Assay Procedure

3.3.3.1. Standard Curve and Samples

One hundred µl of the zero standard was pipetted in duplicate in wells A1 and A2 (blanks). Fifty µl of the zero standard were pipetted into wells B1 and B2 (maximal signal). Each remaining standard solution, 0.25, 0.5, 1, 2, 4, and 8 ng/ml, were pipetted in duplicate, 50 µl each, in wells C1, 2 to H1, 2 respectively. Fifty µl of each sample was pipetted in duplicate in the microtiter plate. Then 50 µl of antibody was added to each standard and sample well except the blank wells, A1 and A2. The plate was then sealed and gently mixed for 30 seconds on a microtiter plate shaker before being incubated in the dark for 1 hour at 4°C. Then the solution in the microtiter plate was discarded and washed 3 times with the rinsing buffer. The conjugate was then pipetted into the wells,

100 µl in each, except for wells A1 and A2. The plate was sealed once again, shaken on the plate shaker, and incubated for another hour in the dark at 4°C. After the incubation, the solution was discarded once again and washed 3 times with rinsing buffer. Then 100 µl of the substrate solution was added into each well including the blank wells, A1 and A2. At this point the plate was incubated for 30 minutes at room temperature (20°C - 25°C) before adding 100 µl of stop solution into each well. The absorbance values were then read immediately by an Epoch BioTek plate reader (BioTek Instruments, Inc., Highland Park, Winooski, Vermont) at 450 nm and recorded for further calculation (Gluten-Tec® ELISA, 2011).

3.3.3.2. Calibration Curve

The optical density (O.D.) of the wells containing the standards and the samples were obtained by subtracting the mean O.D. of the blank wells, A1 and A2. The mean O.D. values for each of the duplicates were divided by the mean O.D. value of the zero standard (wells B1 and B2) and then multiplies by 100. This makes the zero standard equal 100% or maximum absorbance while the other O.D. values are a percentage out of the maximal absorbance.

$$\frac{\text{O.D. standard or O.D. sample}}{\text{O.D. zero standard}} \times 100\% = \%\ \text{maximal absorbance}$$

The calculated values were then plotted on the Y-axis versus the alpha-20-peptide concentration (ng/ml) on the logarithmic X-axis.

In order to calculate the α20 peptide concentrations of the samples, the α20 peptide read from the curve was multiplied by 1000. The conversion factor for peptide to gliadin is 100. This is based off the correlation of peptide content to gliadin found in wheat (Gluten-Tec® ELISA, 2011).

3.4. Bradford Assay Protein Detection

3.4.1. Sample Preparation and BSA Standard

Dried, pulverized bread samples (prepared in section 2.4) were used for determining protein content. Buffer A was used to extract the protein from the samples and the supernatant was taken after centrifugation for analysis. The Coomassie Brilliant Blue G-250 binds to the polypeptide backbone electrostatically and with hydrophobic interactions (Nielsen, 2010).

Bovine serum albumin (BSA) solutions were used as the reference standard. The standard curve consisted of protein concentrations of 0.0 (blank), 0.125, 0.25, 0.50, 0.75, 1.0, 1.25, and 1.50 mg/mL.

3.4.2. Microplate Procedure

Ten µL of each standard or sample was pipetted in duplicate into the appropriate wells of a microplate. Then 300µL of Coomassie Plus Reagent was added to each well and mixed on a plate shaker for 30 seconds. The plate was then incubated for 10 minutes at room temperature before measuring the absorbance at 595nm in an Epoch BioTek plate reader (Nielsen, 2010).

3.4.3. Calculations

The average for the Blank read was subtracted from all standard and sample reads. The standard curve was drawn by plotting the Blank-corrected measurements for each BSA standard vs. its concentration in µg/mL. The linear fitted calibration curve was then used to determine the protein concentrations of each unknown sample (Nielsen, 2010).

3.5. Ethanol Extraction

Prolamins (mostly gliadin and some glutenins) from the samples were extracted by mixing 0.125 g of the pulverized bread with 5 mL 60% (v/v) ethanol in a rotary shaker for 1 hour at room temperature (21°C, 70°F). The samples were then centrifuged at 10,000 x g for 10 minutes and the supernatant was separated. The precipitate was then extracted for a second time by adding 2.5 mL of 60% (v/v) ethanol, mixed in the rotary shaker for another hour at room temperature (21°C, 70°F), and then separated by centrifuge at 5000 rpm for 10 minutes before removing the supernatant for further

analysis (De Angelis et al., 2006). The first and second extractions were measured for protein content using the Bradford method.

3.6. Headspace Gas Analysis

Gas chromatography was used to analyze the volatiles associated with the various sourdough samples, which help determine the extent of fermentation. In order to prevent further fermentation prior to the analysis, dough samples were taken out of the freezer no more than 30 minutes before running the test. This allowed the sample to thaw such that 8 g could be separated and placed into the glass vial.

Samples were analyzed with a gas chromatograph mass selective detector system (Hewlett Packard HP 6890 Series gas chromatograph and Hewlett Packard 5973 mass selective detector) fitted with a headspace sampler capillary column (Agilent Technologies 7697A, 30.0 m $\times$ 250 μm $\times$ 0.25 μm). Prior to analysis, the sealed vials containing the dough samples were heated to 80°C for 20 minutes in the HP 7694. For each sample run the GC oven started at 35°C for 5 minutes, then increased 7°C per minute until it reached 100°C, followed by an increase of 10°C per minute until 230°C was reached. The total runtime was 32.29 minutes (Paucean et al., 2013). The plotted chromatograms where used to calculate gas percentages from peak areas.

3.7. Statistical Analysis

The experiment was replicated 3 times using 3 different batches of sourdough. A general linear model was used to compare all holding time treatments of room temperature (0, 4 and 12 hours) and refrigerated temperature (14, 26 and 50 hours). For parameters that were found to be significantly affected by the treatments ($P \leq 0.05$) the means were separated using the least significant difference command of SAS (SAS, 2016).

4. Results and Discussion

4.1. Loaf Height

Measuring the height of each bread loaf can indicate peak fermentation time, a reflection of maximum gas retention in the bread. Over fermentation can be identified by a loss of volume and reduced gas production due to inactivity of microbes, often caused by a lack of adequate nutrients but can also be affected by hold temperature. In Batch #1 the peak fermentation time for 26°C samples was 8 hours, in Batch #2 the peak was closer to 4 hours, and in Batch #3 the peak was around 2 hours. In the samples held at 4°C, the peak was at 48 hours for Batch #1, 36 hours for Batch #2, and 48 hours for Batch #3. One factor that may have affected these observations is the scoring before baking. The loaves must be scored to allow steam to escape during baking in order to prevent large air pockets. Although all samples were scored in the same manner, some lost more volume than others. The greatest decrease in volume caused by scoring was seen in the samples that had been fermented the longest.

4.2. Moisture Analysis

In general, as the fermentation time increased, the bread moisture increased regardless of hold temperature (Table 5). At 26°C fermentation the difference in bread moisture for the 0 and 12-hour treatments was not significant ($p>0.05$), although there was an average increase of 0.94%. A significant change ($p\leq0.05$) was observed at 4°C fermentation where the moisture increased by an average of 1.87% from 12 to 48 hours. This increase in moisture could be caused by metabolic activity of microbial and endogenous enzymes that result in water as a byproduct. Fermentation also increases water binding compounds that contribute to dough hydration and prevent staling (Gänzle, 2014). This may prevent the dough from losing water via evaporation during the baking process.

4.3. Gluten-Tec® ELISA Protein Quantification

There was no consistent data indicating the reduction of wheat proteins throughout the fermentation process. The large variation within batches indicated the assay was not well suited for sourdough bread as three dilutions were needed to reduce the protein content into the testing range. This process may have greatly reduced the accuracy and precision of the test.

This analysis technique quantifies the gliadin and gliadin fragments of a sample through a correlation factor to the amount of peptide that is detected. The protocol multiplies the alpha-2-peptide concentration by 100 to estimate gliadin content. Confounding this conversion factor, research has found an accumulation of peptides and amino acid metabolites in sourdough fermentation due to proteolysis (Gänzle, 2014) possibly resulting in an inaccurate estimation of gliadin content.

4.4. Bradford Assay Protein Quantification

No statistically significant results were found from the protein quantification using the Bradford method. The Bradford method quantifies the presence of three basic amino acid residues, arginine, lysine and histidine. Under acidic conditions the Coomassie dye binds the protein residues and forms a complex (He, 2011). The disadvantage of the Bradford method is that it is only testing for 3 of the 20 amino acids. Depending on the samples being analyzed, the results could be very misleading because different protein sources have different concentrations and combinations of these amino acids. That being said, the Bradford method indicated there was no evidence of significant change in the concentration of these three amino acid residues, arginine, lysine and histidine, throughout the sourdough fermentation process.

4.5. Ethanol Extraction Protein Quantification

When comparing samples, with the same treatment temperature but different fermentation periods, there was a common trend throughout the data set. Within each batch and treatment temperature, the protein extracted with ethanol increased with fermentation. The difference in ethanol extractible protein between the Control and 26-12 were significantly different ($p \leq 0.05$) based on the sample means from the 3 batches (Table 4). Although the trend was consistent throughout the 3 batches for samples 4-14 and 4-50, the difference was not significant ($p > 0.05$).

Research supported by the FEI (Forschungskreis der Ernährungsindustrie e. V., Bonn) of Germany has shown that sourdough fermentation degrades gluten proteins. A decrease in the glutenin fractions will generate an increase in alcohol soluble oligomeric proteins, or gliadins (Wieser et al., 2008). When extracting with ethanol, the supermatant will contain alcohol-soluble polypeptides (De Angelis et al., 2006).

4.6. Headspace Gas Quantification

Gas chromatographic analysis showed a consistent set of 5 compounds with significant trends and these were chosen based on their presence in all of the dough samples analyzed. 3-methyl-1-butanol and 1-hexanol increased with fermentation time in both the room temperature and refrigerator fermented samples (Tables 7, 8, & 9). The increase in these alcohol volatiles is consistent with fermentation yielding alcohol (Liu et al., 2014).

5. Conclusion

Sourdough cultures are a traditional leavening method that in recent years have been rediscovered due to its nutritional significance and digestability.

Sourdough samples were allowed to ferment for various time durations. The height of each loaf was measured and recorded. As a preparation method and for analysis purposes the samples were dried in a Mechanical Convection Oven. A Gluten-Tec® ELISA kit was used to quantify gliadin and gliadin fragments in the bread samples. This did not show any significant ($p>0.05$) variation between samples fermented for different times or at

different temperatures. In order for the samples to be in the gliadin detection range of the kit, many dilutions of 1:1000 had to be made. Each dilution step can contribute to inaccuracy of the results. Bradford assay was used to determine protein content although no significant ($p>0.05$) difference was detected. This method is quantifying arginine, lysine, and histidine. These particular residues are not found in any of the four confirmed toxic motifs of amino acid sequences (Pro-Ser-Gln-Gln, Gln-Gln-Gln-Pro, Gln-Gln-Pro-Tyr, and Gln-Pro-Tyr-Pro) so we cannot conclude that toxicity was not reduced with this test result (Darewicz et al., 2008). Lastly, ethanol (60%) extraction was used to quantify prolamins (mostly gliadin and some glutenins). Analysis showed that as fermentation time increased, ethanol extractable prolamins increased. This data was significant ($p<0.05$) when comparing the Control and 26-12. A study supported by the European Food Research & Technology program found similar results by discovering a decrease in the glutenin fraction that lead to an increase in the alcohol soluble gliadin fractions (Weiser et al., 2008). Flavor volatiles were collected on the dough samples to analyze sourdough quality and treads. An increase in 1-hexanol was observed as fermentation time increased within the Control, samples held at 26°C and samples held at 4°C. This change was significant ($p<0.05$) when comparing the Control, 0, and the 26°C treated samples, 26-4 and 26-12. Research done in The Netherlands on white bread volatiles and enzyme active soya flour has suggested that an increase in 1-hexanol is a byproduct of lipid oxidation caused by the addition of enzymes (Luning et al., 1991). For these same samples, hexanal decreased as fermentation time increased. This change was significant ($p<0.05$) when comparing the control and 26-12. A study done by the *Dipartimento di Scienze Agrarie* in

Italy analyzed the volatile profiles of white wheat sourdough bread and discovered that hexanal was negatively correlated to the content of ethanol (Ripari et al., 2016). An increase in ethanol would suggest there is progression in the fermentation as time increases.

Further research needs to be done to be able to fully understand the extent of protein hydrolysis, as well as the safety of sourdough bread for people sensitive to certain wheat proteins. It would also be beneficial to pin point the sourdough bacteria with the highest protein hydrolysis ability. In addition, there is a need to better understand certain probiotics and their mechanism of assisting in digestive processes.

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Table 1. Sourdough culture activation schedule and dough sample preparation procedure

Feeding #	culture (g)	flour (g)	water (g)	sea salt (g)	ferment time (hr)
1	40	40	40	0	12
2	120	120	120	0	8
3	360	360	360	0	8
4	1080	1080	1080	0	8
dough samples	3000	2400	670	40	(see table 2)

Figure 2.
Flow chart of sourdough culture activation process

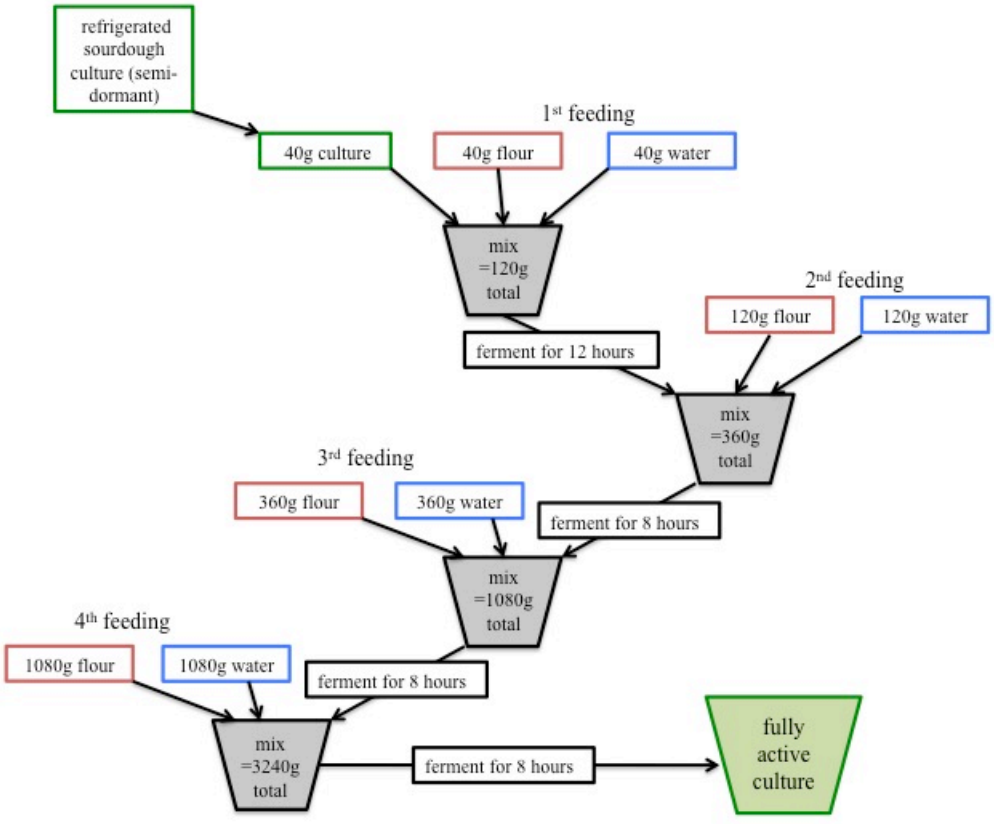

Table 2.
Sourdough loaf sample fermentation schedule

Sample	Dough Sample	Fermentation Time (hours) and Temperature	Repetitions
0 (Control)	0d	0 h	3
26-2	26-2d	2 h @ 80°F	3
26-4	26-4d	4 h @ 80°F	3
26-8	26-8d	8 h @ 80°F	3
26-12	26-12d	12 h @ 80°F	3
4-14	4-14d	12 h @ 40°F then 2 h @ 80°F	3
4-26	4-26d	24 h @ 40°F then 2 h @ 80°F	3
4-38	4-38d	36 h @ 40°F then 2 h @ 80°F	3
4-50	4-50d	48 h @ 40°F then 2 h @ 80°F	3

Figure 3.
Flow chart of loaf and dough sample preparation

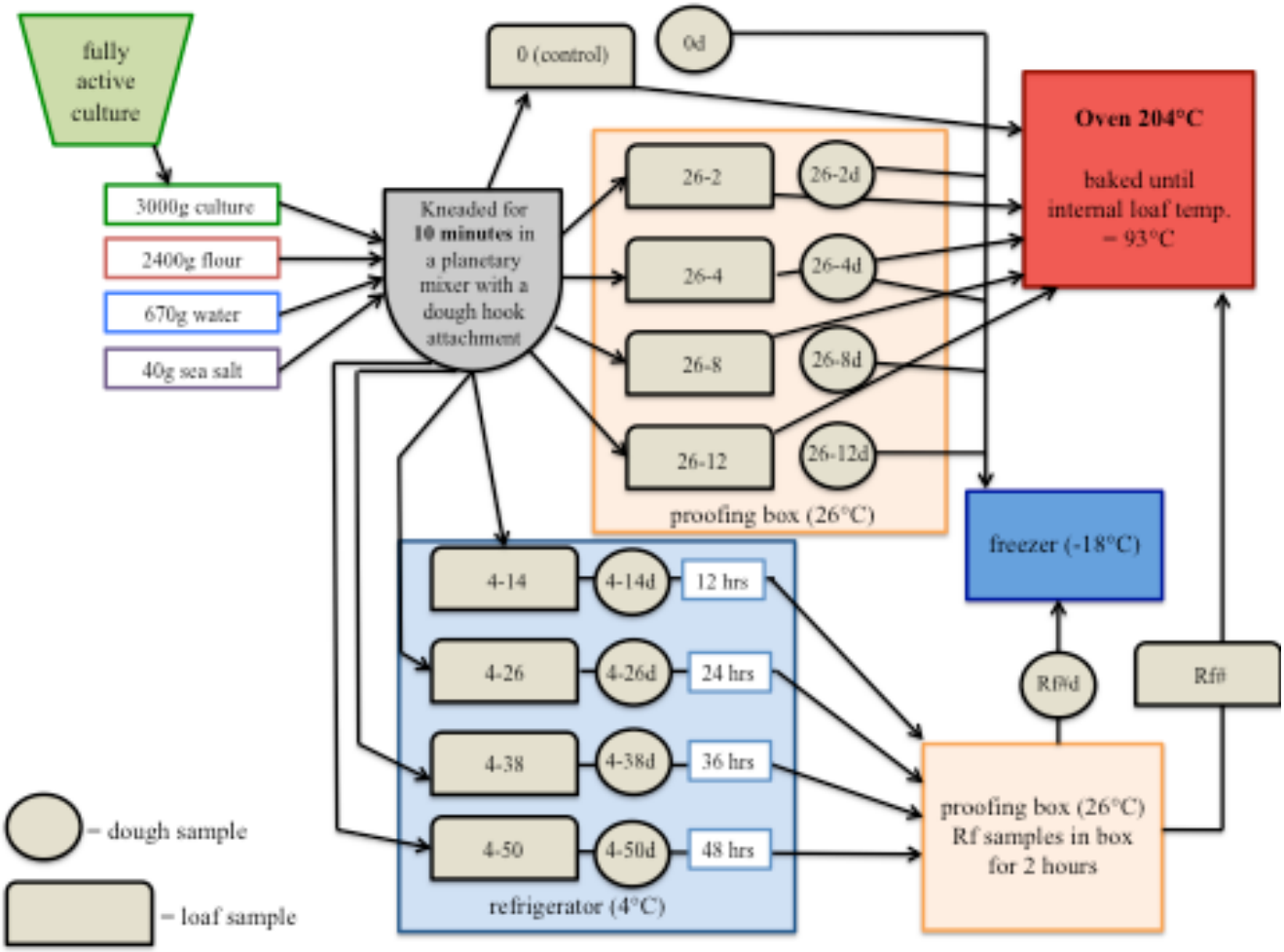

Table 3.
Loaf height of samples fermented at 26°C, n=3

sample	loaf height (cm)	
0	6.8	(4.5, 5.3)
26-2	6.1	(4.7, 7.5)
26-4	6.3	(4.9, 7.1)
26-8	6.1	(5.6, 6.5)
26-12	5.7	(5.5, 6)

Table 4.
Loaf height of samples fermented at 4°C, n=3

sample	loaf height (cm)	
0	8.0	(4.5, 5.3)
4-14	5.9	(4.4, 7.5)
4-26	5.9	(4.5, 7.3)
4-38	6.1	(4.7, 6.8)
4-50	5.9	(4.8, 7)

Table 5.
Percent Moisture in Sourdough Bread at minimum and maximum fermentation times at both 26°C and 4°C

Treatment	Batch	Moisture	Mean
0	B1	39.57	38.72^{B}
	B2	38.36	
	B3	38.24	
26-12	B1	40.39	39.66^{B}
	B2	40.17	
	B3	38.41	
4-14	B1	39.92	38.64^{B}
	B2	37.07	
	B3	38.93	
4-50	B1	40.1	40.51^{A}
	B2	39.96	
	B3	41.48	

*A,B means with different letters are significantly different ($p<0.05$)

Table 6.
Ethanol extracted protein in sourdough bread at minimum and maximum fermentation times at both 26°C and 4°C

Treatment	Batch	µg/mL	Mean
0	B1	236.29	220.56^{C}
	B2	235.99	
	B3	189.41	
26-12	B1	307.78	332.80^{A}
	B2	313	
	B3	377.62	
4-14	B1	192.07	$261.39^{B,C}$
	B2	268.76	
	B3	323.35	
4-50	B1	236.29	$284.53^{A,B}$
	B2	284.33	
	B3	332.98	

*A-C means with different letters are significantly different ($p<0.05$)

Table 7.
Headspace gas volatiles for dough samples held at 26°C

Volatiles	0	26-4	26-12
Acetic Acid	37.8	142.6	130.7
3-methyl-1-Butanol	1092.9	1546.2	3528.8
Hexanal	1307.7^{A}	$1470.8^{A,B}$	782.6^{B}
1-Hexanol	60.2^{C}	132.3^{B}	214.7^{A}
(E)-2-Nonenol	17.1	6.1	7.0
Total Area	2678.2	4095.6	5101.6
Peak Area ×1000	n=3		
*A-C means with different letters are significantly different ($p<0.05$)			

Table 8.
Headspace gas volatiles for dough samples held at 4°C

Volatiles	0	4-14	4-50
Acetic Acid	37.8	113.6	103.8
3-methyl-1-Butanol	1092.9	1438.9	1322.6
Hexanal	1307.7^{A}	507.9^{B}	523.7^{B}
1-Hexanol	60.2^{C}	101.9^{B}	122.9^{B}
(E)-2-Nonenol	17.1	20.3	10.7
Total Area	2678.2	2258.4	2179.7
Peak Area ×1000	n=3		
*A-C means with different letters are significantly different ($p<0.05$)			

Table 9.
Headspace gas average volatile percentages for dough samples held at 26°C and dough samples held at 4°C

Volatiles	0	26-4	26-12	4-14	4-26	4-50
Acetic Acid	1.95%	4.05%	2.96%	4.76%	3.50%	4.59%
3-methyl-1-Butanol	35.56%	40.36%	69.95%	56.22%	34.83%	56.51%
Hexanal	58.82%^{A}	50.8%^{A,B}	21.46%^{B}	32.02%	57.46%	31.52%
1-Hexanol	2.61%	4.64%	5.50%	5.68%	3.83%	6.71%
(E)-2-Nonenol	1.06%	0.15%	0.12%	1.32%	0.37%	0.67%
*A,B means with different letters are significantly different ($p<0.05$)						
n=3						