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South Dakota State University

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THE PARMESAN CHEESE MODEL SYSTEM: ALLEVIATION OF BROWNING DEFECT
IN PARMESAN CHEESE

BY

MEGAN HUEGLI AUBREY

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Dairy Science

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2020

THESIS ACCEPTANCE PAGE

Megan Huegli Aubrey

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree.

Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Llyod Metzger

Advisor

Date

Vikram Mistry

Department Head

Date

Dean, Graduate School

Date

I would like to dedicate this work to my Husband, Jim Aubrey, for his love and support.
As well as my mentor, coach and friend Jeremy Smith for always supporting and guiding
me.

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ABBREVIATIONS

AGE	Advanced Glycation End
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CO ₂	Carbon Dioxide
DHAP	Dihydroxyacetone Phosphate
EMP	Eden Meyer Pathway
G3P	Glyceraldehyde-3-Phosphate
GRAS	Generally Regarded as Safe
HMF	Hydroxymethyl Furfural
HTST	High Temperature Short Time
LAB	Lactic Acid Bacteria
LTB	Low Temperature Browning
MG	Methylglyoxal
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide
NSLAB	Non-Starter Lactic Acid Bacteria
PDO	Protected Designation of Origin
PEP	Phosphoenolpyruvate
PTS	Phosphotransferase System
S.E.	Standard Error
StDEV	Standard Deviation
TA	Titrateable Acidity
UDP	Uridine Diphosphate Glucose

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ABSTRACT

THE PARMESAN CHEESE MODEL SYSTEM: ALLEVIATION OF BROWNING
DEFECT IN PARMESAN CHEESE

MEGAN HUEGLI AUBREY

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In cold temperature storage, Parmesan cheese can develop an undesirable browning defect. Certain pyrazine compounds converted from methylglyoxal have been identified and associated with the brown pigmentations in Parmesan cheese samples. This study focused on creating a cheese model system to study variables that caused browning and variables that helped reduce or alleviate browning. The cheese making steps for the cheese model system were monitored by measuring pH and TA and the final composition for % fat, % moisture, % salt and pH. Additionally, the cooling curve was studied to ensure all wheels of cheese made were cooled similarly. Cheese made in the experiment were then used in a slurry as part of the cheese model system to test variables such as aging temperature, heat abuse, addition of NSLAB, addition of adjuncts, addition of galactose, addition of methylglyoxal and modified %salt and pH. This study identified variables that contributed to browning which included: aging temperature (55°F), addition of methylglyoxal, NSLAB addition, heat abuse and time. Increased browning was observed over time, the most after 3 months, at 55°F in the presence of methylglyoxal and heat abuse. These findings were verified by testing these variables at different ages using a colorimeter. The variables studied to understand how to reduce browning included addition of NSLAB (*Lb. helveticus*), addition of adjuncts (*lb. plantarum* and *Lc. lactis diacetylactis*) and

modified environment of high %salt and low pH. The cheese model system had a % salt of 2.19 and a pH of 5.60 which was modified to 3.00% for high salt and 5.20 pH, all samples were aged at 55°F. Reduction of browning was achieved using both adjuncts and modified environments of high salt and low pH. The greatest reduction-in browning was observed when *Lb. helveticus* and *Lb. plantarum* were added together as a treatment and had a small Delta E (color change) of 0.92. The adjunct, *Lc. lactis diacetylactis* also reduced browning, average Delta E of 1.89 in the presence of *Lb. helveticus*, high %salt and low pH. The most effective way to reduce browning was achieved using adjuncts and modified environments of high salt and low pH.

OBJECTIVE

Undesirable brown pigmentation in Parmesan cheese occurs during storage. Studies have shown, according to UW-Madison certain pyrazine compounds have been identified and associated with the brown pigmentations in Parmesan cheese samples. These pyrazines are believed to be converted from methylglyoxal and is naturally occurring. To address this defect, the use of reducing agents to attenuate methylglyoxal were proposed. These reducing agents, all food grade, were Sodium bisulfite, glutathione and erythorbate. Sodium bisulfite and glutathione were more effective at inhibiting the browning defect. There may be other ways to help reduce browning in parmesan cheese and were explored more in this research study.

In this study, the first objective was to create a cheese model system which provides a consistent cheese platform to test different variables. Parmesan was manufactured using common practices and added to a sodium chloride solution to make a slurry. This slurry was utilized in two different phases. Phase 1 was intended to create browning using variables theorized to cause browning, some of which have been proven in the literature (i.e., methylglyoxal). Other variables studied in phase 1 included: aging temperature, heat abuse, galactose addition and galactose negative NSLAB addition (another known starter microorganism for parmesan). Phase 2 studied other variables / treatments that could potentially alleviate browning (i.e., adjuncts and modified conditions of salt and pH) in combination with confirmed variables to cause browning (i.e., aging temperature, positive confirmed reference NSLAB). All samples generated from the cheese model system were analyzed for color using a colorimeter to understand the change in color over time.

CHAPTER I: Review of Literature

1. Introduction

In the United States, parmesan is a common and popular style of cheese. It is often used grated on Italian dishes or pizzas to add a flare of flavor. Parmesan cheese is a hard-style cheese originating from Italy and is known as the “King of Cheeses.” In Italy, parmesan is commonly referred to as Parmigiano-Reggiano. Parmigiano-Reggiano cheese dates back nine centuries, with historical evidence of its existence in 1200-1300s (Parmigiano Reggiano , 2018). Parmesan in the United States cannot be called Parmigiano-Reggiano due to European law that protects the designation of origin (DOP) or *Protected Designation of Origin* (PDO). The Italian name Parmigiano-Reggiano can be used only if it is manufactured in the provinces of Parma, Reggio Emilia, Bologna, Modena, and Mantua Italy. The unique feeding regimen including only locally grown forage, excluding silage and fermented feeds, gives Parmigiano-Reggiano its trademark flavor and name (Parmigiano Reggiano , 2018).

Many cheese companies in the United States have manufactured the knock off Parmigiano-Reggiano, parmesan, utilizing similar cheese manufacturing concepts and meeting the required standards of identity. In the United States, in order to meet standards of identity, parmesan cheese must be less than 32% moisture and aged at least 10 months (Code of Federal Regulations, 2018). Other than that, manufacturers can make parmesan by different processing parameters, compositional milestones and following a recipe; cheese making is truly an art with a lot of complexity. There are many variables that can affect the final product: breed of animal, milk composition and

quality, processing parameters (times, temperatures, pH etc.), starter culture, non-starter lactic acid bacteria (NSLAB) and aging time and temperature to name a few.

As aforementioned, parmesan is typically aged 10 to 24 months, which provides a degree of variation of products in the market place. All that time allows for maturation of different flavors, aromas and other metabolic compounds such as color compounds. The formation of color compounds in parmesan has been of interest to parmesan cheese manufacturers, because the pigmentation's effect on an undesirable appearance, as seen in figure 1. Notice in table 1, the composition between the brown and white portion of cheese vary, especially in %moisture and %salt.

2. Lactose

Lactose (beta-D-galacto-hexopyranosyl-(1-4)-D-gluco-hexopyranose) is the primary disaccharide in milk (C₁₂H₂₂O₁₁). In bovine milk, it accounts for 4.8 - 5.0% of total milk volume. Lactose is composed of two monosaccharides; glucose and galactose that are connected by a β -1,4 glycosidic bond. This bond is located between the aldehyde group of galactose and the 4-carbon of glucose (refer to figure 2).

2.1. Enzymatic Breakdown of Lactose

Lactose is hydrolyzed into the two monosaccharides, glucose and galactose, by an enzyme called β -galactosidase. B-galactosidase is a glycoside hydrolase enzyme, that cleaves lactose at the 1, 4 glycosidic bond (refer to figure 3).

2.2. Glucose vs Galactose

Glucose and galactose are diastereoisomers because they share the same molecular formula of $(C_6H_{12}O_6)$ but have different atomic arrangements. This means they are optical isomers of each other but are not mirror images of each other and are not superimposable on each other. In figure 4, notice that these monosaccharides differ in their C-4 configuration where galactose does not contain a hydroxyl group, $(-OH)$ (Ernest, 2015). The differences in configuration between these two monosaccharides give them different affinities to other molecules. Glucose is an unmodified monosaccharide making it less susceptible to attaching to lipids or proteins, thus the formation of glucoconjugates. This means that glucose can stay in its original form as oppose to being converted to other molecules. This can also make glucose more stable, energetically favorable and easier to metabolize. Galactose can easily form ester linkages with phosphates or sulfate ions. Because of this, galactose is not found in nature in large quantities.

2.3. Functional Groups of Glucose and Galactose

Glucose and galactose consist of a six-carbon chain with multiple hydroxyl functional groups (OH) and one aldehyde functional group $(CH=O)$ at C-1. The aldehyde portion of glucose or galactose has reducing capabilities and the ability to donate an electron to other molecules.

3. Amino Acids

There are 500 naturally occurring amino acids, but only 20 are encoded within the genetic code of DNA (Wagner, 1983). Amino acids are organic molecules consisting of an amine N-terminus (NH_2) and a C-terminus of a carboxylic acid (COOH). Additionally, there is a side chain called an R group which is specific to each amino acid. These amino acids are grouped into categories of charge, polarity or hydrophobicity where their R groups can be aliphatic, acyclic, aromatic, and or contain hydroxyl or sulfur groups. These amino acids can then covalently attach to other amino acids creating peptide bonds through condensation. Eventually, peptide bonds will begin to interact with other peptide bonds to form different types of proteins. Essentially, amino acids are the building blocks of protein. In figure 5, is a basic chemical structure of an amino acid: (Nutrientsreview, 2016)

3.1. Amines

As aforementioned, an amine is positioned at the N-terminus of an amino acid. Amines can be classified as primary, secondary or tertiary depending on what hydrogen atom is displaced. The chemical structures of these amines are RNH_2 , R_2NH and R_3N , respectively, as seen in figure 6 (Anderson L. C., 2002).

3.2. Functional Groups of Primary Amines

Primary amines contain two hydrogen atoms bound to a nitrogen atom (nucleophilic amino group of the amino acid) and an alkyl or aromatic group.

3.3. Milk Proteins

In milk, there are two types of proteins casein and whey. In bovine milk, these proteins account for 3.3-3.4% of total milk volume, of those proteins 80% is casein and 20% whey. Casein is found in milk in suspension (net negative charge) as a micelle consisting of calcium caseinate plus phosphate, magnesium and citrate and made up of four protein sub-units: k-casein, b-casein, α_{s1} -casein and α_{s2} -casein. Whey protein, the serum portion of milk, and consists of four protein sub-units: α -lactalbumin, β -lactoglobulin, blood serum albumin and immunoglobulins.

4. Color Defects in Cheese: Maillard Reaction in Mozzarella

Browning of mozzarella on pizza during baking is commonly caused by the Maillard reaction (Hui, 2006). The Maillard reaction is a non-enzymatic chemical reaction that occurs at 80-120°C. A reducing agent contains a free aldehyde (aldoses) or ketone (ketoses) group, the active functional group, which interacts with the nucleophilic amino group of an amino acid. A reducing sugar, typically galactose in cheese, acts as a reducing agent. A reducing sugar becomes oxidized as a result of donating an electron to reduce another molecule (i.e., amino acid in Maillard reaction). As mentioned earlier, the aldehyde group of glucose or galactose serves as the reducing agent for the Maillard reaction.

4.1. Chemical mechanism of the Maillard Reaction

The Maillard reaction occurs in three main steps which can be seen in figure 7. First, the nucleophilic addition of a non-protonized amine group to an electrophilic carbonyl of a reducing sugar initiates the Maillard reaction and creates a Schiff base, an N-substituted glycosylamine and water. Secondly, the N glycoside undergoes an Amadori rearrangement where an immonium ion is formed and then isomerized forming a ketosamine. Lastly, ketosamine products then either: (The Maillard Reaction, 2018)

- dehydrates into reductones and two water molecules essentially forming caramels;
- diacetyl, pyruvaldehyde, methylglyoxal and other dicarbonyl products, which are short chain hydrolytic fission products that then undergo strecker degradation or;
- produce brown nitrogenous polymer and melanoidins.

4.2. Rate and color intensity of the Maillard Reaction

Different sugars react faster with molecules than others, for example, pentose sugars react faster than hexoses. Meanwhile, disaccharides are slower to react than hexoses. This is due to size, because smaller sugars tend to react faster than larger sugars (surface area). Additionally, the type of protein the sugar interacts with causes differences in pigmentation and flavor. For example, lysine has two amino groups which increases the reaction rate and causes darker

pigmentation (figure 8). Cysteine, however, contains a sulphur group and is responsible for distinct flavors, but less color pigmentation. (University, 2017)

5. Low Temperature Browning

The browning found in parmesan may not be a result of the Maillard reaction but may follow a similar mechanism at a slower rate. Brown pigmentation in parmesan cheese is typically not observed until late in storage well after the residual sugar is depleted. The Maillard reaction doesn't seem to be the plausible cause for browning, because it requires heat, in parmesan primarily because residual sugars are depleted or relatively scarce after 10 months and parmesan is aged at considerably lower temperatures (50-60°F) (R.D. Divine, 2013). Therefore, understanding low temperature browning (LTB) and the different microbiological and chemical pathways that occur at lower temperatures is necessary.

6. Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are highly utilized in fermented dairy products. In cheese, LAB typically are used as primary and secondary starters for acid production and for flavor. LABs are gram-positive bacteria that are part of the Firmicutes phylum. Most LAB used for cheese making fall under order I, that contain two orders, of the Firmicutes phylum. Order I include the *Bacillales* comprised of 12 families and Order II contains *Lactobacillales* comprised of 6 families (Wood, 2014).

LAB produces ATP via substrate-level phosphorylation and has two distinct fermentation groups, homolactic or heterolactic. Homolactic LAB such as *Lactobacillaceae* and *Streptococcaceae* (excluding one *Lactobacillus* genus) use

glycolysis / EMP pathway (Embden-Meyerhof-Parnas Pathway) for metabolizing glucose. The EMP pathway converts glucose to lactic acid to 2 ATPs of energy per molecule of glucose. *Leuconostocaceae* and some *Lactobacillus* are heterolactic LAB that utilize the phosphoketolase pathway which produces 1 molecule each of lactic acid, CO₂ and ethanol and only one ATP from a single molecule of glucose (Wood, 2014).

7. Transportation of lactose

Cheese milk contains lactose which must be transported into the LAB cell for fermentation, thus energy creation and acid development. There are two systems requiring energy that LAB can use to transport lactose, permease or phosphotransferase (PTS). The permease system utilizes ATP and lactose is transported across the cell membrane. The PTS requires phosphoenolpyruvate (PEP) is generated from a series of reactions involving two enzymes (EI and EII) and a heat-stable protein, as seen in Figure 9 (Wood, 2014).

7.1. Permease System of Lactic Acid Bacteria via Lac Operon

In the presence of lactose the *lac* operon is induced in Lactic Acid Bacteria and is an effective way of digesting lactose. The Lac operon is composed of three genes: *lacZ*, *lacY*, and *lacA*. *LacZ* encodes β -galactosidase which catalyzes the cleavage of lactose into glucose and galactose, as mentioned earlier. *LacY* encodes β -galactoside permease, which is an enzyme that is responsible for transporting lactose into the cell through the cytoplasmic membrane. Finally, *lacA* encodes β -galactoside transacetylase which is an enzyme that displaced an acetyl group from acetyl-CoA to galactosidase,

glucosides and lactosides. However, β -galactoside transacetylase does not transfer an acetyl group to β -galactosidase, which is encoded by *lacZ* (Wang XG, 2002).

7.2. Phosphotransferase System of Lactic Acid Bacteria

The phosphotransferase system (PTS) is typically utilized by *Lactococcus*. The PTS uses phosphoenolpyruvate (PEP) to phosphorylate lactose and transfer of lactose-6-P across the membrane. There are four protein domains for the PTS phosphorylation cascade (EI, HPr, EIIA and EIIB) that regulate interactions between PEP and sugars (Josef Deutscher, 2006).

8. Embden-Meyerhof-Parnas Pathway (EMP)

Once the glucose is in the cell, the EMP pathway is utilized. The EMP pathway should be thought of in two phases: preparatory phase and payoff phase as seen in figure 10. In phase one, five enzymes (steps) are utilized using two molecules of ATP: hexokinase, phosphoglucose isomerase, phosphofructokinase, fructose biphosphate aldolase and triose phosphate isomerase. First, glucose is phosphorylated by hexokinase to D-glucose-6-phosphate using 1 molecule of adenosine triphosphate (ATP). D-glucose 6-phosphate is reversibly converted to fructose-6-phosphate via phosphoglucose isomerase. From here, fructose-6-phosphate is phosphorylated to fructose 1,6-biphosphate using 1 molecule of ATP via phosphofructose kinase. Fructose biphosphate aldolase splits fructose 1,6-biphosphate into two three-carbon sugars; dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P). DHAP can be converted to G3P via triose phosphate isomerase or may convert to

another chemical that are later discussed. G3P will continue to the next phase of glycolysis; the pay-off phase. Glyceraldehyde-3-phosphate dehydrogenase and the reduction of NAD^+ to NADH plus H^+ oxidize and phosphorylate G3P to 1, 3-bisphosphoglycerate. The newly phosphorylated 1, 3-bisphosphoglycerate donates a phosphate group to ADP to make ATP and 3-phosphoglycerate via phosphoglycerate kinase. Phosphoglycerate mutase isomerizes 3-phosphoglycerate to 2-phosphoglycerate. 2-phosphoglycerate is dehydrated to phosphoenolpyruvate (PEP) by enolase. PEP donates its phosphate group to ADP to create ATP and is converted to pyruvate via pyruvate kinase.

In summary the EMP is made up of ten enzymatic steps, seven which are reversible (figure 10). At the end of glycolysis there are two ATP molecules, two NADH and two pyruvate molecules. Lastly, pyruvate will react with NADH to form lactic acid and NAD^+ (Academy, 2018).

9. Galactose Fermentation

Galactose fermentation occurs inside the cell after lactose-6-p has been transported into the cell via PTS or permease systems. Not all Lactic Acid Bacteria can break down galactose since they do not contain the enzymes for the Tagatose-6P or Leloir pathways (The Medical Biochemistry Page, 2018).

9.1. Tagatose-6P Pathway

The tagatose pathway depicts the metabolism of galactose-6P from the phosphotransferase system (PTS). The tagatose pathway begins when galactose and lactose are both transported and phosphorylated by PTS. From there,

Lactose 6-phosphate (lactose-6P) is hydrolyzed intracellularly by phospho- β -galactosidase to galactose-6P and glucose. Galactose-6P is converted to DHAP by a series of reactions involving the three enzymes; galactose-6-phosphate isomerase, tagatose-6-phosphate kinase, and tagatose diphosphate aldolase (Anderson B. a., 1974)

9.2. Leloir Pathway

The Leloir pathway depicts the metabolism of galactose typically from the permease system. The Leloir pathway is comprised of five essential enzymes: mutarotase, galactokinase, uridylyltransferase, UDP-galactose 4-epimerase and phosphoglucomutase. Most galactose is present as β -D-galactose but is converted to α -D-galactose via galactose mutarotase. Next, α -D-galactose is phosphorylated to D-galactose 1-phosphate via galactokinase. The enzyme uridylyltransferase then generates UDP-galactose and glucose 1-phosphate from interaction of UDP-glucose and galactose 1-phosphate via double displacement reaction. Lastly, UDP-galactose 4-epimerase transforms UDP-galactose to UDP-glucose and phosphoglucomutase converts the D-glucose 1-phosphate to D-glucose 6-phosphate (Wong LJ, 1974).

10. Methylglyoxal Research

A study was conducted at the University of Wisconsin Madison to understand different chemical pathways that occur at low temperatures in cheese. Some of these pathways include; metabolism of residual sugars such as galactose, lipid oxidation products, byproducts of fermentation (i.e., methylglyoxal) or enzymes reacting with

primary amines (Rankin, 2013). Since methylglyoxal is known to be a byproduct of glycolysis and also converted into brown pigments via the Maillard reaction, it was theorized to be an intermediate formation of brown pigments at colder temperatures at a slower rate (R.D. Divine, 2013). Studies have shown that the concentration of methylglyoxal decreases during aging. This suggests that methylglyoxal is produced during cheese making or shortly after and available to react with amino acids that are metabolized after sugars are utilized via glycolysis (McDonald, 1992).

10.1. Methylglyoxal and Melanoidins

Methylglyoxal ($C_3H_4O_2$), a strong reductant, can then interact with other amines yielding polymers called melanoidins, which are brown pigments. These melanoidins contain unsaturated heterocyclic rings that absorb fluorescent and ultraviolet light (Jenness, 1984). Methylglyoxal is known to react with various amino acids such as arginine, lysine, cysteine, guanine, adenine and cytosine as seen in Figure 13 (Lo TW, 1994).

10.2. Methylglyoxal reaction with amines

The reaction between an amine and methylglyoxal had a positive correlation with creating browning pigments (pyrazines) which are volatile compounds (R.D. Divine, 2013). The 'brown' colored portion of parmesan cheese releases unique volatile compounds that were identified using gas chromatography. Two pyrazines associated with 'brown' parmesan cheese were identified; 2,3,5-trimethylpyrazine and 3,5-diethyl-2-methylpyrazine

(figure 11). These pyrazines have a brown pigmentation and support the theory that at low temperatures methylglyoxal reacts with an amino acid to form aminoacetone which condenses to form the pigmented pyrazines (R.D. Divine, 2013).

10.3. Formation of Methylglyoxal

Methylglyoxal (MG) as a result of sugar metabolism with or without the presence of a primary amine. Methylglyoxal can be produced as a biproduct from the amadori rearrangement in the Maillard reaction. Also, through the metabolism of glucose or galactose resulting in the biproduct of DHAP that can be further converted to MG (figure 12).

10.4. Pathways for Methylglyoxal

In theory it is believed that the heat induced Maillard reaction speeds up the reaction of primary amines and reducing sugars. However, it may be plausible that methylglyoxal can be formed as a result of the interaction between proteins and reducing sugars at colder temperatures for parmesan, but at a slower rate. In the Maillard reaction, the amadori product can be dehydrated into 1-amino-1-deoxy-2-ketose which enolizes into 1, 2 – eneamino or 2, 3-enediol. From these products, 5-hydroxymethyl-furfural (HMF) and then α -dicarbonyl intermediates can be formed, which is typically pH sensitive (Jenness, 1984). Some of these intermediates include pyruvaldehyde, acetol, diacetyl and methylglyoxal (figure 14). These intermediates can then enzymatically convert to form brown pigments.

Methylglyoxal can also be formed after glycolysis, where glucose or galactose is converted to dihydroxyacetone phosphate (DHAP) and glyceraldehyde phosphate by enzyme aldolase (G3P). G3P will continue to be metabolized into pyruvate which would not cause browning. However, DHAP can undergo two different routes. First, triose phosphate isomerase may convert DHAP to GAP thus creating pyruvate. Or methylglyoxal synthase may convert DHAP to methylglyoxal. Methylglyoxal can be converted to pyruvate or may react with an amino acid to form aminoacetone via strecker degradation. Aminoacetone can then condense to various pyrazine compounds which have brown pigmentation as seen in Figure 13. There are likely other pathways that methylglyoxal can be formed, but these pathways are the most understood today.

11. Role of Lactic Acid Bacteria in Cheese Making

In cheese making, lactic acid bacteria are used to create acid to decrease the pH closer to the iso-electric point of casein micelle. The casein micelle is made up of hundreds of amino acids with different hydrophobic and hydrophilic tendencies. This causes the casein proteins to fold around the hydrophobic (non-water loving) amino acids and form a micelle that has a negative charge and repels other micelles. By decreasing the pH of milk from 6.7 neutralizes the outside amino acids of the micelle, making the protein less soluble. Therefore, the production of acid helps precipitate the protein that makes cheese. In addition to decreasing pH, heat and enzymes induce the precipitation of casein. Once the lactic acid bacteria have utilized all the glucose, galactose metabolism begins. As mentioned earlier, as part of stage two in glycolysis,

two 3-carbon sugar phosphates are generated: G3P and DHAP. Not all DHAP is converted into G3P to make pyruvate and then lactic acid.

High levels of DHAP are induced by an influx of sugars like glucose or glucose-6-phosphate. The formation of methylglyoxal is regulated by levels of DHAP, the principal route. When abundant, DHAP is dephosphorylated into methylglyoxal via methylglyoxal synthase. As part of the detoxification of methylglyoxal, it is then enzymatically converted into either D-lactate or L-lactate. This detoxification system is a glutathione dependent glyoxalase I-II (G. P. Ferguson, S. Totemeyer, M. J. MacLean, I. R. Booth, 1998). The conversion of methylglyoxal to L-lactate requires both methylglyoxal reductase and aldehyde dehydrogenase yielding lactaldehyde and ultimately L-lactate. The glyoxalase pathway must be employed to convert lactoylguatathione to D-lactate. Both L-lactate and D-lactate convert into pyruvate and enter the Krebs cycle.

11.1. Purpose of Methylglyoxal for Bacteria

Methylglyoxal does not yield ATP, but rather recycles phosphate (Russell, 1998). Therefore, it is believed that bacteria produce methylglyoxal as a means of survival. Unfortunately, at high concentrations methylglyoxal is believed to cause cell death because it's a toxic electrophile that interacts with macromolecules like DNA and inhibits protein synthesis (G. P. Ferguson, S. Totemeyer, M. J. MacLean, I. R. Booth, 1998). Additionally, the reaction between methylglyoxal and amino acids can form advanced glycation end products (AGE). AGES are either

proteins or lipids that are covalently bound to sugars and can cause many degenerative diseases like diabetes and others. Therefore, the formation of methylglyoxal especially when abundant poses as a food safety risk.

11.2. Prevention of Methylglyoxal using Reducing Agents

The formation of methylglyoxal in parmesan cheese is not only an attribute defect but also a food safety risk. Therefore, it is important to study ways to alleviate browning in parmesan, thus reducing the production of methylglyoxal. Some research has been conducted to study reducing agents and their ability to reduce browning by inhibiting methylglyoxal and amino acid interactions. Reducing agents such as sodium bisulfite and glutathione inhibit methylglyoxal production. Sulfites and sulfur-containing amino acids are strong nucleophiles because of their strong tendencies for reduction and quenching free radicals (Friedman, 1996). The use of the reducing agents, strong nucleophiles, creates thiohemiketals and thioketals on the reactive carbonyl in methylglyoxal (Rankin, 2013). According to the Food and Drug Administration, these reducing agents are generally recognized as safe (GRAS). However, addition of non-traditional or non-authentic ingredients is not always favorably perceived by customers.

12. Conclusions and future work

Based on the literature, there has been some research published that have verified the metabolic pathways and precursors responsible for cold storage browning. There hasn't been, however, a lot of research conducted on ways to prevent browning other than using reducing agents. These reducing agents have been helpful agents to reduce the amount of browning we see in products. There is however, a gap in trying to understand how cheese making can be used to prevent formation of methylglyoxal that can then be converted pyrazines. Therefore, introducing a cheese model system to create a consistent substrate to test different variables is important. First this cheese model system needs to brown that browning can occur. After that, identifying different factors that can be introduced during cheese making to help prevent browning can be validated. Some of these variables include; modifications to salt and pH levels as well as the use of adjuncts and different storage temperatures. Additionally, some other work could be done to understand the lactic acid bacteria added as starter and their phenotypic outputs (i.e., production of methylglyoxal) using metagenomics. From here, design starter that do not produce methylglyoxal. This component was not studied in these experiments but could be an innovative way to help prevent cold storage browning.

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Table 1

Characteristics	% Moisture	% Fat	pH	%Salt
Isolated Brown Piece	30.69	29.50	5.36	2.50
Isolated White Piece	33.95	28.00	5.40	3.00
Homogenous	34.25	28.00	5.29	2.99

Table 1. Final Composition from Source Cheese with Browning Defect. Sample had white and brown patches, that were tested.

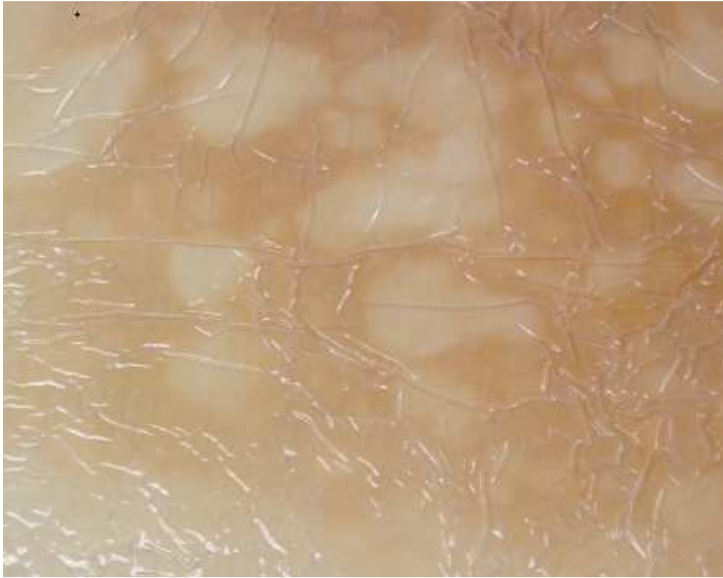
FIGURES

Figure 1. Undesirable browning appearance in Parmesan Cheese captured in 2016. Ingredients for this cheese included: Pasteurized Part Skim Milk, Cheese Culture, Salt and Enzymes.

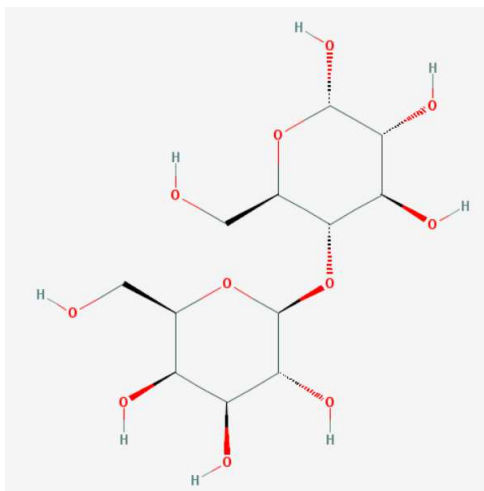


Figure 2. Chemical Structure of Lactose (Information, 2018)

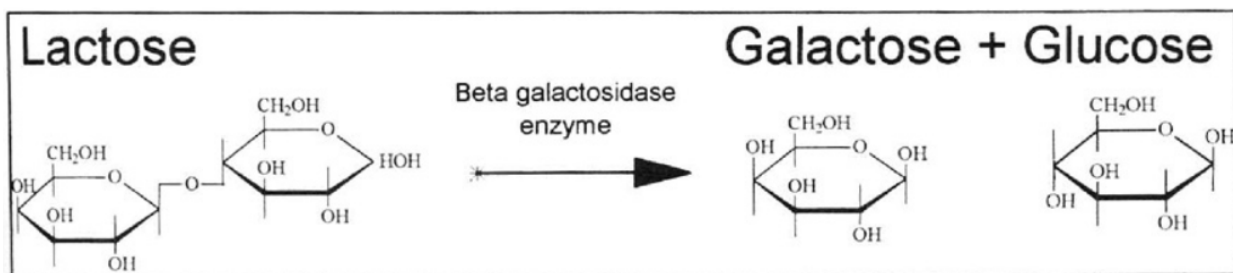


Figure 3. Action of beta-galactosidase (Gene Regulation I, 2011)

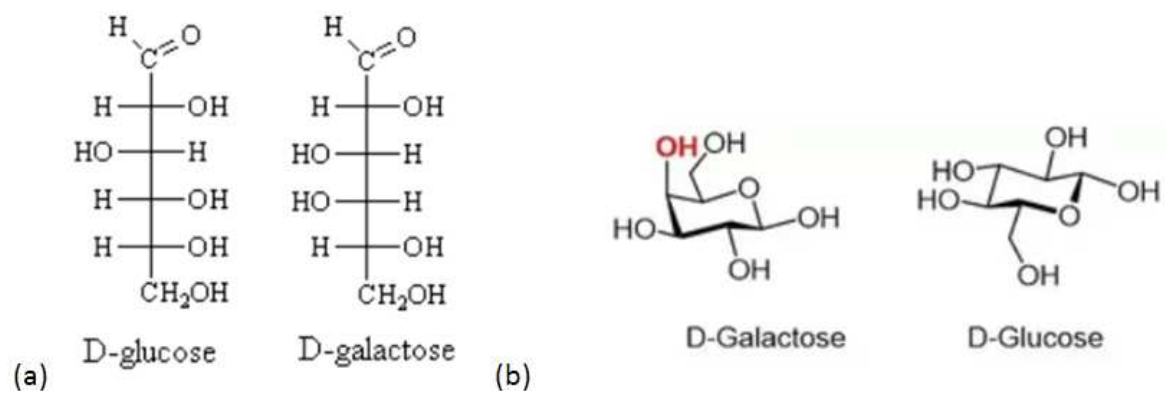
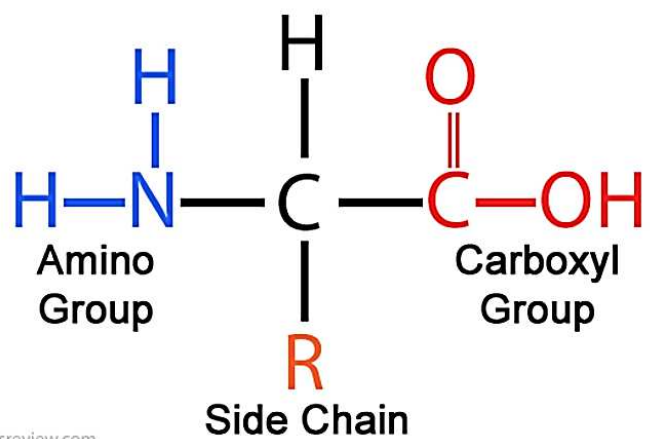


Figure 4. The structures of glucose and galactose, a. (Ernest, 2015) and b. (Christian, 2016)

Amino Acid Structure



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Figure 5. Basic Amino Acid Structure

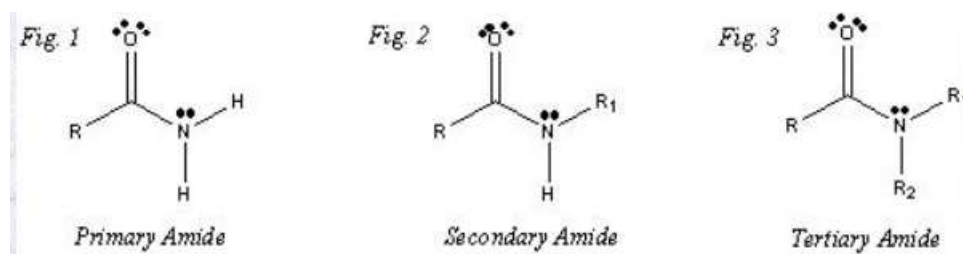
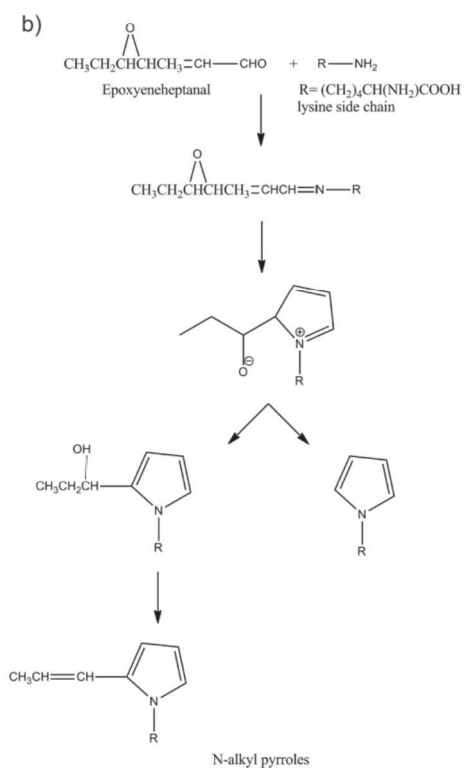
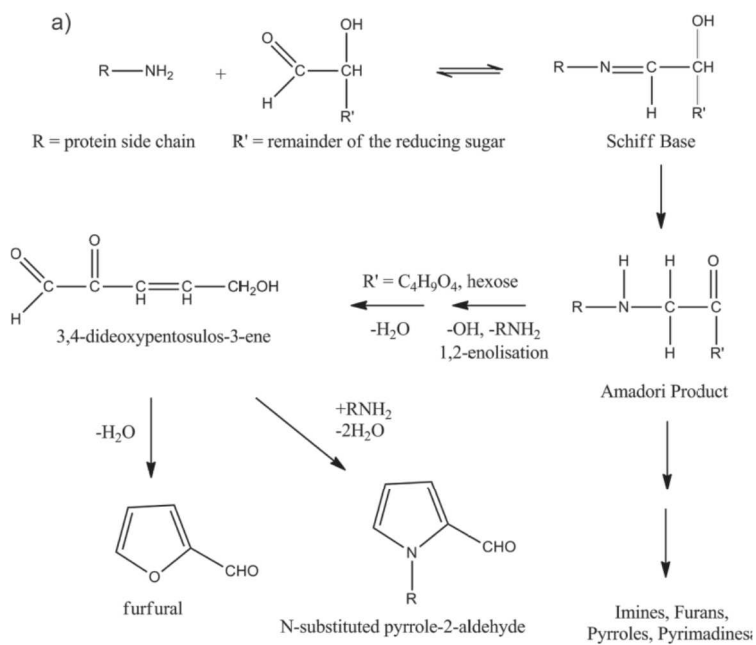


Figure 6. Different Chemical Structures of Amines; Primary, Secondary and Terti
(BioLabs, 2018)



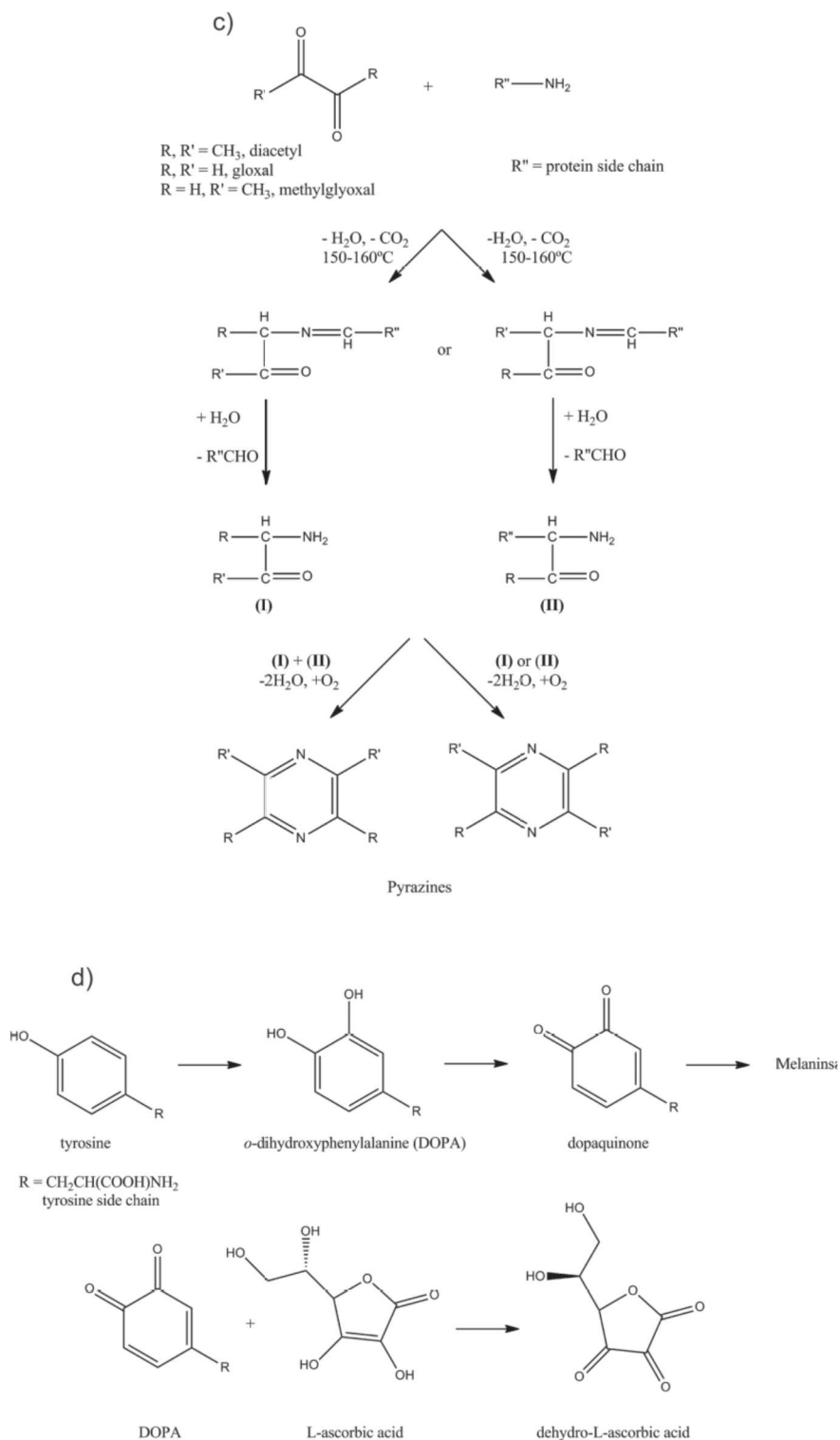


Figure 7. Browning of Parmesan Cheese During Storage Hypothesized volatile browning end products. (a) General form of sugar-protein interaction, showing the volatile furans and pyrroles that can result (Smith et al., 1994; Nursten, 2005); (b) example of a lipid oxidation product browning reaction with epoxyeneheptanal

reacting with lysine to form N-alkyl pyrroles (Hidalgo and Zamora, 1995; Friedman, 1996); (c) reaction of several diketones with primary amines to represent interaction between fermentation products and protein (Rizzi, 1972); (d) enzymatic hydroxylation of tyrosine to DOPA, which can then produce nonvolatile melanins or react with l-ascorbic acid to form dehydro-l-ascorbic acid (Friedman, 1996). Hypothesized volatile browning end products. (a) General form of sugar-protein interaction, showing the volatile furans and pyrroles that can result (Smith et al., 1994; Nursten, 2005); (b) example of a lipid oxidation product browning reaction with epoxyeneheptanal reacting with lysine to form N-alkyl pyrroles (Hidalgo and Zamora, 1995; Friedman, 1996); (c) reaction of several diketones with primary amines to represent interaction between fermentation products and protein (Rizzi, 1972); (d) enzymatic hydroxylation of tyrosine to DOPA, which can then produce nonvolatile melanins or react with l-ascorbic acid to form dehydro-l-ascorbic acid (Friedman, 1996).

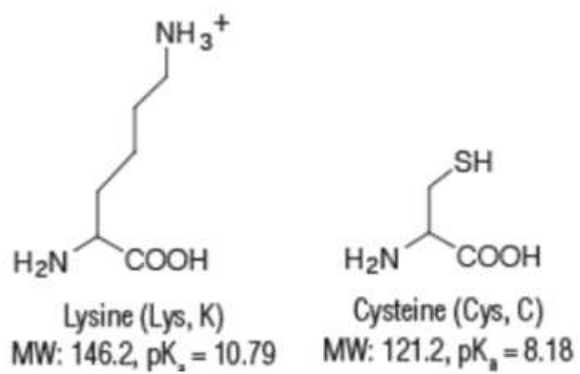


Figure 8. Chemical Structure of Lysine and Cysteine (BioLabs, 2018)

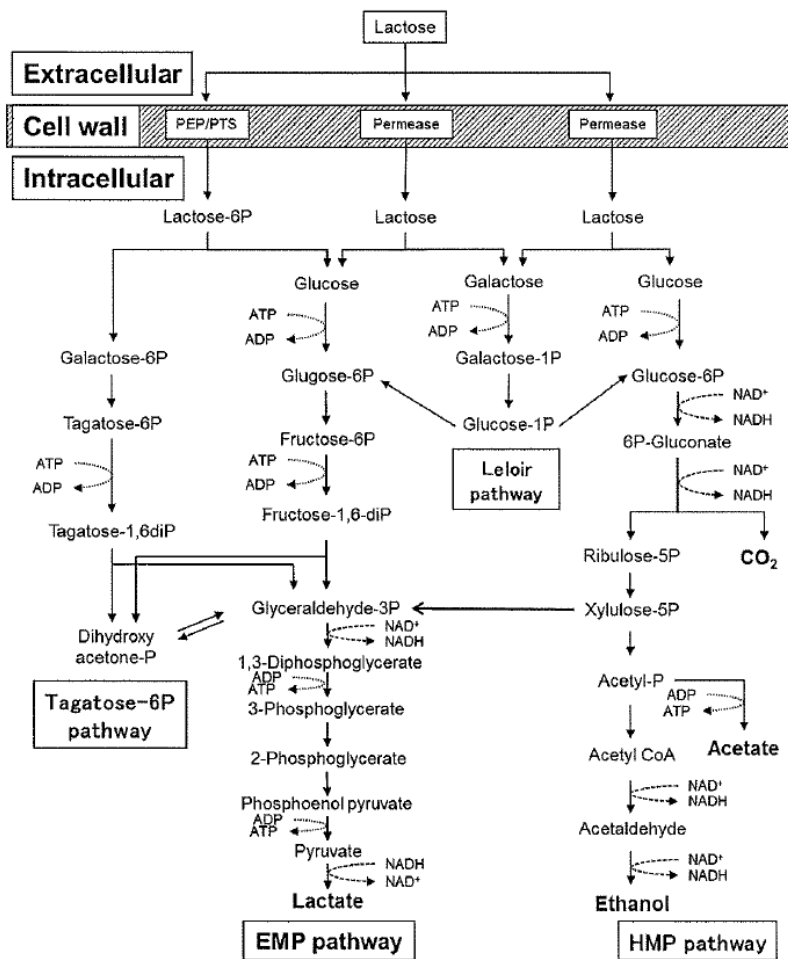


Figure 9. Glycolytic and Phosphoketolase pathway of lactose metabolism in lactic acid bacteria (Patrick F. Fox, 2000)

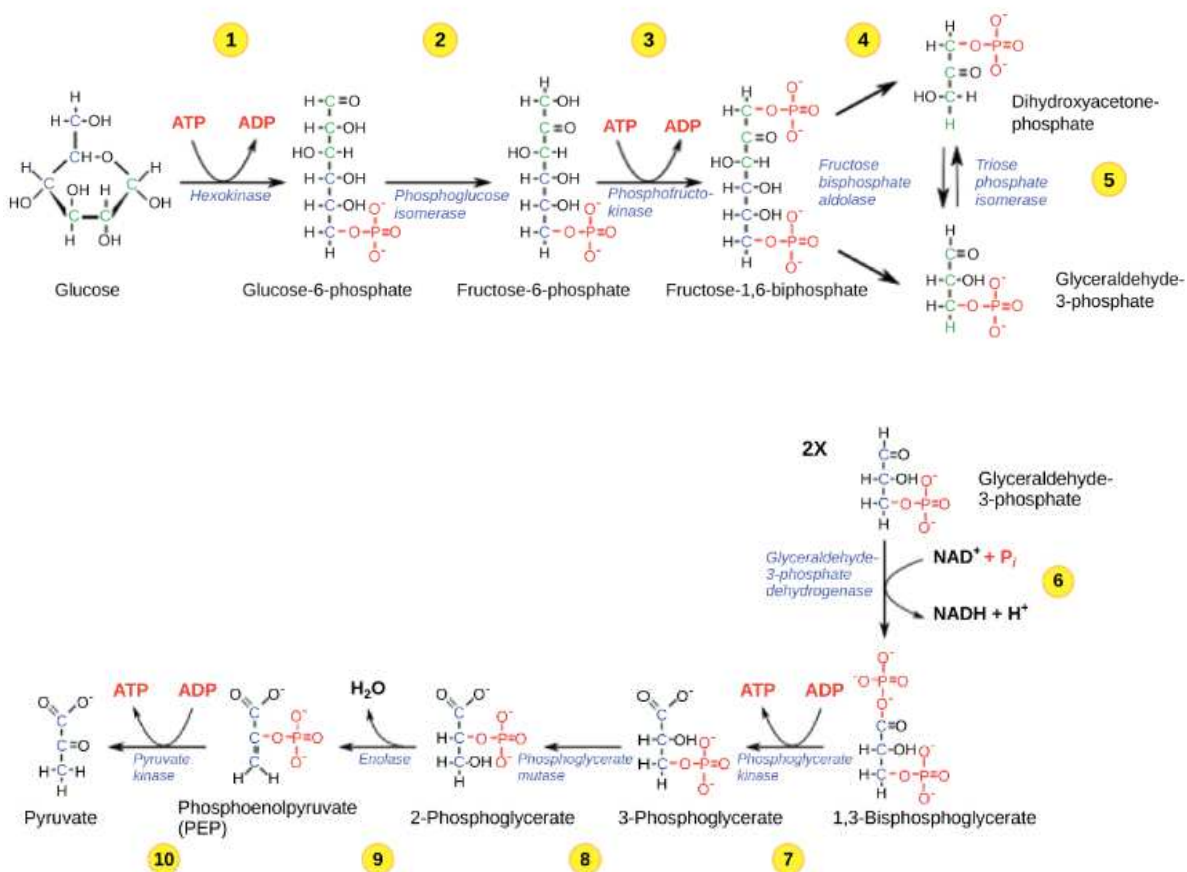


Figure 10. The EMP Pathway series of reactions (Academy, 2018)

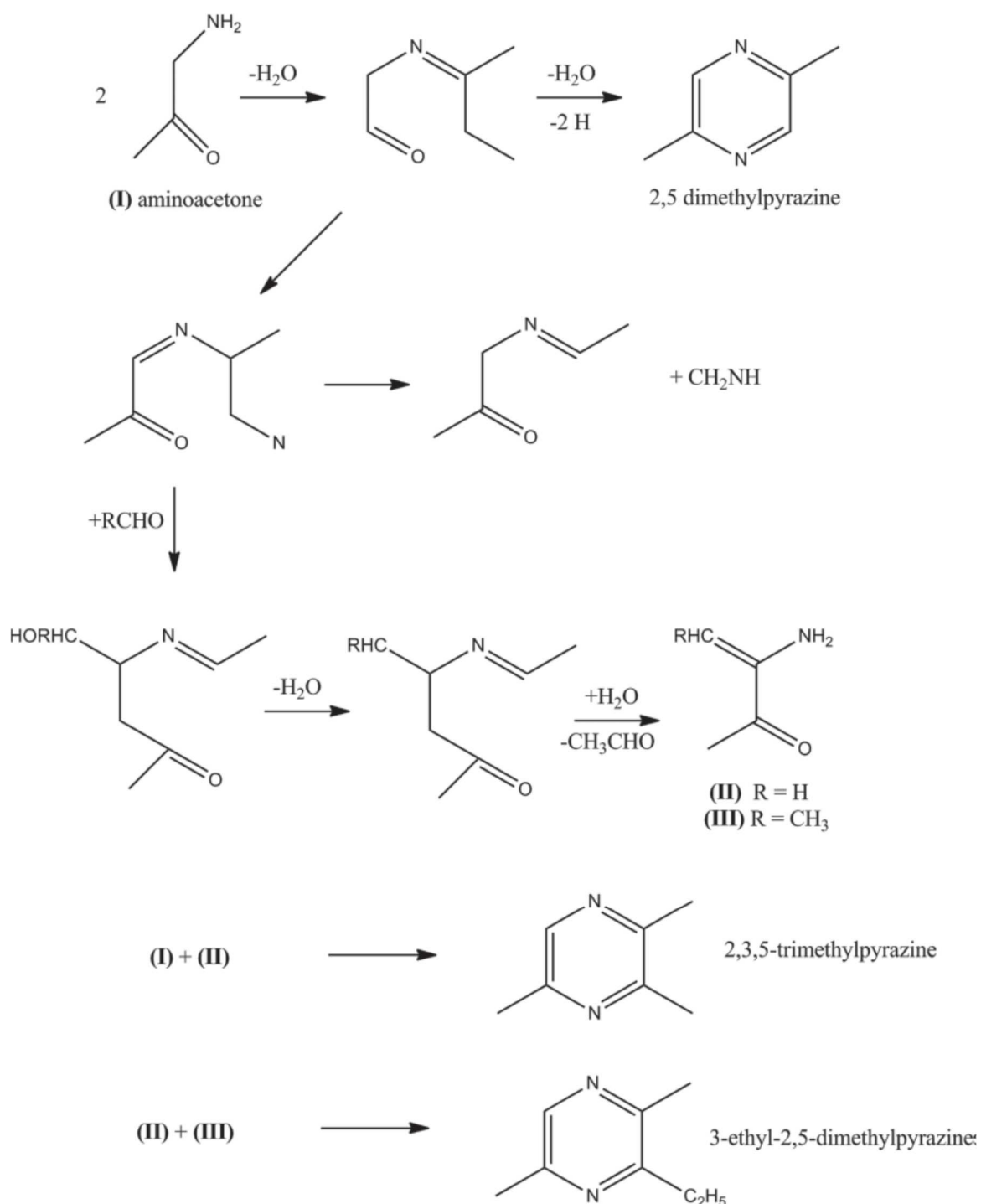


Figure 11. Formation of 2,3,5-trimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine from an aminoacetone pathway (Rankin, 2013)

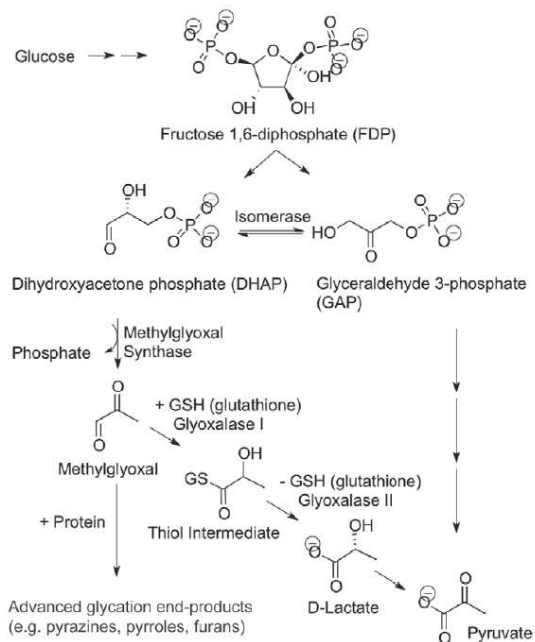


Figure 1. Methylglyoxal formation and metabolism (Russell, 1998; Silva et al., 2005).

Figure 12. Methylglyoxal formation and metabolism (Rankin, 2013)

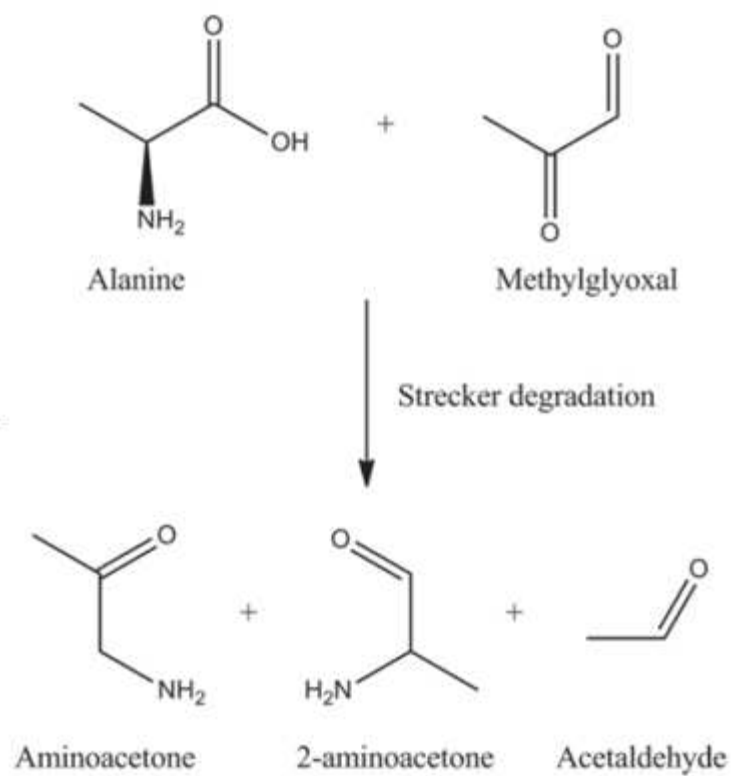


Figure 13. The Strecker degradation of alanine and MG to form aminoacetone

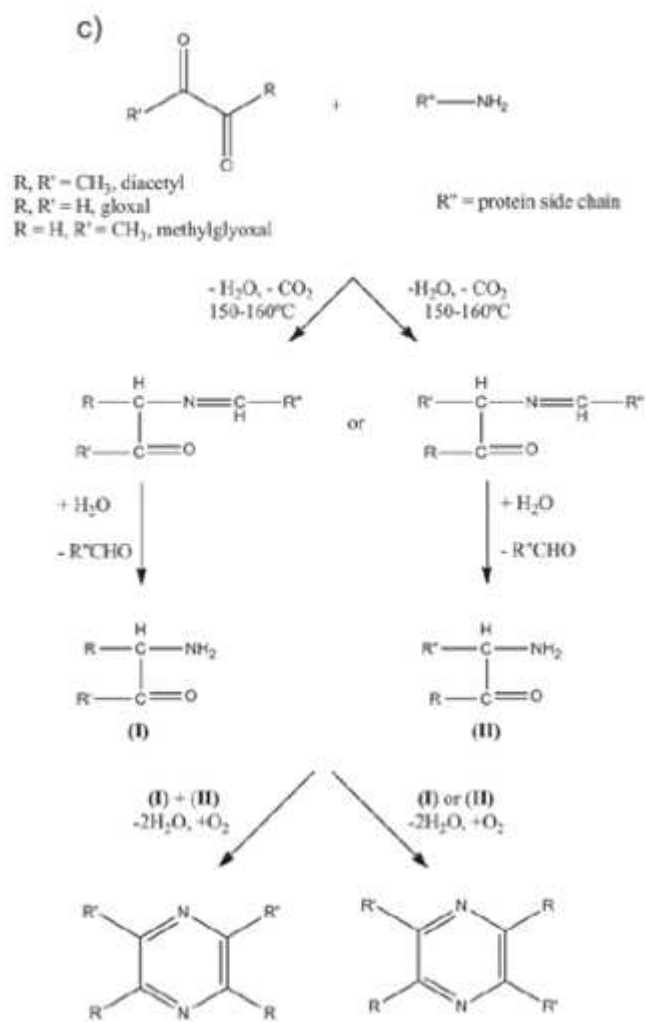


Figure 14. Browning of Parmesan Cheese During Storage

CHAPTER II: Parmesan Cheese Model System.

1. Introduction

A cheese model system helps bring order to the complexity of cheese chemistry and serves to try to understand what is going on in the cheese during aging. The cheese model system was created in order to create a consistent cheese platform to test different variables relative to postulated theories on browning in Parmesan. This model system was made up of parmesan cheese made from common in industry manufacturing processes that was mixed into a slurry, a combination of cheese and sodium chloride solution, and treated with different variables to understand their impact on browning.

2. Parmesan Cheese Make

The cheese for the model system in this experiment was created with the intentions to mimic the most common manufacturing process in the industry. The intention is to create an ideal cheese, from a manufacturing perspective, so the findings from this experiment can be used by most manufactures in the United States. Below are some manufacturing objectives that were utilized in the cheese model system and are consistent with the most common practices in the industry.

- The standardization of milk using a fat to protein ratio was utilized in order to obtain target final composition.
- High Temperature Short Time (HTST) pasteurization was used to destroy any harmful pathogenic microorganisms and or other microorganisms that may

affect ripening which are consistent with industry standards in the United States.

- A mixed strain of starter culture with symbiotic associations was inoculated in milk to help create acidity and start proteolysis.
- A bovine rennin was used to coagulate the casein micelles to the cheese making process to help the aggregation of casein and entrapment of fat.
- A double cut using $\frac{1}{4}$ inch knives was used in order to get smaller curd particles which helps decrease moisture. A smaller curd particle has greater surface area and less ability to trap in moisture. Additionally, the final curd size can affect salt absorption and cooling rate.
- The curds could heal after cutting in order to form a skin so that yield was not compromised.
- The main goal of cooking is to help reduce moisture and increase acid production. These both occur during most of the process but is maximized in this step. A ramped cooking step was used in order to allow the lactic acid bacteria to optimally acclimate, propagate and create acid in the cheese milk.
- A post cook stir step was utilized to allow for additional moisture release by the physically colliding curd particles.
- The whey drain step allows for the curd mass to be separated from the serum protein.
- The curd was cheddared in order to remove more moisture and knit the curd into one mass.

- The double mill and wash step was used to create higher surface area cheese and to wash off any residual sugars in the cheese. The washing of the curd may affect the final pH but provides a simpler environment to test variables with. The experiment will focus on comparing galactose levels.
- Dry salting was used in two steps because it's common for manufacture to dry salt and to maximize salt absorption. Salt is not only a flavor enhancer, but an antimicrobial. The prevention of NSLAB propagation in this experiment is important because these will be studied.
- Curd was pressed in order to maintain a wheel structure.
- Product was vacuum sealed in non-oxygen permeable bags to limit mold growth
- Product was individually stacked for consistency at 38°F cooler to start the cooling profile.

A cooling curve study was conducted to ensure all wheels manufactured were cooled as quickly and efficiently as possible. Controlling the micro-environment, especially in aged cheeses, is particularly important for the quality and consistency of the product. This is because the cheese micro flora creates various chemical compounds, thus affecting the quality particularly the sensory attributes (Gilles, 1987). An important factor for controlling the micro flora is cooling after the curd leaves the vat. Studies have shown slow cooling increases thermophilic LAB growth and lactose hydrolysis (P.F.FOX, 1987). Therefore, fast cooling is favorable because it limits the proliferation of micro flora in the cheese. Excessive growth of micro flora, particularly non-starter micro flora, could contribute to sensory defects in color, flavor and mouthfeel. Additionally, it's pertinent to have a standardized procedure for cooling so that all products are cooled with the same

efficiency to decrease variability. Some manufacturers use brining to cool the wheels, where others dry salt the wheels and use cold air (coolers). Most manufacturers dry salt Parmesan to save cost, so cooling using cold air is typically employed. Therefore, it's pertinent to understand what environment the wheels should be in in order to cool to refrigeration temperatures and as quickly as possible to limit growth of bacteria, thus undesirable flavor defects.

In this study, a method for cooling Parmesan cheese with dry salting was employed in order to establish the cooling curve rate and parameters. The study had three main objectives. The first objective was to identify the make procedure for the Parmesan cheese to ensure all wheels are consistent; cheese model system. The second objective was to measure the cooling curve under a certain condition; in this study cooling media (air) and cooler temperature (38°F). Included in this objective was the task of connecting the temperature probes to the Parmesan wheels. Also included in this objective was the task of understanding the software for these probes and setting them up for the experiment and to use the data in statistical analysis. The third objective was to understand how consistently and efficiently all the wheels were cooled down to a targeted core temperature. Consistency will be measured using a linear regression model to understand the regression equation. Efficiency will be measured by recording the temperature and determining if the wheels cooled to $\leq 40^{\circ}\text{F}$ in ≤ 24 hours from when the wheels are placed into the cooler.

3. Cheese Slurry

Factorial designs were created by John Bennet Lawes and Joseph Henry Gilbert who were associated with the Rothamsted Experimental Station in the 19th century (Mather, 1963). A factorial design is an experiment design that correlates multiple factors with different values and or levels and tests all possible combinations. Testing all possible combinations helps correlate the effect of each factor and their values or levels on the dependent variable. In statistics, factorial designs can then be analyzed via ANOVA and or regression analysis. An ANOVA (Analysis of Variance) is used to compare two or more means by examining the differences in the means by looking at their respective variances and asking are the differences explained by the variances or not; thus it is the same as a t-test run on multiple pairs, but accounts for the relationships of all the means, not just the two. A regression explains the effect of independent (explanatory) variables on a dependent variable, again with multiple regression techniques, accounting for all the relationships. ANOVA and regression are closely related; an ANOVA is one specific type of a regression just like a t-test is a single case of ANOVA.

The factorial designs testing different variables will done using the cheese model system in a slurry. A slurry is a mixture of water and insoluble matter (Merriam-Webster, 2018). In this study, cheese slurries were used as a platform to test the variables in Phase 1 and 2. The combination of cheese, water and test variables can be properly mixed using cheese slurries and is intended to replicate the cheese ripening process. Parmesan is a low moisture cheese, in order to properly replicate the aging conditions, a 2:1 ratio of water to cheese was used in order

replicate the ripening process. This ratio provided enough water to fully mix the cheese, but not have excessive moisture. A 3.12% NaCl solution was used to get the salt compositions of the slurries to the target salt of 2.50%. This was calculated based on a 2:1 ratio of cheese to moisture based on the W. James Harper and T. Kristofferson method (W. James. Harper, 1970).

4. Materials and Methods

4.1 Parmesan Cheese Make

4.1.1 Milk Standardization

The milk was standardized to a fat to protein ratio of 0.79. This was done by blending 3.10% protein and 2.45% fat. The fat to protein ratio is calculated knowing the target fat to protein ratio and percent protein tested in the milk. From there, butter fat is then skimmed off to reach the desired fat to protein ratio.

$$(\% \text{protein}) * (\text{fat: protein ratio}) = \% \text{ butter fat}$$

4.1.2 Heat Treatment of Milk

The milk was then heat treated at 162°F (68°C) for 16 seconds (HTST pasteurization). The milk was then pumped into four open 800-pound conventional vats (all numbers / calculations assume 800 pounds of milk).

4.1.3 Starter Culture Addition

The milk prior to starter addition had a titratable acidity of 0.16 and a pH of 6.65. Two cultures from Chris Hansen were inoculated into the vats; 48.8 grams of TDMN (*Lactobacillus helveticus*) and 38.4 grams of TD35 (*Streptococcus thermophilus*). The amounts of cultures used were suggested by Chris Hansen based

on amount of milk in vat. The culture was slowly agitated for 40-50 minutes at 90°F.

4.1.4 Rennet Addition and Cutting

Chymax Extra, bovine chymosin (Chris Hansen), was added to milk in the vat for a 20-minute set time (22.7 grams). The newly formed coagulum was cut two times, up and down, using ¼ inch knife to obtain a very small curd particle size. The curd could heal for 10 minutes and then the curd was ramped cooked for 30 minutes.

4.1.5 Cooking/Ripening

The ramped cooking included 15 minutes where the curd was cooked at 108°F and then 122°F for the remaining 15 minutes. Prior to whey drain, there was a 30-minute post cook stir.

4.1.6 Cheddaring / Milling / Rinsed Curd

After draining the whey, the curd was cheddared for 60 minutes where the curd was flipped every 10 minutes (total of 6 flips). The curd was then double milled and washed with 21 gallons of water at 115-120°F water for 5-10 minutes to decrease galactose accumulation in the curd.

4.1.7 Dry Salt Addition

Excess water was drained (approx. 7 minutes) and the curd was dry salted in two stages targeting a 2.2% salt. The two stages of salt addition both included a 5-minute stir of 0.90 pounds (409 grams) of salt.

4.1.8 Pressing / Packaging

The curd was then shoveled into a hoop where it was pressed at 20psi for 45 minutes and again at 30psi for 45minutes. The wheels were then vacuumed sealed in non-oxygen permeable packaging where temperature probes were inserted into the sides of each wheel. Each wheel was then placed in a 38°F cooler individually stacked on racks. A total of eight wheels were manufactured in this production. The final composition targets are listed in table 1. The cheese making steps can be summarized by the procedural figure 1.

4.1.9 Cooling

Temperature probes (Mesa Labs) were set up to collect temperature data over a week date range. These probes were labeled with numbers A, B, C.... etc. to track the wheels (8 total wheels were produced). The temperature probes, 6 inches in length, were inserted into all eight wheels that were 13.5 inches in diameter. The

wheels were placed in a 38°F cooler for 5 days. The data from the temperature probes were collected, compiled and analyzed using Minitab.

4.2 Cheese Slurry Procedure

This method utilized 500 g of cheese / 250 ml of 3.12% or 4.62% NaCl, to achieve 2.50% or 3.00% salt respectively. Using NaCl can help maintain the same salt concentration in cheese as well as not changing the ionic strength. In other words, it buffers the cheese environment. In this experiment, the average salt of all the wheels was 2.19% (target was 2.50% in phase 1 and 3.00% in phase 2). The following calculation was used to obtain a 2.50% NaCl percentage per 1000 ml of water.

$$\frac{((2 (2.19 \% \text{ salt}) + x \% \text{ NaCl}))}{3} = 2.50$$

$$x = 3.12\% \text{ NaCl}$$

Each wheel of Parmesan produced weighed approx. 22-pounds Each slurry tested contained approximately 417.13 grams of cheese. Based on this, 208.5 ml of 3.12% or 4.62% NaCl solution was used to achieve the targets salt for each slurry.

5. Results and Discussion

5.1 Parmesan Cheese Make

The cheese made for the model system was all manufactured the same to ensure that differences observed in color were truly due to the natural chemistry and microbiology associated with the treatments. All pH/TA milestones and cooling curves were obtained during cheese making that used the cheese model system. The final composition for %moisture was high (approx. 37% avg) and low %salt (2.19%). In chapter 3, the cheese slurries will be adjusted to different salt levels using sodium chloride. Moisture will not be modified but will be managed to ensure there is a homogenous mix without increasing the moisture too much. It is important to note, that not all factors, such as moisture, can be managed through a slurry, but all slurries will have the same composition to ensure consistency.

On average, all wheels were cooled to $\leq 40^{\circ}\text{F}$ in approximately 12.25 hours after being placed in the 38°F cooler. Efficient cooling was achieved using at 38°F cooler, for all wheels, because the target temperature was achieved prior to the expiration of 24 hours. Below is a depiction of the cooling curves for all eight wheels, refer to graph 1.

All wheels follow a similar regression equation and are best fit by a quadratic line. The cooling curve lines for all wheels, followed the same cooling profile with limited variability. Consistent and efficient cooling in all

wheels was achieved in order to reduce wheel to wheel variability and ensure the product quality from a microbial perspective (figure 2).

The main drivers in the cheese making was to achieve the right composition and ensure consistency throughout the make procedure. A fat to protein ratio was used to standardize fat and protein which is affected by seasonality. Cows put less fat and protein in milk during the summer months because of the warm temperature; and they are more likely to drink water, thus diluting solids. Fat to protein ratio, is a calculation that helps cheese makers manage the solids in milk to help blend in or remove fat based on the protein concentration in milk. This helps reduce variability and creates consistency in cheese making, and so cheese makers can account for consistent yield. This also helps achieve desired target composition.

Heat treatment or pasteurization of milk is a legal requirement to ensure food safety. For high heat short time, the legal requirements indicate that milk should be heated to 161F for 15 seconds and then rapidly cooled. This is achieved using a heat exchanging mechanism; extremely warm water is pumped through one side of the plates of the pasteurizer. As milk crosses on the other side of the plates, a heat exchange occurs allowing the milk to be rapidly heated to pasteurized limits. These parameters may change based on the amount of fat or other solids put into the milk; more concentrated solids will require more heat and longer time to ensure all pathogens are killed. Pasteurization is a means to eliminate pathogens and extend shelf life but does not kill all thermophilic bacteria. Pasteurization works to kill bacteria by

denaturing their enzymes, so they no longer work and damages bacteria's cell walls, proteins and fats that are stored within the cell.

Starter culture is added to milk to help make cheese. Starter Culture's role is to convert lactose to lactic acid, and potentially other molecules that can help with flavor, texture, etc. Starter culture produces acid, which makes it hard for NSLAB or spoilage bacteria to grow since most do not grow optimally at lower pH. In parmesan, a mixed strain of rods and cocci are used for their symbiotic relationship. The cocci work to convert lactose to lactic acid which stimulate the rods to break down proteins, which create more energy for the cocci. The cultures in parmesan are also heat tolerant, thermophiles and can survive and proliferate at warmer temperatures utilized throughout the cheese making.

Rennet, extracted from calves, is a processing aide in cheese that is composed of multiple enzymes to help precipitate casein out of solution. For cheese, the main enzyme to help with precipitating casein out of solution is chymosin, which is an aspartic endopeptidase. Chymosin works by cleaving the 105-106 amino acids of K-casein, methionine and phenylalanine, that removes the negative charge on the casein micelle making them more neutral. The micelles then join entrapping fat and proteins, and the serum protein whey is separated (the first stage to removing moisture/syneresis).

Curd is cut using $\frac{1}{4}$ inch knives to get a small particle size. Smaller particles have greater surface area, which allows more moisture to be removed. It is

important to ensure cutting is consistent, using sharp knives and a uniform cutting procedure. Manual cutting is difficult to reproduce and introduces a lot of variability. Because of this, often different sizes of curd are formed which contribute to variability and limited control over moisture. Therefore, automated steps help reduce this variability making it easier to achieve desired moistures.

Cooking is a step, also known as ripening has multiple benefits. First, it helps to remove moisture from the cheese, which is necessary for Parmesan since it is a low moisture cheese. Warm temperatures in cooking increase the hydrophobic interactions and kinetics between casein micelles, making them squeeze together and expel moisture. Secondly, the starter culture added to the milk work optimally at warmer temperatures which allows them to proliferate in milk to produce lactic acid and other molecules that contribute to the symbiotic relationship mentioned earlier and flavor molecules. Additionally, when the starter is creating acid, this is dropping the pH. As the pH decreases more calcium phosphate is being solubilized which has effects on texture and buffer capacity. During cooking, there is agitation and a post cook stir that help physically drive out moisture by colliding

The cheese is then cheddared to allow the casein micelles to knit together. Again, this helps with syneresis to achieve desired moisture by physically (weighted) removing it. Cheddaring is the most traditional way of knitting curd together, but some manufacture has large equipment to help this and prevent the manual process of flipping cheese. Milling cheese, again, helps

with reducing moisture because it increases surface area of knitted curd allowing for syneresis. When curd synereses, moisture, whey, sugars, fat soluble vitamins and bacteria are removed.

Salt can be added in one of two ways, by brining or dry salting. Brining occurs when after curd is pressed, and the wheel or block is submerged in high salinity brine for an extended period (hours). Salt then migrates through the wheel and eventually equilibrates during aging. Brining is also beneficial for managing cooling rates, especially if the system allows for each wheel to be consistently cooled. Additionally, salt can be added to the milled curd in the dry form. Typically, dry salting is conducted in two stages, because salt also helps to remove moisture based on osmotic relationships. Because of this, some of the salt may expel off too. Salt is beneficial to cheese to help remove moisture, serve as an antimicrobial (inhibits microbial growth), controls the NSLAB population, serves as a preservative because of its ability to be an antimicrobial and control bacterial growth and enhance flavors that develop in the cheese during aging. Salt is a flavor enhancer and works to compliment and synergize flavors that are created overtime in parmesan.

As aforementioned, parmesan cheese needs to be pressed and can be done before or after salting based on how salt is added. Pressing the cheese uses mechanical force to continue to knit cheese together and remove moisture. It is typically done in two stages to get form stability. Additionally, cooling can be done in the brine, or by using cold ambient air/coolers. Cooling wheels or blocks consistently helps improve the microbial quality of the cheese. Cheese

that is cooled fast could result in textural issues in the center (hard centers) because moisture will be rapidly removed from the middle, however, will reduce the amount of proliferation of microflora in the cheese. This could affect the cheese in terms of flavor development that could have negative or positive effects depending on the bacteria present and what compounds are being produced. Additionally, cooling too slow will increase the amount of time bacteria can proliferate at optimal temperatures. This too can affect the flavors or other compounds, pigmented compounds, produced in the cheese by increasing the rate they are produced creating more of them. Cheese flavor can be muddled and excessive overtime because of the accumulating of flavor molecules and there is a higher likely hood of pigmented compounds to be produced. Therefore, controlling the cooling profile that is optimal for the targeted flavor and appearance profile is recommended.

5.2 Cheese Slurry

A cheese slurry is a medium used to test variables from a base cheese. This medium serves as a similar platform the cheese itself by having a similar composition, microflora environment, salt and pH environment and cheese derived from similar make procedures in Parmesan. In cheese making, everything has a ripple effect and correlations. Therefore, using a slurry that is as close to parmesan as possible was desired. The objective of the cheese model system was to design it with specific characteristics in mind:

- Utilizes common manufacturing practices,

- Develop a base cheese that is free of factors that could affect observations (i.e., washing of residual carbohydrate),
- A flexible system that allows manipulation to incorporate and test potential variables (a slurry provides this),
- Represents the primary composition variables in parmesan cheese and
- Allows simulation of cheese ripening and maturation.

6. Conclusion for Cheese Model System

This study focused on creating a cheese model system that will be to study variables that caused browning and variables that helped reduce or alleviate browning. The cheese model system was needed to ensure all variables started with a homogenous substrate. The procedure to make Parmesan was consistent with traditional cheese making in the United States with the most common ingredients for starter culture and rennet. The cheese making steps were monitored by measuring pH and TA and the final composition for % fat, % moisture, % salt and pH. Additionally, the cooling curve was studied to ensure all wheels of cheese made were cooled similarly. Ensuring the cheese cooled similar helps reduce differences in the microbial population of the cheese. Additionally, adding the parmesan to a slurry will create a cheese model system to test variable in order to understand their effect on browning.

Modifications to the cheese model system could have utilized brining as oppose to dry salting, to help achieve cooling and the cheese and the utilization of different starter.

In this cheese model system, *Lb. helveticus* was used as the starter because it is galactose positive but was also identified as a positive control. Therefore, the cheese model system could have used *Lb. bulgaricus* as the starter. All the studies in chapter three, used the cheese model system created in this experiment. This allows for true comparisons of variables to assess impact on color defects. Additionally, a 3:1 or 4:1 ratio could have been used in the slurry, but the risk regarding getting an adequate and homogenous mixture. There were main characteristics identified for the cheese model system for it to be considered a successful model system. A cheese model system is an effective way to model because it serves as a near real simulation of what is occurring chemically and microbiologically in cheese during aging. Additionally, it is very cost effective (reduces), safe and reduces the noise/variability in cheese making by making many samples from one batch of cheese. This serves more as a bench top and modeling, that can serve as a simulation to the large-scale production of cheese making. Its likely, that the findings from the model system will be translatable in a large-scale production, especially if the variables tested to relieve browning can be controlled or contained through cheese making (i.e., %salt, pH, use of adjuncts, etc.). Additionally, the dependent variables derived from the cheese model system that are correlated to variables tested can be statistically analyzed very quickly, in a large sample size. There are however, limits to the simulation since the cheese model system itself cannot account for all aspects of parmesan cheese aging. For example, a slurry combines sodium chloride with cheese, ultimately increasing the moisture (above standards of identity). However, based on the literature, increased browning in the maillard reaction is a result of evaporation of water in baked conditions. Therefore, if browning occurs in the samples from the cheese model

system and are statistically different than those deemed white, one could assume the effects would be more extreme if the moisture was lower. Additionally, the samples will not be aged in a wheel or block but rather jars or petrie dishes which does not represent the aging conditions in terms of surface area, formation of rind and head space. Lastly, the samples were only aged to 3 months and could have been aged to 10 or more months. It is assumed that if browning occurred from 0-3 months that it will remain a visible defect in the cheese through its entire shelf-life. However, these are assumptions and would need to be verified.

7. Further Readings

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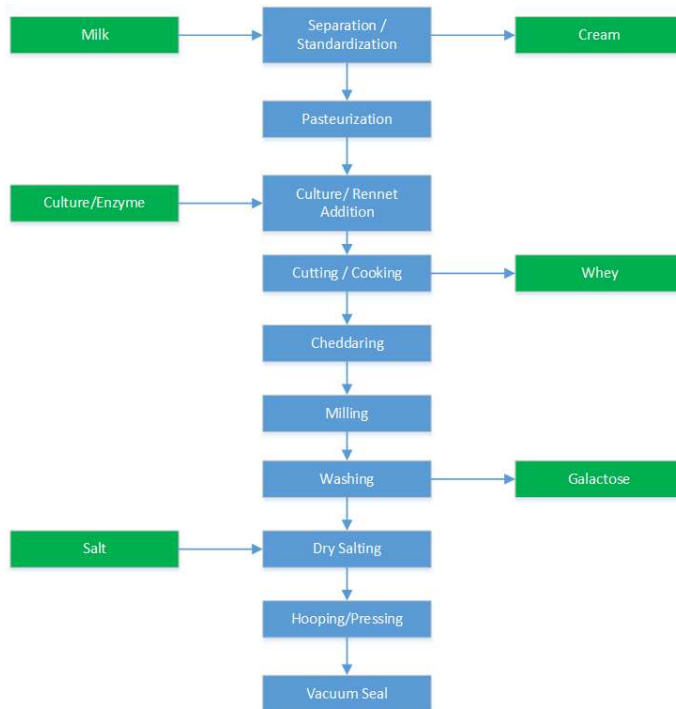
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TABLES

Parmesan Target Composition				
Moisture	Salt	Butterfat	pH	*FDB
<32.00%	2.50%	27.00%	5.35	39.70%

FDB calculation is calculated as; $FDB = \frac{\%Fat}{100 - \%Moisture}$

Table 1. Cheese Model System Parmesan Target Composition numbers for salt, butter fat, pH and salt.

FIGURES**Figure 1.** Parmesan Cheese Process Flow Chart

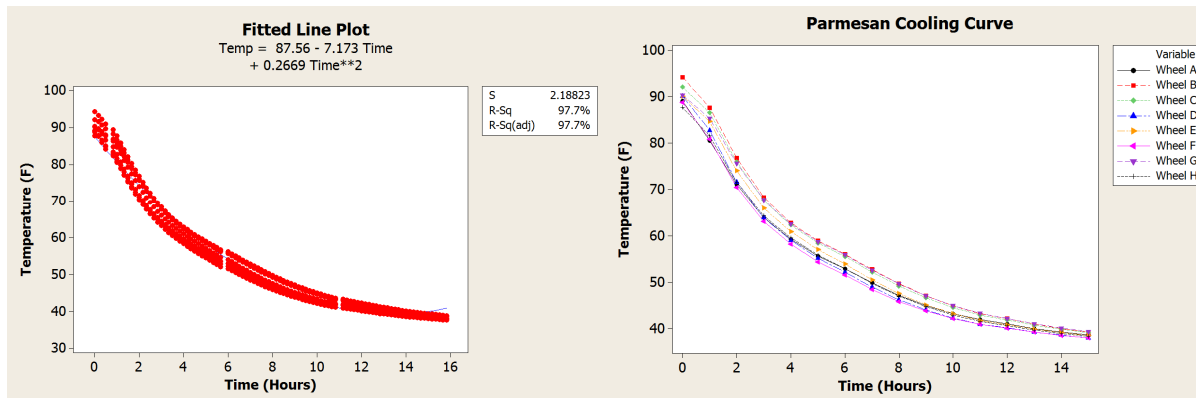


Figure 2. Parmesan Cooling Curve Time Series Plot & Fitted Line Plot (Quadratic Equation)

CHAPTER III: Use of Cheese Model System to Test Variables That Cause or Inhibit Browning.

1. Introduction

1.1. Natamycin Testing

Mold tends to grow in warm aerobic conditions and is air-borne in the form of spores. These spores can land on Parmesan and begin to propagate, thus creating a mold aroma and appearance on cheese. A way to alleviate this problem, especially for aged cheeses that are aged long enough to have noticeable effects of mold growth, is to treat the cheese with a mold inhibitor. A common mold inhibitor used in the cheese industry is natamycin because it replaces chemical preservatives, gives a neutral taste and is not pH dependent. Natamycin is created via fermentation by a bacterium called *Streptomyces natalensis* (Pharmacists, 2018). Natamycin prevents the growth of mold through the inhibition of amino acid and glucose transport across the plasma membrane of a mold cell by binding to ergosterol (te Welscher YM1, 2012). Ergosterol, is a sterol found in the cell membrane in mold that acts like cholesterol in animals. Ergosterol helps mold with structural integrity and fluidity. Since Natamycin is a common processing aide, ruling this out as a potential browning variable is appropriate. Additionally, natamycin will be used in trial plans to prevent mold growth of samples. Therefore, it's important to know that natamycin is not causing the browning when considering other variables and correlations using a factorial design.

1.2 Phase I in Cheese Model System

The variables studied using the cheese model system slurry in Phase I include: galactose addition, methylglyoxal addition, NSLAB (Non-Starter Lactic Acid Bacteria), Aging Temperature, Age and Heat Abuse. The accumulation of galactose in Parmesan cheese is common due to the inability of *S. thermophilus* to metabolize galactose to lactic acid. As mentioned earlier, galactose is a reducing sugar that tends to react with primary amines that can be enzymatically converted into pigments. Additionally, any bacteria present in cheese that can metabolize galactose can convert galactose into other compounds like methylglyoxal that can be enzymatically converted into brown pigments. Therefore, testing the tendency of galactose to be converted into brown pigments in the cheese model system environment is essential.

Methylglyoxal has been proven to be a precursor to browning; therefore, testing this variable in the cheese model system is essential. *Lactobacillus helveticus* has been shown to produce methylglyoxal. 2.0 to 3.5 ug of methylglyoxal/g is necessary for the formation of brown pigmentation during aging (McDonald, 1992).

The starter culture used to make cheese typically die after the manufacture of cheese. Their cells rupture releasing enzymes into the cheese which still contribute to ripening. In aged cheeses, like Parmesan, non-starter

lactic acid bacteria begin to grow slowly during aging, as seen in figure 1 (Cheese Science, 2018).

The NSLAB, *Lactobacillus bulgaricus*, was added to the Parmesan cheese slurries and is galactose negative. This means *Lactobacillus bulgaricus* is not able to digest galactose because it does not have the enzymes for the galactose metabolism. The starter culture used in the cheese model system was *Lactobacillus helveticus*, which is galactose positive.

The cheese model system, particularly the wash step in the make process, should rid the slurry of any residual galactose because of the starter and the rinse step. The addition of galactose and or *Lactobacillus bulgaricus* to the cheese slurry allows these variables to be studied for effect independently. This can help to understand the method for methylglyoxal production for browning.

Cheese treatments were aged in three different temperatures to understand aging temperature's effect on browning. The aging temperatures studied in this phase include; 45°F, 55°F and 65°F. Most aged cheeses, like Parmesan, are aged between 50-55°F. These three temperatures offer two extreme viable options and a reference at 55°F. This study did not focus on relative humidity, but samples were aged in jars in incubators (not exposed to the environment). When cheese ages, different biological and chemical processes occur (i.e., proteolysis). Supplying different temperatures will increase or decrease the rate of biological and chemical reactions in the cheese. Therefore, understanding the rate of browning in Parmesan and if it is affected or induced by different

temperatures is essential. Additionally, testing products at different ages against other variable will help to understand the rate of browning development. It is likely, that with more age, more browning will be observed. Some cheese treatments were subjected to heat abuse to understand the cheese model system more and use heat abuse to increase reactions and results. The temperatures for heat abuse are not practical for cheese aging but help correlate the variables at a quicker rate or can mimic impractical storage of product out in the marketplace.

1.3 Milk Experiment to determine Positive Control Microorganism for Phase II

A milk screening was used as a rapid way of detecting the tendency of bacterium to produce browning which provides a positive control for phase 2 studies. Additionally, inoculating milk with different bacterium that does not have the tendency to produce browning could help prevent browning by competing for substrates. This experiment focused on growing up eight strains of microorganisms to understand NSLAB (1), adjunct culture (2) and starter cultures (3) abilities to produce browning, these included: *Pediococcus acidilactici*(1), *Lactobacillus brevis* (1), *Lactobacillus rhamnosus*(1), *Lactobacillus plantarum* (2), *Lactococcus lactis diacetylactis* (2) *Lactobacillus paracasei* (2) *Lactobacillus bulgaricus* (1,3) and *Lactobacillus helveticus*(1,3).

1.4 Phase II in Cheese Model System

Common NSLAB found in different cheeses include (C.M. Lynch, 1999): *Pediococcus acidilactici*, *Lactobacillus Rhamnosus*, *Lactobacillus bulgaricus*,

Lactobacillus helveticus and *Lactobacillus brevis*. These bacteria typically survive pasteurization and could metabolize residual sugars after cheese making when the starter culture has died, and lysed enzymes as seen in figure NSLAB typically grow slowly reaching 10^7 - 10^8 cfu/g within 2-3 months (Cheese Science, 2018). Controlling the wild strain NSLAB by ripening temperature and cooling rate after pressing could help eliminate any browning defects (P.F. Fox, 1998). It's also important to understand the starter culture impact on browning. Even though starter culture dies off, their cells rupture, and their enzymes are released into the cheese. The release of enzymes could affect ripening; therefore, *Lactobacillus bulgaricus* and *Lactobacillus helveticus* were included in this screening (Cheese Science, 2018).

Other bacterium studied could serve as adjunct cultures that can help metabolize residual sugars which include: *Lactobacillus plantarum* and *Lactococcus lactis diacetyl*. *Lactobacillus plantarum* is commonly found in fermented food products as well as silage because of its ability to out compete other organisms by producing lactic and acetic acid via EMP (Jae-Han Kim, 2010). If *Lactobacillus plantarum* is not a brown producing bacterium, it may serve as a good adjunct to out compete NSLAB who do product brown pigments. *Lactococcus lactis diacetyl* is typically used as an aroma producing strain that metabolizes glucose and citrate to produce diacetyl and acetoin. The development of acid from converting glucose to lactic acid activates the metabolism of citrate not in terms of pH, but the depletion of pyruvate. *Lactococcus lactis diacetyl* will then begin to convert citrate to pyruvate

(Nieves Garcia-Quintans, 2008). *Lactococcus lactis diacetyl* can metabolize glucose to form pyruvate which is then converted to diacetyl. Most cheese contains less than 0.05 mg. of diacetyl per 100 g. of cheese. The diacetyl content of the Cheddar cheese ranged from 0.016 mg to 0.335 mg per 100 g of cheese (Harold E. Calbert, 1949). As mentioned earlier, in order to make pyruvate bacterium must product G3P. Therefore, addition of an adjunct to cheese known to produce pyruvate to make diacetyl could decrease shift the concentration of G3Ps. This means, there is a greater chance of G3P being created as oppose to DHAP, thus decreasing the chance of creating methylglyoxal as seen in figure 3.)

Other compositional factors such as salt and lactic acid were utilized in conjunction with the other variables listed above. Since different %salt or pH levels can be achieved through cheese making, perhaps changing the cheese environment could play a significant role in browning defect observed in Parmesan. Salt is effective at preventing microbial growth because it reduces the water activity of food by binding to water molecules. Also, the addition of salt induces osmotic pressures in bacteria cells causing them to lose water which may lead to cell death or stunted growth. In cheese making, salt is introduced after the LAB has produced acid. Therefore, increasing the %salt in Parmesan may help reduce the proliferation of NSLAB (Jane E Henney, 2010). The effects of low (2.50%) and high (3.00%) salt will be studied in phase 2. Just like salt can inhibit microbial growth, pH is also known to have antimicrobial capabilities. Most bacteria have an optimal growth at a pH near

7.00. In phase 1, the target pH was 5.60 which are standard in the industry. In phase 2, the pH is much lower targeting a 5.20. Bacteria contain enzymes responsible for metabolizing energy, that work optimally at specific pH. Since most bacteria do not grow that well under a pH of 5.50, the enzymes will not work as well, thus stunting growth as seen in figure 4. By decreasing the pH of parmesan using lactic acid created by LAB, NSLAB growth could be limited during aging (The Effects of pH on Microbial Growth, 2018).

For phase two, four variables were tested in combination to make a total of twenty-four treatments per trial. These variables one NSLAB, two adjuncts, normal vs low salt and normal vs low pH. The variables included can be seen in the factorial design in table 1. Additionally, greater detail regarding the factorial design's sub variables in terms of NSLAB types, adjunct types and compositional factors can be found in table 2.

1.5 Colorimeter Analysis

The Colorflex colorimeter contains an optical sensor for spectrophometry readings of color. This equipment works by taking a quantitative measurement of the wavelength based on the reflection or transmission of a sample. There are different outputs the colorimeter can generate, however, this experiment focused on L*a*b* outputs.

L*a*b* are color coordinates that were developed in the 1950-1960s to create a uniform color scale (HunterLabs, 2008). This scale was more visually uniform than the XYZ color scale because of the cubic organization. The XYZ

color system provides a colorimetric distance between samples, but not the perceived color difference (Explanation of the LAB Color Space, 2018). The L*a*b color system provides a 3-D depiction of color, as seen below in figure 2 (HunterLabs, 2008).

As seen in figure 2, the 'L' value runs top to bottom giving numbers ranging from 0-100 depicting the relative luminance (lightness) in terms of white or black. The color channels are depicted by a* and b* values, green-red and blue-yellow respectively. When values a* and b* equal zero, this represents a true neutral grey value. For the a* value, a positive number of correlates to red and a negative number of correlates to green. For the value b*, a positive number of correlates to yellow and a negative number of correlates to blue. The numbers generated from each sample provides can provide infinite three-dimensional coordinates for color (CIELAB color space, 2018). These numbers can then be compared to an original reference number. In this experiment, fresh cheese (reference cheese) free of the browning defect can be compared to variable treated cheese in terms of DeltaE (change of color). The letter E in DeltaE (ΔE) stands for Empfindung, a German word for sensation (Labs, 2018). A Delta E of 1-2.5 is the lowest threshold of humanly perceived difference and would be considered a match. A Delta E of 3-6 is a commercially acceptable color change, but color difference can be perceived (View Sonic, 2019). In this study, product that is less than 4.5 will be considered white and anything greater than 4.5 is brown / greater than normal color change due to aging. The control samples that were aged at 45F with no heat abuse or addition of MG, galactose

and NSLAB had a Delta E of less than 4.5 throughout aging. The calculation for ΔE is represented in the equation below;

$$\Delta E = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$$

A Delta E of 1 is the lowest threshold of humanly perceived difference. A generally considered to be barely perceptible by the average human observer; a Delta E between 3 and 6 is typically considered an acceptable match in commercial reproduction on printing presses.

2 Materials and Methods

2.1 Source Cheese Natamycin Testing Procedure

One-hundred grams of both white and brown cheese from the Source Cheese with the brown defect were collected and placed in a whirl-pack and ice cooler. Samples were sent to Cherney, a Microbiological service for natamycin testing using the spectrophometric MoA 099 method.

2.2 Phase I: Factorial Design

A factorial design was created to test out five variables in different combinations. These five variables included: heat abuse, aging temperature, galactose addition, methylglyoxal addition and Non-starter Lactic Acid Bacteria (LAB) addition. These variables were all chosen to help understand different substrates and or environments that could induce browning in Parmesan. This factorial design was tested for browning at three different ages (1, 2 and 3 months), which is an additional variable (table 1).

2.3 Phase I: In Cheese Model System Procedure

The parmesan wheels were shredded up using Hobart equipment and thoroughly mixed. The shredded cheese was separated into two trials conducted in triplicate, heat abuse vs no heat abuse. For each trial, 24 samples of cheese were weighed out (417.3 grams / treatment) and stomached for 30 seconds in a whirl pack bag at a 2:1 ratio (cheese: NaCl) with a 3.12% NaCl solution that was autoclaved at 121°C for 12 minutes to make a cheese slurry. In addition to the mixture of cheese and NaCl solution other variables were added based on the

factorial design. These variables include galactose (2.1g/cheese slurry), methylglyoxal (0.003g methylglyoxal/cheese slurry) and NSLAB (3.79ml NSLAB / cheese slurry). Samples in trials that did not include heat abuse were placed in 45°F, 55°F or 65°F coolers / incubators for aging. The three other trials were placed into 100F bread ovens for 48 hours for heat abuse. After heat abuse, the samples were placed in 45°F, 55°F or 65°F coolers / incubators for aging. Product was aged for three months. Product was sampled three times over three months for colorimeter results, three samples were created for each treatment and tested in the colorimeter 4 times (n=36 / each treatment). Fresh samples were taken to create a baseline for color in the colorimeter. After each sampling, product was sprayed with natamycin.

2.3.1 Phase I: Galactose Addition Calculation

Parmesan cheese that is 6-9 months of age typically contains .021 g galactose / 100 g Parmesan (Sandra C. Van Calcar, 2014). Fresh Mozzarella contains 0.8 g galactose / 100 g of cheese which should be slightly higher than young parmesan due to higher moisture. Therefore, 0.5 g galactose / 100 g cheese of galactose was added to each specified trial (see conversion calculation below);

$$\left(\frac{(417.13 \text{ g of Cheese})}{(100 \text{ g of Cheese})} \right) (0.50 \text{ g galactose}) =$$

2.1 g galactose per 417.13 g of cheese

2.3.2 Methylglyoxal Addition Calculation

This experiment called for 0.003g of methylglyoxal to be added to 417.13 grams of cheese (see conversion calculation below);

$$\begin{aligned}
 & (2.50 \text{ ug/ g methylglyoxal} * 417.13 \text{ g of cheese}) \\
 & = 1043.25 \text{ ug / g methylglyoxal per trial} \\
 & = 0.00104325 \text{ g methylglyoxal per trial}
 \end{aligned}$$

A 40% methylglyoxal solution was used

$$\begin{aligned}
 & \frac{0.00104325 \text{ g of methylglyoxal}}{0.40} \\
 & = 0.003 \text{ g methylglyoxal per trial}
 \end{aligned}$$

2.4 Milk Experiment for Positive Control: Freeze-dried Culture Propagation

Procedure

Eight single strains of different microorganisms with different abilities to produce browning were selected for a rapid screening test for browning. These cultures were purchased from ATCC or freeze dried in house. These strains included: *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactococcus lactis diacetyl* and *Lactobacillus helveticus*. All strains were freeze-dried, the ATCC procedure for opening and propagation of cultures was utilized. First the freeze-dried strains needed to be removed from their glass vial. The tip of the outer vial was heated over a Bunsen burner with the addition of a few water drops that would allow the glass to crack. While

over the Bunsen burner, a tip of a pen was used to strike the end of the vial. Once the glass from the tip was removed, the insulation and inner vial was removed with a sterile tweezer.

The bacterial strains were thawed upright. Immediately after thawing, the minicryovial was wiped down with alcohol and the entire contents were aseptically transferred to 10mL of MRS broth. The empty vials were then discarded. The bacterial strains were then incubated overnight anaerobically at their optimal temperatures:

- *Pediococcus acidilactici* at 38°C,
- *Lactobacillus paracasei*, *Lactobacillus Rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactococcus lactis diacetyl* and *Lactobacillus helveticus* at 42°C and
- *Lactobacillus brevis* at 30°C.

Note: NSLAB in phase I and II as well as adjuncts in phase II were propagated using the same procedure.

2.4.1 Milk Experiment for Positive Control: Phosphate Buffer and skim milk medium Procedure

For the rapid screening test (E.I. Shannon, 1969), one-hundred milliliters of reconstituted 13% non-fat skim milk was dispensed into ten 200-ml glass prescription bottles. Samples were autoclaved at 121°C for 12 minutes. Twenty-ml of phosphate buffer (pH of 7.0) were dispensed into ten 200-ml glass prescription bottles and autoclaved at 121°C for 12

minutes. The milk samples were aseptically transferred to the bottles containing the phosphate buffer. The ten phosphate-skim milk media were inoculated at a rate of 1% with the bacterial strains, methylglyoxal and a control (nothing added). Milk samples were visualized for browning via colorimeter after one week.

2.5 Phase II in Cheese Model System Procedure

All cheese slurries were made with a 2:1 ratio of cheese to an autoclaved NaCl solution (500g cheese / 250 ml NaCl solution in whirl pack bags). There were two solutions made, high and low. The high solution was made by mixing 4.62% NaCl with 1000ml of water to target a 3.0% salt concentration in the cheese slurry based on the average salt of the source Parmesan being 2.19%. The low solution was made by mixing 3.12% NaCl with 1000ml of water to target a 2.50% salt concentration in the cheese slurry. All NSLAB and adjuncts were grown up to 1.0×10^6 cfu/ml. For the cheese slurries that studied NSLAB or adjuncts, 0.1ml NSLAB or adjuncts / 11 g of cheese were added to the whirl pack containing the slurry. From there, all slurry solutions were checked for pH and standardized with lactic acid using a sterile pipettor and a pH meter. Once all the contents for all slurries were added, the whirl packs were mixed using a stomacher for 30 seconds. Additionally, one cheese slurry was made with the addition of just 0.003g of methylglyoxal / 417.3g of cheese as a positive control. Once all samples were thoroughly mixed, they were poured into four petri dishes, labeled, vacuum packed and stored at 55°F for aging. A reference color sample was collected using the LAB

setting of the colorimeter. Samples were color tested once every month for three months.

2.6 Hunter Lab Color Flex Calibration Procedure

The HunterLab Colorflex unit was turned on a display screen popped up. The word “standardize” was selected to begin the standardizing process. First, a black glass tile was placed on the reading surface of the unit and then a white tile. The unit automatically calibrated the darkness and whiteness range based on these tiles. These numbers for black and white were included below:

$$\text{Black} = (L^*)0.15 (a^*) 0.13 (b^*) -0.24$$

$$\text{White} = (L^*)92.98 (a^*) -1.33 (b^*) 0.53$$

After, scroll through options using the arrow button until the option LAB appears. This tells the Colorflex to generate $L^*a^*b^*$ values.

2.6.1 Phase I: Hunter Lab Color Flex Sample Run Procedure

After the Colorflex was calibrated, three petri dishes of cheese, without treatments, were collected and tested for baseline color using the colorimeter. Each petri dish was measured four times by dividing the plate into quadrants and rotating clockwise. The average reference color $L^*a^*b^*$, untreated fresh Parmesan included: $(L^*) 87.82 (a^*) -2.29 (b^*) 16.11$. Once the reference colors were collected four replicates of each trial (total of 24) were collected for 6

wheels for 3 ages (1, 2 and 3 months). The numbers collected were entered excel where ΔE was calculated.

3 Results and Discussions

3.1 Defective Market Cheese Natamycin Testing

Mold inhibitors are commonly used on aged products. Because of this, defective cheese samples were sent in to Cherney, a Microbiological service for natamycin in order to ensure the defect is not a result of mold inhibitor. This defect appears to be patchy brown and white spots spread throughout in the defective cheese. Samples from white and brown patches were collected and submitted for testing. For the brown patch 0.00 ppm of natamycin were detected and 1.09 ppm of natamycin was detected for the white patches. It does not appear that natamycin is causing browning because there was not a lot of natamycin present in the results. If the concentrations were higher in the brown cheese, one may conclude that natamycin can affect the chemistry of browning or be pigmented itself. Additionally, since there was more natamycin found in the white cheese, its likely it doesn't contribute to browning in the conditions it was made and stored. Therefore, natamycin can be a treatment used to prevent mold growth in the cheese slurries.

3.2 Colorimeter values as Dependent Variable

The colorimeter provides three variables that can then be correlated to treatments. These values, as aforementioned, are L^*a^*b . These values were analyzed to ensure that when a sample was considered brown that the L values lower and the a^*b values were higher than the samples deemed

white. In order to get one dependent variable, the $L^*a^*b^*$ values were converted into one single ΔE value that could then be correlated to variables tested in phase I and II. This single ΔE value for each treatment would be compared to all other treatments that had multiple variables tested as a factorial design and needed to have a ΔE greater than 4.5 to be deemed brown.

3.3 Phase I: Brown vs. White Colorimeter Studies Descriptive Statistics

The colorimeter values $L^*a^*b^*$ define color in terms of lightness vs darkness (L^*), red vs green (a^*) and yellow vs blue (b^*). Differences in L^* are depicted as a higher number being lighter and a lower number being darker. For the a^* value, a positive value is seen as more red, and a negative value is greener. Of course, the more positive the more negative a value the that color is expressed. As for the b^* value, a positive value indicates yellow and a negative value indicates blue. Again, the more positive or negative a value the more that color is expressed. The results from the colorimetry tests showed differences in the mean, standard deviation (StDEV), standard error (S.E.) and range from $L^*a^*b^*$ and ΔE (change in color) values comparing white and brown cheese as seen in table __. Brown colored cheese corresponds to lower L^* values and higher a^* and b^* values. This means that brown colored cheese is more dark, green and yellow compared to the white colored cheese. Brown colored cheese was defined as having a $\Delta E \geq 4.5$ based on the average of the entire population set. The variables studied in Phase I included, aging

temperature, galactose addition, methylglyoxal addition, NSLAB addition and heat abuse to understand the source of browning.

One-way ANOVAs were run comparing the entire population of samples to compare brown cheese vs white cheese based on Delta E, L*, a* and b* values to determine if brown and white cheese are statistically different in color based on their overall change in color, delta E, L*, a* and b*. Statistical differences were observed for color (p-value <0.05) comparing brown cheese vs white cheese for Delta E, L*, a* and b* as seen in graph 1. Brown cheese had a larger Delta E, which means it changed more from the reference product compared to the white cheese. As aforementioned, the white cheese had higher L* values and lower a* and b*, that were statistically different than the brown values.

3.4 Phase I: One-Way ANOVA White Cheese Delta E vs Variables

One-Way ANOVAs were run on white cheese data for Delta E vs variables (aging temperature, galactose addition, methylglyoxal addition, NSLAB addition and heat abuse. No statistical differences (p-values > 0.05) were observed between 'white' cheese Delta E vs Variables. Data from this sample set was removed from future analysis because these values do not correlate to browning of cheese. Additionally, this data supports the definition of browning to have a Delta E ≥ 4.5 , since there were not statistical with product less than this value (Table 5). The samples that were deemed white, most control samples, were typically aged at 45°F with no galactose, MG or

NSLAB addition and they were not heat abused. It is likely that product aged at 45°F would need to be aged longer in order to observe the browning defect. This is likely based on optimal growth conditions for bacteria proliferating in the cheese and their tendencies to break down proteins at this temperature. For browning to occur in parmesan at colder temperatures there needs to be an accumulation of amino acids that can react with reducing agents like methylglyoxal or sugars. The 45°F temperature may produce browning over time at a slower rate or there are different molecular pathways being utilized at this temperature and age. Over the 45°F temperature is the least extreme aging temperature treatment and would be considered greener in terms of its complexity relative to the other aging conditions. Based on the results, however, aging temperature is not the largest lever and is not linear (i.e., 55°F had more browning than 65°F). It makes sense that the samples that did not receive MG and NSLAB addition would have less pigmentation since MG is a known precursor to browning and the NSLAB added is galactose negative. Since the NSLAB is galactose negative, there will be an accumulation of galactose in the cheese for other NSLAB to utilize and potentially form pigmented compounds. Therefore, these samples likely did not have an accumulation of substrates available in the cheese because of the wash step employed in the cheese model system to remove extra carbohydrates. Additionally, the sample did not contain heat abuse which was tested to mimic heat adulteration in the market place or accelerated ripening and is a warmer condition that could speed up the reaction of molecules like the maillard

reaction. This did only, however, test cheese up until 3 months and browning may occur overtime and was not accounted for here. With this in mind, however, it can be assumed that in this condition browning does not occur within three months. In an applied sense, manufacturers could produce parmesan at a lower temperature, utilize a wash step and use *Lb. bulgaricus* as part of their mixed strain. However, there would be investments needed to do this in terms of storage and capacity because maturation of cheese would take longer to achieve the desired flavor for parmesan and its complexity. It is unlikely a manufacturer would want to do this. Additionally, it would need to be verified that browning does not occur throughout storage and flavor analysis would need to be conducted to understand if the desirable flavors are achieved in a lower aging temperature. It should be assumed the flavor profile would be different.

3.5 Phase I: One-Way ANOVA Brown Cheese Delta E vs Variables

One-way ANOVAs were run for Brown cheese Delta E vs. aging temperatures, galactose addition, methylglyoxal addition, NSLAB addition to understand what is driving color differences. Based on the data, all variables had statistically significant differences in browning (Table 6).

As seen in graph 2, the different variables have different effects on the Delta E of brown cheese. To summarize, aging temperature increases the average Delta E, but more so at 55°F. There are no real perceivable differences between the addition of galactose in the Phase I study. The addition of methylglyoxal

and NSLAB increase the average Delta E. Additionally, product that received heat abuse, had increased average Delta E. Product with the variables were studied at three different ages: 1, 2 and 3 months. As seen below, the average Delta E increases with increasing age. The regression model that best depicts the data for Phase I includes $\Delta E = \text{Aging Temperature} + \text{Methylglyoxal} + \text{NSLAB} + \text{Heat Abuse} + \text{Age}$. The adjusted R-squared number for this equation was 17.78%, which is a fairly low number. As aforementioned, aging temperature does not appear to be linear. It seems that a browning at a specific temperature for low temperature browning is likely driven more by either the chemistry that is induced at this temperature or the metabolic tendencies of the microflora present in the cheese at this age. However, browning could significantly increase in the other aging temperatures (i.e., 45°F or 65°F) with more age depending on the concentrations of molecules produced at those temperatures. Additionally, browning was observed at all temperatures, but was more pronounced at 55°F; and it can be assumed browning pigmentation will remain in cheese over time since the reaction from aminoacetone to pyrazines is not reversible. However, could pyrazines be further converted to other molecules is a fair question to ask and learn more about. For reasons mentioned earlier, MG addition causes browning and was deemed a precursor to pyrazine formation in other studies done at UW-Madison. Additionally, the galactose negative NSLAB addition to the cheese would cause an accumulation of galactose in the cheese for another microflora to utilize. It is unsure, why galactose addition was not statistically different for browning compared to

those samples that did not have galactose addition. However, the DeltaE values for both were greater than 4.5. This would suggest that galactose may or may not cause browning and there are greater driving forces or tendencies for galactose to be utilized in other ways, for example, energy creation. As opposed to being utilized for phosphate recycling. However, in the presence of MG there is a greater likelihood for that molecule to be converted to pyrazines because of its ability to reduce amino acids that are accumulating in aged cheeses because of proteolysis and energy creation of NSLAB proliferation. Additionally, MG is more reactive than galactose because it has one more reactive functional group. It is possible, overtime that galactose addition could have had more of an effect, but this was an extreme case of adding galactose to cheese. It makes sense that heat abuse caused more browning because heat increases the kinetics in the cheese making reactions between molecules happen quickly. Also, the bacteria present in parmesan are NSLAB or LAB thermophiles that optimally grow at warmer conditions.

3.6 Phase I: Brown Cheese Delta E vs Aging Temperature (45°F, 55°F, 65°F)

Statistical differences were observed between aging temperatures (45°F, 55°F and 65°F) at different ages as seen in graph ___. The three ages studied include one, two and three months. At one month, 45°F was statistically different than 55°F and 65° with a mean Delta E of 8.11, 8.52 and 8.70, respectively. Therefore, 45°F temperature is least brown and 65°F the brownest of the sample set. This would suggest that increased temperature would induce browning. At two months, no statistical differences were observed between

temperatures 45°F (Delta E, 8.95), 55°F (Delta E, 9.22) and 65°F (Delta E, 9.25). However, the Delta E values after 2 months have increased since the previous month. At three months, statistical differences were observed between all temperatures that had different degrees of browning; 45°F (Delta E, 8.32), 55°F (Delta E, 10.67) and 65°F (Delta E, 9.34). After 3 months, product aged at 55°F had the greatest amount of color change, thus browning. Based on increasing Delta E values, as product ages there is an increased amount of browning. However, it appears that browning formation may be induced at a specific temperature. After 3 months, product aged at 55°F had the highest amount of browning. As aforementioned in the methods section, most manufactures age Parmesan between 50-55°F. This means, that the common aging temperature may exacerbate browning in Parmesan. Perhaps aging Parmesan at a lower temperature, at 45°F, would slow the browning formation down, but could delay ripening time and flavor development. Additionally, the delta E did increase at 45°F, and would likely have visible browning still. Aging at elevated temperatures, 65°F aging temperature did not produce as much browning but is still high enough to notice visible different. Therefore, aging temperature may help reduce or slow down browning formation, it does not eliminate browning to a significant level on its own. In phase II, additional studies will focus on aging product at 55°F but adding in different variables to alter the parmesan environment (Graph 3). The fact that statistical differences were observed in different ways at different ages, suggests that there is a lot of chemistry and microbiology that is dependent on temperature. Certain

compounds are produced based on the chemistry and microbiology that is induced at a specific temperature. Based on this, accelerated ripening is not recommended because it appears that browning will occur, but different temperatures will have different rates and degrees of browning. These aging temperatures could be studied at a longer time to better understand their impact at sale age.

3.7 Brown Cheese Delta E vs Galactose Addition

Brown cheese samples Delta E values were correlated with and without galactose addition. No statistical differences were observed when comparing these two treatments, but browning formation increased with age as seen in graph 4. This suggested that increased amount of galactose introduced into the cheese does not induce browning, but browning will occur overtime. As aforementioned, galactose accumulates in parmesan because of lactose being broken down into glucose and galactose. Glucose is readily used, and the residual galactose is left to be metabolized by NSLAB or bacteria that can break down galactose. Therefore, galactose can be a precursor to browning, but an increased amount of galactose does not increase browning. Therefore, understanding how galactose is metabolized to so it so not converted overtime to different compounds leading to browning. This is an area for refinement and optimization in the phase II study.

3.8 Brown Cheese Delta E vs Methylglyoxal Addition

Samples with methylglyoxal addition had statistically larger Delta E values at all ages, thus more browning; one month (Delta E, 8.66), two months (Delta E, 9.48) and three months (Delta E, 9.85). Again, Delta E values increased with time suggesting browning increases with time as seen in graph 5.

Based on the data, delta E values increased with or without methylglyoxal addition. This suggests that more than one factor contributes to browning. The addition of methylglyoxal vs no methylglyoxal addition is statistically different and increases over time as seen in graph 6. Therefore, the addition of methylglyoxal regardless of other factors, increases the occurrence of browning. Based on the literature, methylglyoxal is known to be a precursor to browning and create ,3,5-trimethylpyrazine and 3,5-diethyl-2-methylpyrazine, which are brown pigments. Therefore, understanding how methylglyoxal is produced to counteract its development is essential, and will be studied more in phase II.

3.9 Brown Cheese Delta E vs NSLAB Addition

The NSLAB added during phase I was *Lactobacillus bulgaricus*, which is galactose negative. Statistical differences were observed with the addition of NSLAB at two months only. Samples with NSLAB addition at two months had higher Delta E values of 9.33, thus more browning (graph 7). However, Delta E values increased overtime with and without NSLAB addition. This suggests, the NSLAB other than *Lactobacillus bulgaricus* causes browning.

3.10 Brown Cheese Delta E vs Heat Abuse

During Phase I, some product was heat abused and was held at 100°F for 48 hours. Heat abuse was studied to understand if browning formation can be induced by heat. As seen in the aging temperature study, aging temperature does increase the average Delta E. This would suggest that higher temperature over a long period of time would increase browning. However, heat abuse studies a short period of time of exposure to heat, at an exacerbated level. Based on the data, increased temperature for a short period of time does induce browning as seen in graph 8.

The Delta E values were compared for aging temperature and age with heat abuse. The largest average Delta E value was 12.26 observed with 55°F aging temperature for 3 months with heat abuse. As seen in the graph 9 below, consistent with what was observed with aging temperature, the 55°F aging temperature has the highest degree of browning and after 3 months with heat abuse. Therefore, both heat abuse (short term and aging at 55°F (long term) can increase the rate / production of browning.

3.11 Milk Experiment: Positive Control

Increased browning was observed with the addition of NSLAB in the cheese model system. The NSLAB added to the cheese model system was *Lactobacillus bulgaricus* because it's a common starter strain in addition to *Lactobacillus helveticus*. A milk experiment was conducted to identify a

positive control microorganism (common starters and NSLAB) that would produce browning and microorganism ‘adjuncts’ who may produce less browning. Methylglyoxal has been identified as the precursor to browning, therefore a positive control microorganism would have the tendency to produce methylglyoxal. As stated in the literature, methylglyoxal production is induced by an influx of glucose, glucose-6-phosphate, lactate or glycerol. These substrates are then converted to DHAP and then to methylglyoxal via methylglyoxal synthase. Methylglyoxal production is regulated by the levels of DHAP, the more DHAP the more methylglyoxal. Therefore, regulating the production of methylglyoxal is important and will be studied more in Phase II. To move to phase II, some other microorganisms were tested as adjuncts to see if they produced less browning in the same environment and addition of methylglyoxal (serves as another positive control). These microorganisms may tend to utilize the DHAP pathway less and could out compete bacteria that need to utilize the DHAP pathway for survival. Much like adjuncts are added to improve flavor, there may be potential opportunities to add them to reduce/eliminate appearance defects such as browning.

A total of eight microorganisms were inoculated in milk and then analyzed for Delta E color differences. Additionally, methylglyoxal was added to milk as well. A one-way ANOVA and Duncan grouping was conducted on the sample set comparing Delta E vs bacteria source, as seen in table ___. Notice, the methylglyoxal sample had the highest Delta E value, highest total color change. Whereas the control, nothing but milk, had the lowest change in color.

Additionally, *Lactobacillus helveticus* had a larger Delta E than most and is a common starter for Parmesan and serves as a good positive control. In fact, it was the starter used for the cheese model system. For this reason, *Lactobacillus helveticus* was studied more in phase II. *Lactobacillus bulgaricus* had the lowest Delta E and is also a common starter and was the NSLAB added to phase I studies. This suggests that additional NSLAB are more responsible for browning, than if *Lactobacillus bulgaricus* was added as a starter culture. *Lactobacillus brevis* and *Lactobacillus fermentum*, in other studies, have been identified as potential microorganisms to reduce browning by their abilities to modulate concentrations of methylglyoxal (Gandhi NN, 2018). This other study focused on how these microorganisms reduced methylglyoxal to 1,2-propanediol which decreased methylglyoxal concentrations and prevented browning. In addition to these findings above, Phase II studied *Lactobacillus plantarum*, a known probiotic and strong competitive microorganism used in meats to outcompete foodborne pathogens. As seen in table 7, *Lactobacillus plantarum* produced lower a lower Delta E (less than 4.5). Additionally, *Lactococcus lactis diacetylactis* had lower Delta E (less than 4.5) and was studied more in phase II. *Lactococcus lactis diacetylactis* is a known flavor producer, particularly for producing diacetyl. Diacetyl is created through the glycerol-3-phosphate pathway. If *Lactococcus lactis diacetylactis* is added to Parmesan, it could potentially outcompete NSLAB and have a higher tendency to utilize the glycerol-3-phosphate pathway as oppose to the dihydroxyacetone phosphate (DHAP) which is known to produce methylglyoxal.

3.12 Phase II: Brown vs. White Colorimeter Studies Descriptive Statistics

For phase II, colorimeter data was collected for all samples. Again, the colorimeter values $L^*a^*b^*$ represent the relative lightness and colors of red vs green and yellow vs blue as stated earlier. Again, in Phase II, samples deemed brown had $\Delta E \geq 4.5$ based on the average of the entire population set. As seen in table 8, the average ΔE for brown cheese is lower than Phase I and the average is less than 4.5. Additionally, the number of observations of brown cheese decreased in Phase II. This suggests, the treatments used in Phase II has helped reduce browning. The $L^*a^*b^*$ values for brown cheese are also very similar to the white cheese, suggesting an overall decrease in brown samples. Also, the lowest ΔE for white cheese is zero, meaning no color change and was observed in treatments with the addition of both *Lactobacillus helveticus* and *Lactobacillus plantarum*. The treatments studied in Phase II included modified salt, modified pH, NSLAB addition (positive control) and use of adjuncts to help reduce browning all aged at 55°F.

3.13 Phase II: One-way ANOVA of Delta E vs Treatment

To understand what variables produced less browning, both brown and white cheese data was kept for Phase II analysis to compare the ΔE values to treatment. As seen in table 9, statistical differences were observed with NSLAB addition, adjunct addition, high salt, low pH, methylglyoxal addition

and galactose addition. Of those variables, presence of NSLAB, adjunct, higher salt and lower pH helped reduce browning in Phase II. In phase II, all treatments other than galactose and MG addition provided less browning than the control samples. This is likely due to the combination of treatment and their effects on each other that resulted in less browning. Remember, that phase II focused on the reduction of browning and variables postulated for this that could easily be applied in cheese making. The NSLAB studied in phase II, was *Lb. helveticus* which was also identified as a positive control microorganism in milk. It is galactose positive, so it made sense being a positive control microorganism because it can breakdown galactose which is typically the reducing sugar that accumulates in cheese. It appears, however, *Lb. helveticus*, is more susceptible to the environment rather than having a great tendency to produce browning. Therefore, *Lb. helveticus* can produce browning or produce the substrates for browning because it can metabolize galactose that can be converted to DHAP. However, under certain conditions like pH and salt or in the combination with other dominant microflora it has more of a tendency to create compounds that do not potentially create pyrazines. This makes sense, because milk and cheese have different compositions (i.e., moisture, salt and pH). Milk is a more neutral pH of 6.7 vs a pH of 5.20 or 5.60 in the cheese model system. Additionally, the salt concentration in the cheese model system was much higher than in milk and the moisture was much higher in milk than in cheese. This further supports that the chemistry and microbiology in the cheese is very sensitive and elusive when it comes to browning formation. Additionally, *Lb. helveticus* was either

outcompeted by other adjuncts added or they had a symbiotic relationship. From the results, a theory would be that *Lb. helveticus* breaks down galactose to DHAP or G3P and the adjuncts added had stronger competing forces that allowed for DHAP to be isomerized to G3P and there was a greater conversion to different molecules that yield energy. Especially, since the adjuncts used are known to outcompete other bacteria likely because they out create energy and a means to live in fermented foods.

3.14 Phase II: Main Effects for Sample Population vs Delta E

As seen in graph 10, a main effects plot was generated to understand the impact of different treatments and their effect on Delta E (average). Delta E average was lower in product 'deemed' white because it had a Delta E less than 4.5. Again, with increasing age increased browning is observed, however, to a less extent in Phase II. *Lb. helveticus*, was identified as a positive control in milk, but seemed to produce less browning than the control product. Both *Lc. lactis diacetylactis* and *Lb. plantarum* had less browning than the control product, this suggests these adjuncts helped reduced browning. In some cases, treatments had both *Lb. helveticus* and an adjunct added to it; reduction in browning was observed in these treatments and will be discussed more later. A higher salt concentration and lower pH had less browning than the normal condition. The normal condition was the same salt % and pH as phase I. Both methylglyoxal and galactose

addition had higher degrees of browning. Product deemed ‘white’ had lower degrees of browning than product ‘deemed’ brown.

3.15 Phase II: Treatment combination and Reduction of Browning

In phase II, combinations of variables were applied to the cheese model system to understand their impact on browning and these were called ‘treatments’. The combination on variables were studied to understand if the treatments had a larger decrease in browning than just the variable itself. The treatment combinations included a control (no addition of NSLAB, adjunct or modified salt of pH), NSLAB addition, Adjunct addition, and modified % salt (high 3% vs normal 2.19%) and pH (low 5.2 vs normal 5.6). Below is table 10, which depicts the average Delta E for different treatment combinations. Notice, the table is grouped 1-8 and each category (besides category 1), has one treatment in bold which depicts the lowest average Delta E. Of all categories, the treatment with the addition of *Lb. helveticus* and *Lb. plantarum* only had the lowest average Delta E, 0.92. This would suggest that *Lb. plantarum* is successful at out competing *Lb. helveticus*, the positive control identified in the milk study. Additionally, *Lb. helveticus* combination with high salt and low pH alleviated browning producing an average Delta E of 1.97. When *Lb. helveticus* is combined with *Lc. lactis diacetylactis* a high salt and low pH environment is needed to help reduce browning (avg. Delta E 1.89). Lastly, the treatment with *Lb. helveticus*, *Lb. plantarum* and *Lc. lactis diacetylactis* helped reduce

browning too, Delta E 2.17. Therefore, *Lb. helveticus* tendency to produce browning can be controlled through use of adjuncts and or modified environments of high salt and low pH. It appears that *Lb. plantarum* is more effective adjunct than *Lc. lactis diacetylactis*. *Lc. lactis diacetylactis* seems to reduce browning only in the presence of *Lb. helveticus* (double dose, since starter culture was *Lb. helveticus*), which may suggest a symbiotic relationship. Whereas, *Lb. plantarum* will reduce browning with and with the addition of *Lb. helveticus*. For example, *Lb. plantarum* with high salt produced an average Delta E of 1.73. All the Delta E values are less than the control (no addition of NSLAB or adjunct or modified environment of %salt and pH) where the Delta E is 2.60. The average Delta E for just *Lb. helveticus* is 2.51 which is very similar to the control. When adjuncts were not used and just modified environments were used on the control, the lower pH of 5.2 helped to reduce browning. Therefore, the use of adjuncts and modified environments have helped to reduce the browning defect associated with the cheese model system and product with a positive control microorganism.

4. CONCLUSION

Phase I helped identify what variables contributed to browning which included: aging temperature (55°F), addition of methylglyoxal, NSLAB addition and heat abuse. All samples in phase one were tested for color changes via colorimeter at 1, 2 and 3 months. With increasing age, there was an increase Delta E thus browning. There was increased browning observed at 55°F that had a Delta E of 9.22, which interestingly the most typical aging temperature for Parmesan. Therefore, all product studied in

phase II were aged at 55°F to understand if variables tested reduced browning at a temperature most browning was observed. The addition of methylglyoxal correlated with increased browning and had an average Delta E of 9.85 after 3 months.

The NSLAB added in Phase I was *Lb. bulgaricus*. Statistical differences were observed for browning at only two months with an average Delta E of 9.33. Because of this, the impact of NSLAB addition was not fully understood. Since browning increased overtime, this suggests that some other NSLAB is responsible for producing intermediates for browning. Increased Delta E was observed in both heat abuse and overtime with age. Parmesan cheese samples were heat abused by placing them in 100°F for 48 hours and then stored at 45°F, 55°F and 65°F. Of the samples heat abused, product aged at 55°F had the highest Delta E 12.26 at 3 months. This suggests that increased browning is observed at specific temperatures (55°F) and maybe exacerbated if heat abused prior.

Phase II studied variables thought to help reduce browning that could be done during cheese making using product from the cheese model system. The variables studied in Phase II included addition of NSLAB (*Lb. helveticus*), addition of adjuncts (*lb. plantarum* and *Lc. lactis diacetylactis*), modified environment of high %salt and low pH. The microorganisms studied in phase II were selected based off a milk experiment. This milk experiment tested eight microorganisms to observe the degree of browning produced after a week. The milk experiment identified *Lb. helveticus* as a positive microorganism because it produced an average Delta E of 5.6. Both *Lc. lactis diacetylactis* and *Lb. plantarum* produced lower Delta E 3.6 and 3.3, respectively. These were identified as negative controls (adjuncts) for phase II to test their ability to

limit browning with and without a positive control microorganism and in modified environments of high salt and low pH. The cheese model system had a % salt of 2.19 and a pH of 5.6 which was modified to 3.00% for high salt and 5.2 pH for phase II. Reduction and browning were achieved using both adjuncts and modified environments of high salt and low pH. The greatest reduction in browning was observed when *Lb. helveticus* and *Lb. plantarum* were added together as a treatment, Delta E of 0.92. This suggests that *Lb. plantarum* as an adjunct outcompetes *Lb. helveticus* and has less of a tendency to produce browning intermediates, like methylglyoxal. The adjunct, *Lc. lactis diacetylactis* also reduced browning, average Delta E of 1.89 in the presence of *Lb. helveticus*, high %salt and low pH. If *Lb. helveticus* is used as the starter and no adjuncts are used, a lower pH target of 5.2 can help reduce browning, average Delta E of . In phase I, increased browning was observed overtime, mostly at 3 months. The standards of identity for Parmesan is to be aged at least 10 months. In phase II, browning did not increase significantly from 1-3 months. However, if browning increases slowly over time the use of certain adjuncts can help reduce browning. Because of this, additional studies could be conducted to understand if increasing the amount (%) of adjunct added could help withstand browning overtime.

To summarize, increased browning was observed over time, the most after 3 months, at 55°F in the presence of methylglyoxal and heat abuse. Methylglyoxal is produced via DHAP pathway and converted to specific pyrazines that are brown. Therefore, controlling their production using identified adjuncts with modified environments of low pH and high salt were studied in Phase II. A positive microorganism and adjuncts were identified through a milk experiment. These microorganisms were then tested in

different combinations (treatments) to understand their interactions to induce or reduce browning. The most effective way to reduce browning was achieved using adjuncts a modified environment of high salt and low pH.

Lastly, it is important to understand the impacts of the recommended variables to reduce browning. The use of adjuncts helped to reduce browning, likely due to their abilities to outcompete other microflora in the cheese and create a concentration of G3P environment that could be metabolized to more favorable compounds for flavor and color. However, could there be any ramifications in terms of excessive flavor development or other catabolic reactions as a result of an influx of these flavor molecules. Everything seems to work in an equilibrium, therefore, could other undesirable compounds be created or off flavors as a result of lactic acid accumulation. You could also be hindering the microflora diversity in cheese that maybe responsible for other metabolic reactions for fats or proteins that are quintessential for parmesan target flavor. By having and under-diversified microflora in Parmesan there could be other unintended consequences that need to be studied further. Additionally, increasing the targeted salt in Parmesan helped reduce browning, likely due to its antimicrobial abilities. When you think of this, however, again you maybe hindering the proliferation of bacteria or metabolic pathways that could cause browning; but also hindering metabolic pathways that produce flavors intended for parmesan. Because of this, manufactures may need to age cheese longer in order to get the same intensity and diversity of flavor. On the other hand, salt enhances flavor perception. Therefore, increasing salt may give the perception of more flavor and enhance certain aspects of flavor. The flavor may become peakier and less complex and robust as a result, but

maybe more desired by certain manufactures; this all depends on what the manufactures target for flavor is. Also, decreasing the pH in cheese will alter the metabolic pathway and chemical pathways because enzymes will work at certain pH. Additionally, a more acidic environment may not be conducive to certain microflora, and again the diversity and concentration of some microflora will certainly change. Lastly, there were combination effects observed in phase II and those are harder to explain and understand with out understanding the compounds produced or knowing what's going on in the cheese from a microbiology or chemistry standpoint. Some of this could be further understood by using things like gas chromatography, HPLC or metagenomics. It can, however, be concluded that different combinations of variables studied in phase II have different effects on browning, but most importantly reduced browning.

3. Further Readings

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TABLES

Test 1: No Heat Abuse (4 Wheels)					Test 2: Heat Abuse (4 Wheels)				
<i>Treatment 1 (Starter Mix 1, No Heat Abuse)</i>					<i>Treatment 1 (Starter Mix 1, Heat Abuse)</i>				
<i>24 Samples/one wheel</i>					<i>24 Samples/one wheel</i>				
<i>Trial Number</i>	<i>Aging Temperature</i>	<i>Galactose</i>	<i>Methylglyoxal</i>	<i>NSLAB</i>	<i>Trial Number</i>	<i>Aging Temperature</i>	<i>Galactose</i>	<i>Methylglyoxal</i>	<i>NSLAB</i>
1	45	Y	Y	Y	1	45	Y	Y	Y
2	45	N	N	N	2	45	N	N	N
3	45	Y	Y	N	3	45	Y	Y	N
4	45	N	N	Y	4	45	N	N	Y
5	45	Y	N	N	5	45	Y	N	N
6	45	N	Y	Y	6	45	N	Y	Y
7	45	Y	N	Y	7	45	Y	N	Y
8	45	N	Y	N	8	45	N	Y	N
9	55	Y	Y	Y	9	55	Y	Y	Y
10	55	N	N	N	10	55	N	N	N
11	55	Y	Y	N	11	55	Y	Y	N
12	55	N	N	Y	12	55	N	N	Y
13	55	Y	N	N	13	55	Y	N	N
14	55	N	Y	Y	14	55	N	Y	Y
15	55	Y	N	Y	15	55	Y	N	Y
16	55	N	Y	N	16	55	N	Y	N
17	65	Y	Y	Y	17	65	Y	Y	Y
18	65	N	N	N	18	65	N	N	N
19	65	Y	Y	N	19	65	Y	Y	N
20	65	N	N	Y	20	65	N	N	Y
21	65	Y	N	N	21	65	Y	N	N
22	65	N	Y	Y	22	65	N	Y	Y
23	65	Y	N	Y	23	65	Y	N	Y
24	65	N	Y	N	24	65	N	Y	N

Table 1. Phase I Factorial Design

Phase II Sub Variables			
<i>NSLAB</i>	<i>Adjunct</i>	<i>Salt (adjusted by NaCl)</i>	<i>pH (adjusted by lactic acid)</i>
<i>Lactobacillus helveticus</i> (positive control)	<i>Lactobacillus plantarum</i>	High (3.0%)	Low (5.2)
None	<i>Lactococcus lactis diacetyl</i>	Normal (2.5%)	Normal (5.6)
	None		

Table 2. Phase II Sub Variables

Phase 2: Factorial Design				
	NSLAB	Adjunct	Salt	pH
1	N1	A1	Normal	Normal
2	N1	A1	Normal	Low
3	N1	A1	High	Normal
4	N1	A1	High	Low
5	N1	A2	Normal	Normal
6	N1	A2	Normal	Low
7	N1	A2	High	Normal
8	N1	A2	High	Low
9	N1	NA	Normal	Normal
10	N1	NA	Normal	Low
11	N1	NA	High	Normal
12	N1	NA	High	Low
13	NA	A1	Normal	Normal
14	NA	A1	Normal	Low
15	NA	A1	High	Normal
16	NA	A1	High	Low
17	NA	A2	Normal	Normal
18	NA	A2	Normal	Low
19	NA	A2	High	Normal
20	NA	A2	High	Low
21	NA	NA	Normal	Normal
22	NA	NA	Normal	Low
23	NA	NA	High	Normal
24	NA	NA	High	Low

Table 3. Phase II Factorial Design

Phase I Descriptive Statistics: Brown vs White Colorimeter Data and Delta E							
		N	Mean	Step	S.E	Range (low)	Range (high)
Brown	L*	1679	82.06	1.79	0.00110	74.29	86.23
	a*	1679	-2.02	0.84	0.00050	-3.87	5.59
	b*	1679	22.83	1.94	0.00116	17.66	32.07
	Delta E	1679	9.02	2.17	0.00129	4.54	18.91
White	L*	49	85.00	0.88	0.018	83.85	86.74
	a*	49	-2.60	0.60	0.012	-3.5	-1.14
	b*	49	18.15	0.84	0.017	17.16	20.22
	Delta E	49	3.71	1.00	0.010	2.50	4.46

Table 4. Phase I Descriptive Statistics: Brown vs White Colorimeter Data and Delta E

One-Way ANOVA: White Cheese Delta E vs. Variables	
Variable	Statistically significant (p-value <0.05)?
Aging Temperature (45°F, 55°F, 65°F)	No
Galactose Addition	No
Methylglyoxal Addition	No
NSLAB Addition	No
Heat Abuse (Y/N)	No

Table 5. Summarization of the One-Way ANOVAs of White cheese Delta E vs. variables independently but grouped by age.

One-Way ANOVA: Brown Cheese Delta E vs. Variables	
Variable	Statistically significant (p-value <0.05)?
Aging Temperature (45°F, 55°F, 65°F)	Yes
Galactose Addition	No
Methylglyoxal Addition	Yes
NSLAB Addition	Yes
Heat Abuse (Y/N)	Yes

Table 6. Summarization of the One-Way ANOVAs of Brown cheese Delta E vs. variables independently but grouped by age.

Means with the same letter are not significantly different.			
Duncan Grouping	Mean	N	Bacteria Source
A	29.3382	4	Methylglyoxal Positive
B	6.1013	4	Lactobacillus rhamnosus
C	5.6346	4	Lactobacillus helveticus
D	4.4631	4	Lactobacillus casei
E	3.6393	4	Lactococcus lactis
E			
E	3.4580	4	Lactobacillus pediococcus
E			
E	3.3410	4	Lactobacillus plantarum
F	2.8416	4	Lactobacillus brevis
G	1.5516	4	Lactobacillus bulgaricus
H	0.5208	4	Control

Table 7. Average Delta E for Various Microorganisms in Milk Experiment

Phase II Descriptive Statistics: Brown vs White Colorimeter Data and Delta E							
		N	Mean	Step	S.E.	Range (low)	Range (high)
Brown	L*	65	85.66	2.63	0.04	77.12	86.76
	a*	65	-1.98	0.42	0.006	-2.98	-0.56
	b*	65	18.33	1.44	0.022	14.39	21.27
	Delta E	65	3.67	2.31	0.04	4.51	11.78
White	L*	246	85.67	2.5	0.01	83.50	89.07
	a*	246	-1.99	0.41	0.002	-2.97	-1.47
	b*	246	18.25	1.40	0.01	15.37	20.39
	Delta E	246	3.57	2.21	0.01	0.00	4.48

Table 8. Comparing brown vs white colorimeter data. Reference color of average brown sample and while sample color. *Brown equal to Delta E ≥ 4.5

One-Way ANOVA: Brown Cheese Delta E vs. Variables	
Variable	Statistically significant (p-value <0.05)
Age (1, 2, 3 month)	No
NSLAB (<i>Lb. helveticus</i>) Addition	*Yes
Adjunct Addition (<i>L. lactis</i> and <i>Lb. plantarum</i>)	*Yes
High Salt (higher than phase 1)	*Yes
Low pH (lower than phase 2)	*Yes
Methylglyoxal Addition	Yes
Galactose Addition	Yes
Color (Brown vs White)	Yes

*Less browning associated with this Variable.

Table 9. Statistical analysis for Phase II variables

Phase II: Treatment Combination vs Delta E (avg.)			
Treatment	Sample ID	Delta E	S.E.
Control	1	2.60	0.19
<i>Lb. helveticus</i>	2	2.51	0.19
<i>Lb. helveticus</i> + high salt	2A	4.20	0.19
<i>Lb. helveticus</i> + low pH	2B	2.10	0.19
<i>Lb. helveticus</i> + high salt + low pH	2C	1.97	0.19
<i>Lb. helveticus</i> + <i>Lb. plantarum</i>	3	0.92	0.19
<i>Lb. helveticus</i> + <i>Lb. plantarum</i> + high salt	3A	3.29	0.19
<i>Lb. helveticus</i> + <i>Lb. plantarum</i> + low pH	3B	2.02	0.19
<i>Lb. helveticus</i> + <i>Lb. plantarum</i> + high salt + low pH	3C	4.10	0.19
<i>Lb. helveticus</i> + <i>Lc. lactis diacetylactis</i>	4	3.43	0.19
<i>Lb. helveticus</i> + <i>Lc. lactis diacetylactis</i> + high salt	4A	3.89	0.19
<i>Lb. helveticus</i> + <i>Lc. lactis diacetylactis</i> + low pH	4B	2.80	0.19
<i>Lb. helveticus</i> + <i>Lc. lactis diacetylactis</i> + high salt + low pH	4C	1.89	0.19
<i>Lb. helveticus</i> + <i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i>	5	2.17	0.09
<i>Lb. helveticus</i> + <i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i> + high salt	5A	3.57	0.10
<i>Lb. helveticus</i> + <i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i> + low pH	5B	2.42	0.09
<i>Lb. helveticus</i> + <i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i> + high salt + low pH	5C	2.99	0.09
<i>Lb. plantarum</i>	6	3.52	0.19
<i>Lb. plantarum</i> + high salt	6A	1.73	0.19
<i>Lb. plantarum</i> + low pH	6B	4.10	0.19
<i>Lb. plantarum</i> + high salt + low pH	6C	2.90	0.19
<i>Lc. lactis diacetylactis</i>	7	3.49	0.19
<i>Lc. lactis diacetylactis</i> + high salt	7A	2.95	0.19
<i>Lc. lactis diacetylactis</i> + low pH	7B	3.22	0.19
<i>Lc. lactis diacetylactis</i> + high salt + low pH	7C	3.50	0.19
<i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i>	8	3.51	0.09
<i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i> + high salt	8A	2.34	0.09
<i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i> + low pH	8B	3.70	0.09
<i>Lb. plantarum</i> + <i>Lc. lactis diacetylactis</i> + high salt + low pH	8C	3.20	0.09

Control + high salt	9A	2.80	0.19
<i>Control + low pH</i>	9B	1.94	0.19
<i>Control + high salt + low pH</i>	9C	2.70	0.19

Table 10. Phase II Treatment Combination vs Delta E (avg.)

FIGURES

NSLAB

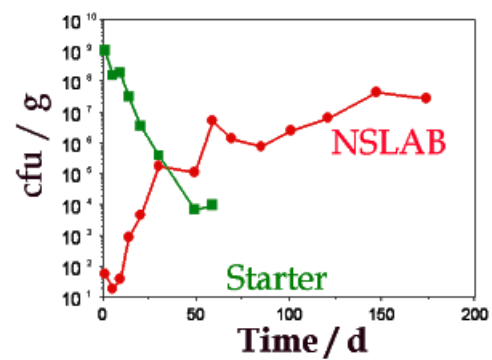


Figure 1. Starter vs NSLAB growth (CFU/g) overtime

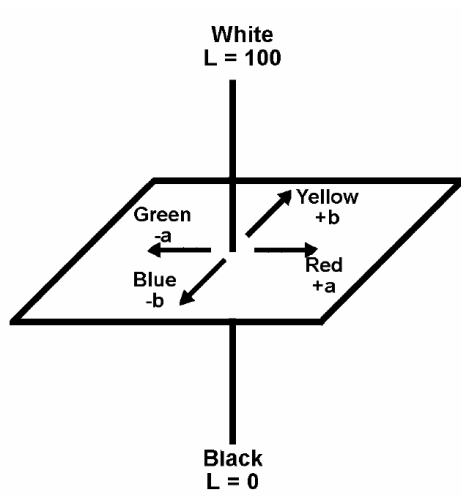


Figure 2. LAB color coordinates (Labs, 2018)

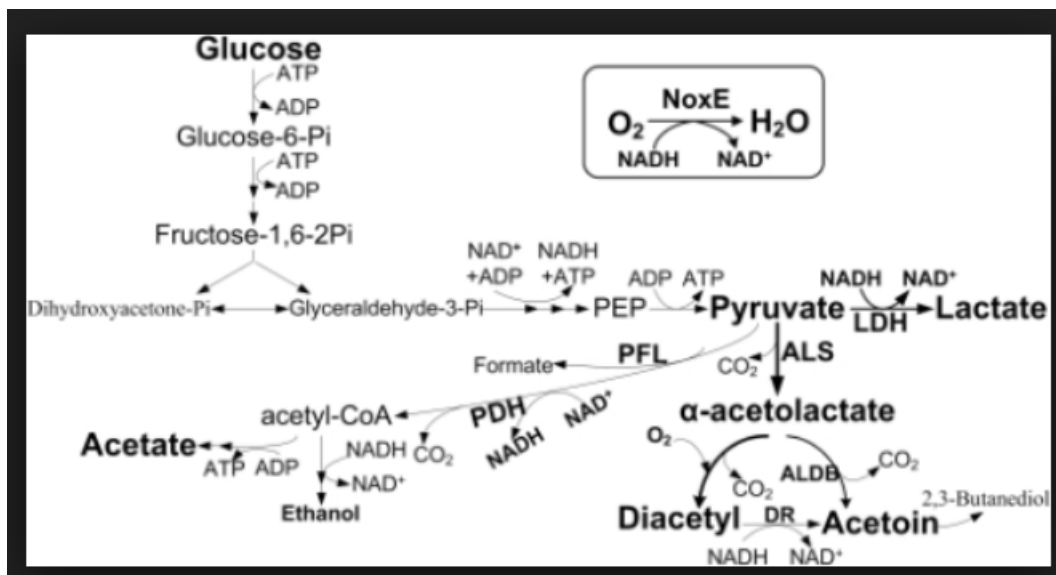


Figure 3. The G3P Pathway (Guo T, 2012)

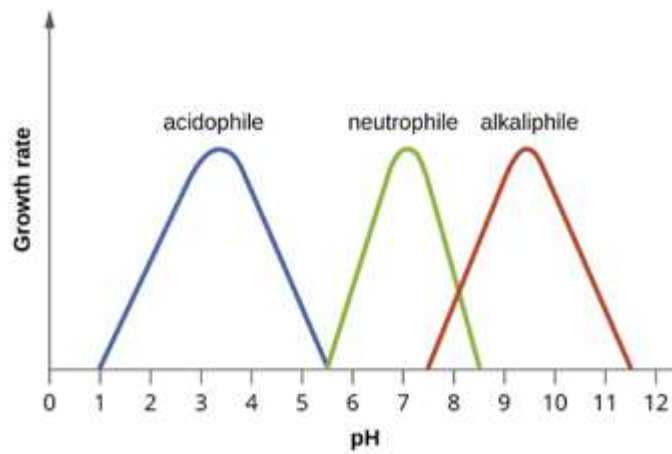
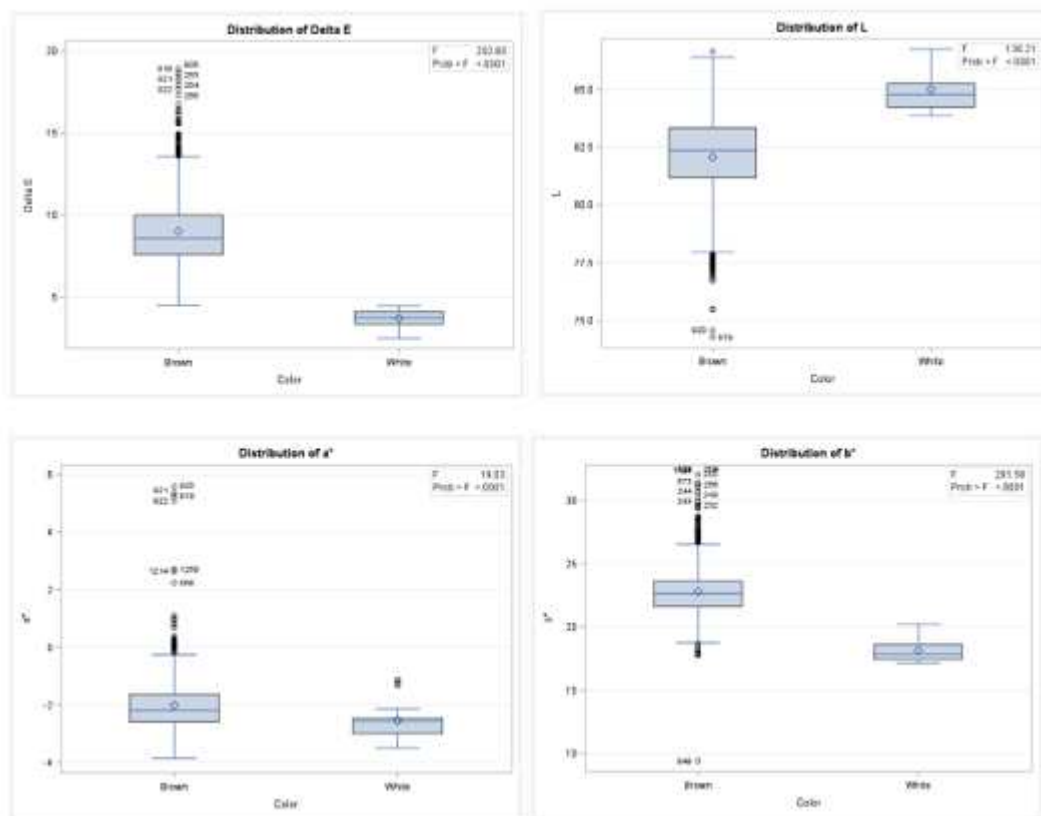
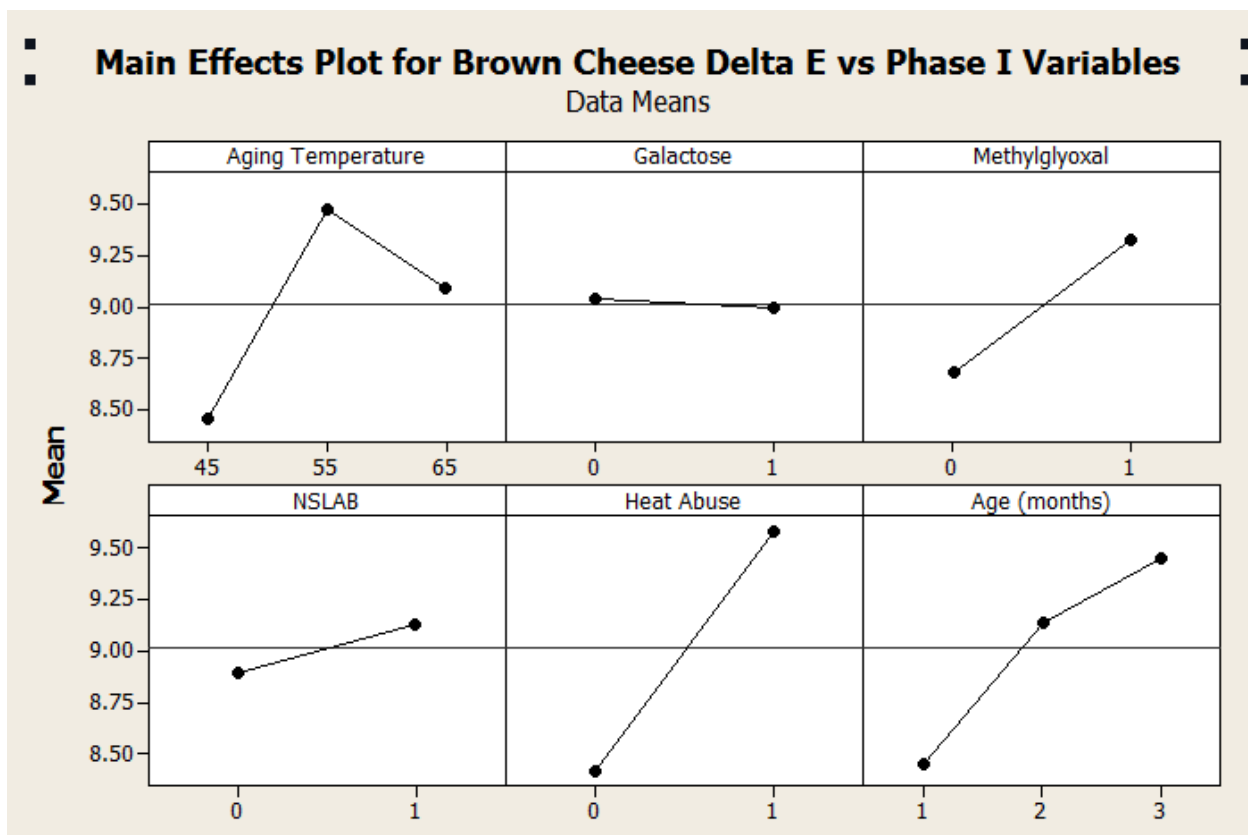


Figure 4. Growth rates of acidophiles, neutrophils and alkaliphiles in differing pH.

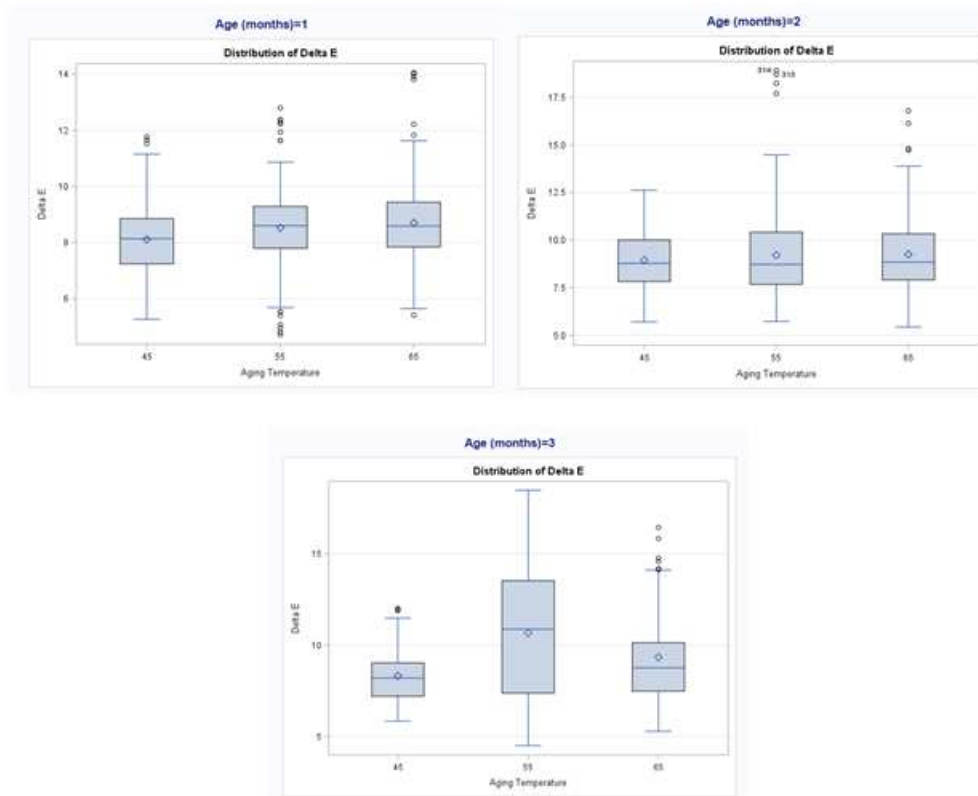
GRAPHS



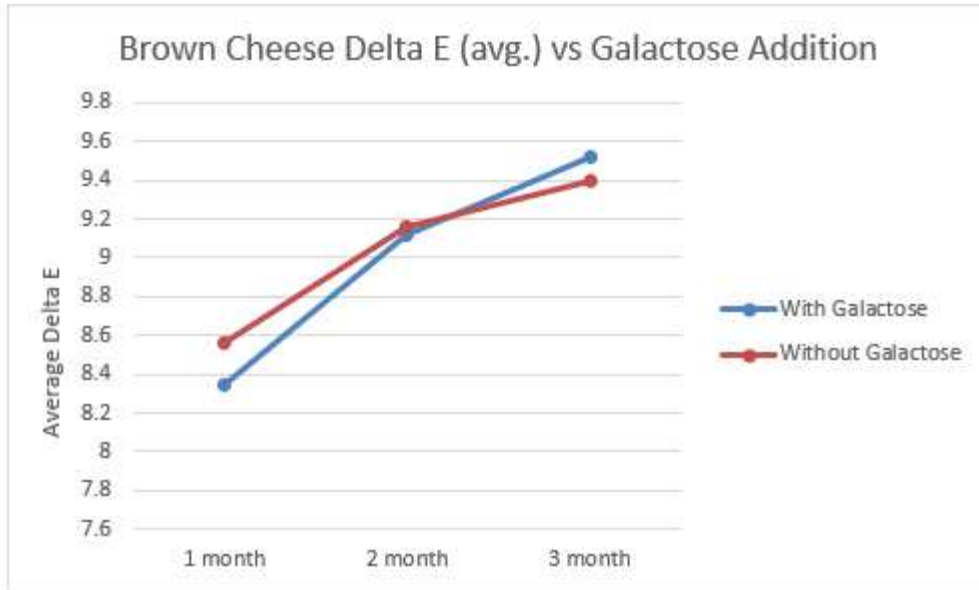
Graph 1. Boxplots of One-way ANOVA comparing brown vs white cheese Delta E, L*, a* and b* values. White cheese has lower Delta E, a* and b* values.



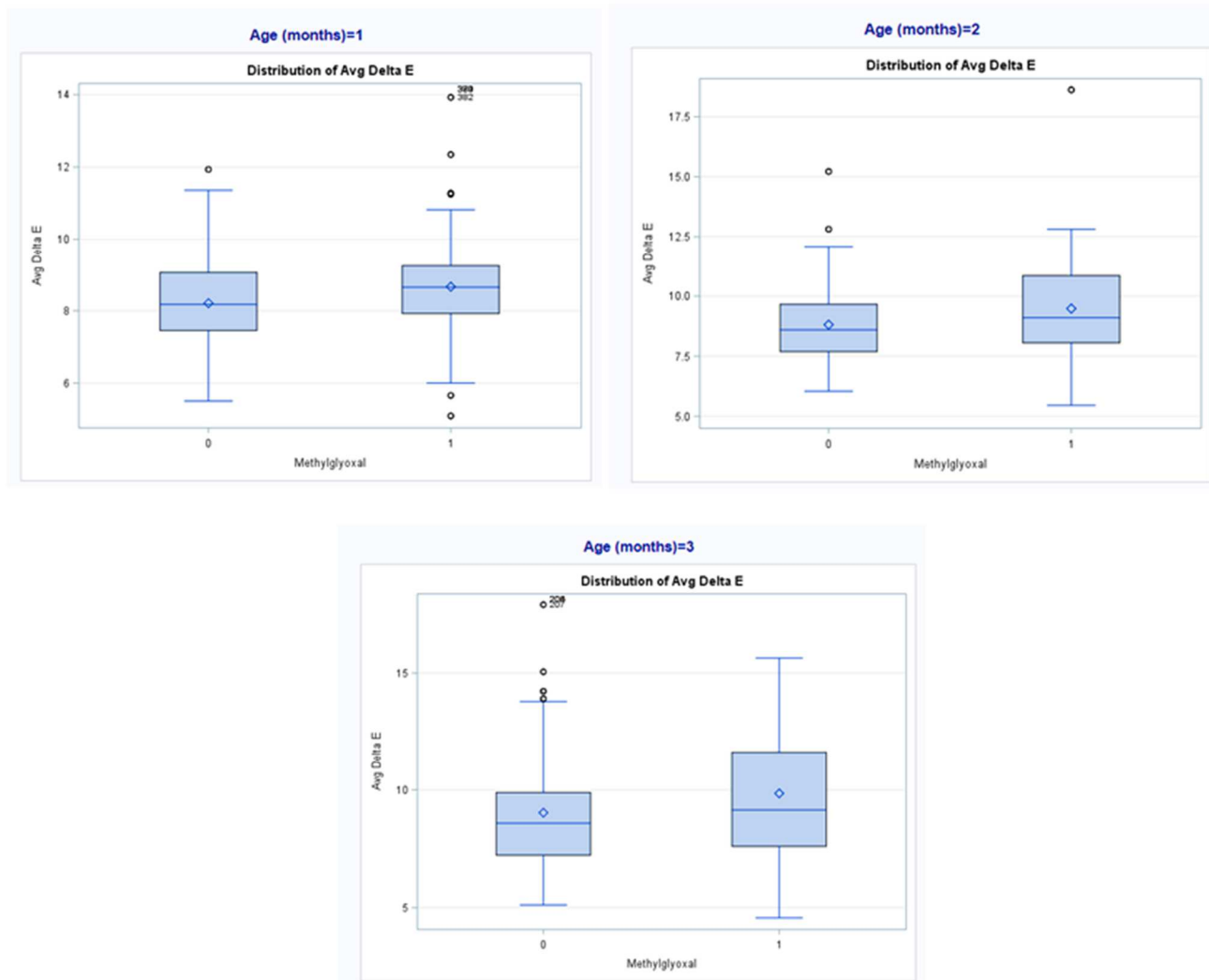
Graph 2. Main Effects Plot for Brown Cheese Delta E vs Phase I Variables



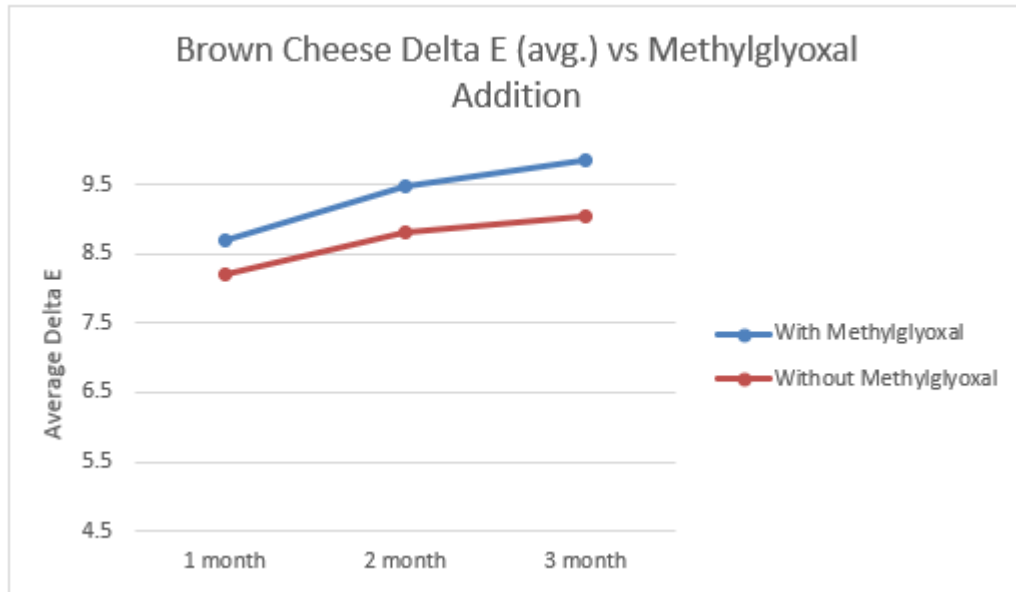
Graph 3. One-Way ANOVA: Brown Cheese Delta E vs Aging Temperature; Age (months) Grouping



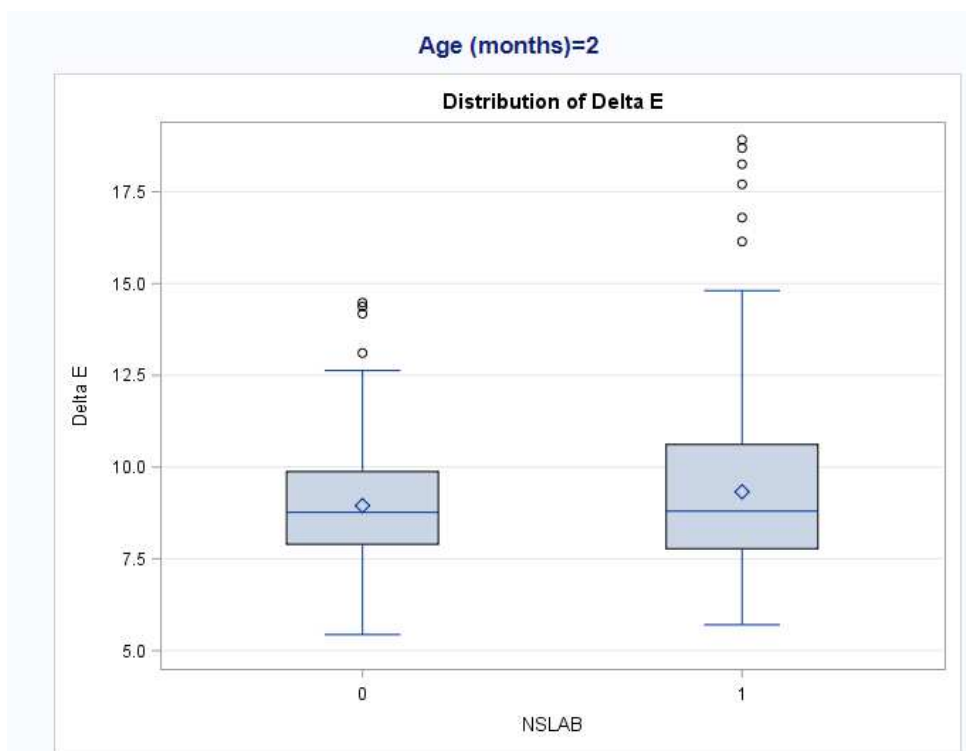
Graph 4. Monthly comparison of Brown Cheese Delta E vs Galactose Addition



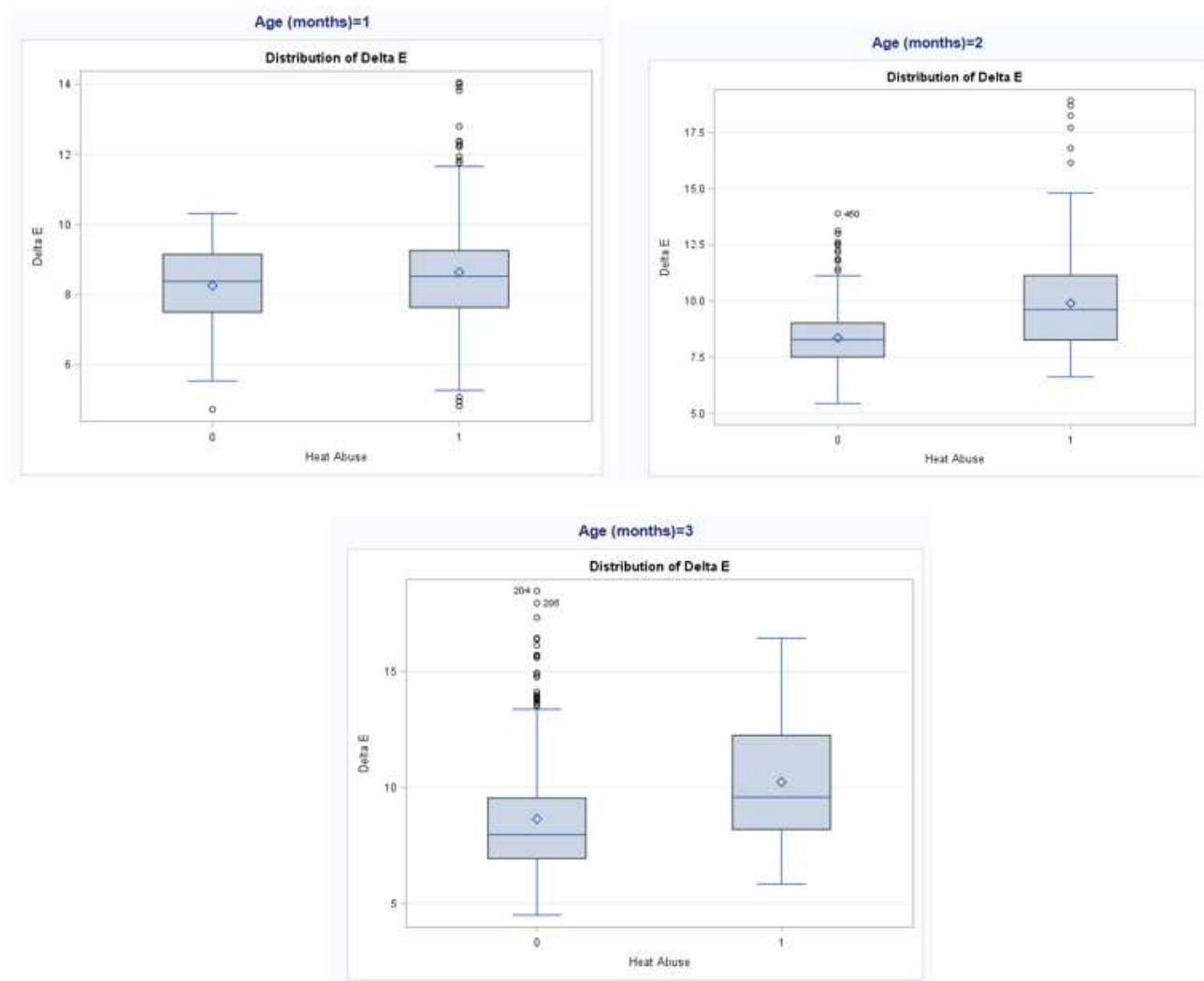
Graph 5. One-Way ANOVA: Brown Cheese Delta E vs Methylglyoxal Addition; Age (months) Grouping



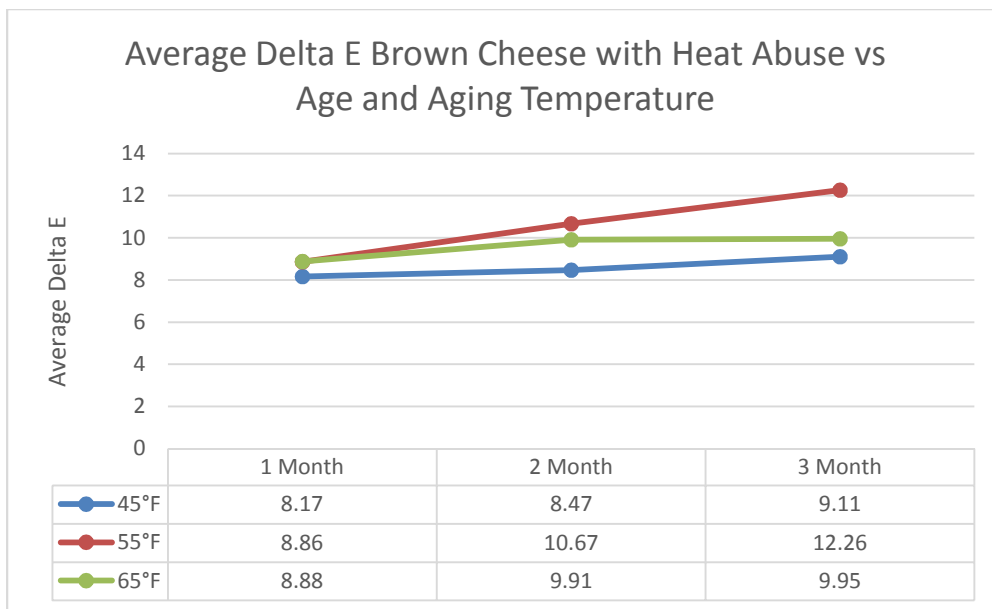
Graph 6. Brown Cheese Delta E (avg.) vs Methylglyoxal Addition



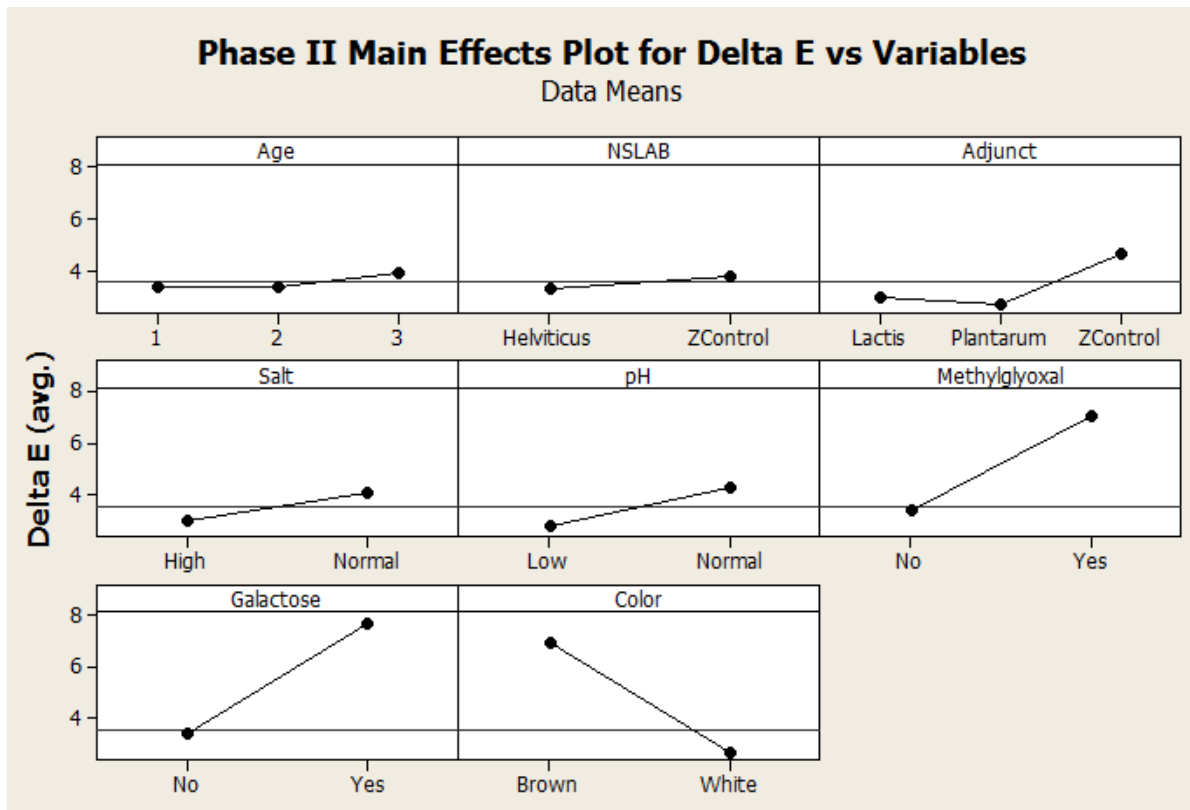
Graph 7. One-Way ANOVA: Brown Cheese Delta E vs NSLAB Addition; Age (months)
Grouping



Graph 8. Delta E as a result of Heat Abuse over time



Graph 9. Average Delta E Brown Cheese with Heat Abuse vs Age and Aging Temperature



Graph 10. Main Effects Plots comparing Phase II treatments to Delta E averages

Chapter IV. Overall Conclusions and Future Work

Overall, this study focused on creating a cheese model system to test variables that have been identified for contributing to browning as well as some other variables and conditions. These variables included: galactose, methylglyoxal, aging temperature, heat abuse, NSLAB addition, adjunct addition, age and modified %salt and pH. The cheese model used created a consistent platform to test the variables to ensure no additional noise or variability skewed data.

Based on previous work and in this experiment, it seems clear that methylglyoxal is a precursor to browning. Therefore, inhibiting the production of methylglyoxal is a key factor for reducing browning observed in Parmesan as a result of aging (cold temperature storage). Some other factors, like aging temperature and heat abuse, can increase the rate of browning as a result of methylglyoxal production. Additionally, with increasing time, more browning occurs. Additionally, use of adjuncts and modified environments of increasing salt and decreasing pH helped reduce the occurrence of browning.

Some additional work can be done to optimize these current theories to provide more data that supports these findings. Additionally, Parmigiano Reggiano from Italy does not seem to have browning defects. Therefore, understanding if browning occurs with different feed, NSLAB and raw vs pasteurized milk could be of interest. Lastly, since methylglyoxal is a result of

glycolysis, understanding bacteria's phenotypic outputs and metabolic pathways using technology such as metagenomics maybe of interest too.