DISSERTATION

SENSORY QUALITY OF CHEDDAR CHEESE MADE WITH BULK STARTER AND DIRECT TO VAT STARTER CULTURE

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ABSTRACT

SENSORY QUALITY OF CHEDDAR CHEESE MADE WITH BULK STARTER AND DIRECT TO VAT STARTER CULTURE

The production of cheese in the world consumes around 35% of the total milk production and has increased on average by 4% per year over the past 30 years (Fox et al., 2017). Cheddar consumption had modestly and steadily increased in the past several years in the United States and has increased 2.22% from 9.87 pounds per capita to 10.09 pounds per capita (*USDA ERS Dairy Data*, n.d.). Over the past several decades modernization of cheddar production in the United States has enabled producers to increase throughput with fewer resources resulting in more efficient production and consistent quality. This includes the common practice of standardizing cheese making procedures on a strict timing basis and using reliable and consistent rate and extent of acidification through culture selection and dosage. One such advancement was the development of defined starter cultures produced in a frozen or lyophilized state to be applied directly to the vat as a direct to vat inoculant (DVI) by the cheesemaker. Previously lactic acid bacterial cultures, defined or natural, were propagated by the cheesemaker prior to cheese production by a preceding fermentation of milk or whey and used to inoculate the milk for cheddar production.

The current research investigated if any differences in cheddar cheese biochemical and sensory characteristics exist among cheeses made with bulk starter and DVI technologies. Cheeses were produced using bulk starter culture technology, DVI technology, and DVI technology with pre-acidification then ripened for 90 days. The rate and extent of acidification in the process was analyzed with cheese composition, extent of the catabolism of protein and fat during ripening, and sensory characteristics of the cheese analyzed. MANOVA model analysis reported that the treatments had a significant effect on

the cheesemaking process (p=0.00381). Coagulation time was the only response found to be statistically significant (p=0.00081) from the process, biochemical, and sensory responses after mixed model analysis was completed. The make data or milk batch was found to have a significant effect on the cheese production process (p=0.00036), biochemistry (p=0.04391), and sensory characteristics (p=0.00002) of the cheeses. Therefore, it can be concluded that there was no difference in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and DVI, and there was no difference in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and DVI culture preparations with recipe adjustment for coagulation. The null hypotheses cannot be rejected.

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CHAPTER 1 Introduction

Cheese is a form of preserved milk which represents an important source of both macro- and micronutrients in the diet. The manufacture of cheese is a process which relies on coagulation and the acidification of milk, often via microbial fermentation, and subsequent syneresis to remove whey as a means of preservation. The production of cheese in the world consumes around 35% of the total milk production and has increased on average by 4% per year over the past 30 years (Fox et al., 2017). Cheddar consumption has modestly and steadily increased by 2.22%in the past several years in the United States from 9.87 pounds per capita to 10.09 pounds per capita (*USDA ERS Dairy data*, n.d.).

Over the past several decades modernization of cheddar production in the United States has enabled producers to increase throughput with fewer resources resulting in more efficient production and consistent quality. This includes the common practice of standardizing cheese making procedures on a strict timing basis and using reliable and consistent rate and extent of acidification through culture selection and dosage. These developments are due, in part, to advances in automation and engineering technology and sanitation practices, but also largely due to advances in biotechnology for the management of starter cultures to ensure stable, consistent, and safe production of cheese. One such advancement was the development of defined starter cultures produced in a frozen or lyophilized state to be applied directly to the vat as a direct to vat inoculant (DVI) by the cheesemaker. Previously lactic acid bacterial cultures, defined or natural, were propagated by the cheesemaker prior to cheese production by a previous fermentation of milk or whey and used as a bulk starter to inoculate the milk for cheddar production.

Cheese quality is influenced by several factors including milk quality, chemical composition of the cheese, culture selection, rennet selection, and microbial composition of the cheese. Consistent and reliable acidification are a requirement for timing-based cheese production to control quality. Rate and

extent of fermentation along with mechanical and thermal processes of cheese production directly influence cheese composition, and therefore quality. Cheese ripening is the process of biochemical changes in the cheese including the catabolism of carbohydrates, proteins, and fats by the starter culture and other flora in the cheese. Texture and flavor develop during ripening and shape the characteristics and quality of the cheese. Culture and enzyme selection influence the biochemical reactions during ripening along with cheese composition.

The cultures for cheddar production are generally sourced from specialized biotechnology manufacturers. Cheesemakers in modern cheddar factories have the choice between two different formats from which to source their starter culture and add it to the cheese milk. Production of bulk cultures for cheddar making is characterized as a process in which inoculum is obtained from the specialized biotechnology manufacturer and the cheesemaker then takes the inoculum and grows a larger volume of starter in a specialized media at the cheese plant to be used as the starter culture added to the cheese. Increasingly more common today is the use of concentrated starter cultures often referred to as direct vat inoculant (DVI) or direct vat starters (DVS) cultures. They are manufactured by specialized biotechnology production sites by propagating the strains in a specialized medium under pH control, concentrating the fermentation to remove water, then selling commercially as frozen or lyophilized starter cultures.

Since its inception direct to vat starter culture technology has been embraced for decades by many cheese producers. Today there still exists a substantial portion of the cheese industry which hold an anecdotal belief that this culture technology produces a cheddar of inferior texture and sensory quality. Little information exists in the literature exploring the cause of these perceived differences by cheesemakers between traditional culture propagation technologies and modern direct to vat technologies.

Bulk starter production at the cheese plant requires specialized knowledge, training, and equipment. This knowledge includes the sterilized preparation of growth media, aseptic techniques, activity testing for determining inoculation dosage, and sanitation practices. Over the past several years there has been a loss of knowledge in the art and science of bulk starter production at the cheese plant due to previous generations of cheesemakers aging out of the workforce, automation in modernized cheese production, and high turnover in employment. Research on the topic of starter culture technology would be useful to inform cheesemakers and cheese producers when choosing the most suitable culture format for their operation for delivering consistent quality cheddar cheese, while protecting against the threat of knowledge loss in cheese production.

The objective of this research was to investigate if any differences in cheddar cheese biochemical and sensory characteristics exist among cheeses made with bulk starter and DVI technologies. The study would test two null hypotheses. First, there would be no difference in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and DVI culture preparations without recipe adjustment for expected differences in coagulation. It was anticipated that the timing of the coagulation step and the pH of the milk at coagulation would differ among the bulk starter treatment and a DVI treatment without adjustments. The second null hypothesis to be tested was that no difference would be observed in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and DVI culture preparations with recipe adjustment for the coagulation step using pre-acidification.

CHAPTER 2 LITERATURE REVIEW

Introduction 2.1

Cheese is a form of preserved milk which represents an important source of both macro- and micronutrients in the diet. Milk is a fertile source of nutrients for bacteria which grow well in an ambient environment. These adventitious bacteria, known as lactic acid bacteria (LAB) naturally contaminate milk from the environment (fundamentals). The genera commonly found include *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Lactobacillus*. Milk contains sugar, in the form of lactose, as a source of energy and produce lactic acid as a byproduct. The lactic acid lowers the pH of the milk, curdling the proteins at their isoelectric point. This acidifying and curdling of the milk can be considered spoilage today when fermentation of the milk is not intentional. In the early history of cheesemaking this natural phenomenon of desirable bacteria present in the milk was harnessed to preserve the milk as a form of stable food supply, what we know as cheese.

Today the use of defined commercial starter cultures is used to control the fermentation process and produce high quality and safe cheese industrially. Historically starter cultures where sourced from the whey of the previous days production and contained undefined species of desirable LAB. Over the past several decades modernization of cheddar production in the United States has enabled producers to increase throughput with fewer resources resulting in more efficient production and consistent quality. These developments are due, in part, to advances in automation and engineering technology, sanitation practices, but also largely due to advances in biotechnology for the management of starter cultures to ensure stable, consistent, and safe production of cheese.

In recent decades innovations progressed in starter culture technology defined strain starters were developed. Single strain defined starters were introduced, but suffered from bacterial virus

infection, which compromised the fermentation and cheese quality and safety. Multi-strain defined starters were then introduced to combat this environmental pressure and ensure fermentation. Initially these defined cultures were propagated by the cheesemaker prior to cheese production by a previous fermentation of milk or whey and used as a bulk starer to inoculate the milk for cheddar production. However the ratio of the multiple strains was difficult to reliably control. The most recent advancement was the development of defined starter cultures produced in a frozen or lyophilized state to be applied directly to the vat as a direct to vat inoculant (DVI) by the cheesemaker. This technology had the potential to extensively use multiple strain cultures with consistent proportions of the strains contained within it.

These recent advancements in DVI defined starter culture technology allowed for dependable acidification and along with advancements in milk quality we now have reliable fermentation which permits modern production to produce cheese on a fixed time schedule and of higher quality (Johnson, 2017). Today there still exists a substantial portion which hold an anecdotal belief that this culture technology produces a cheddar of inferior texture and sensory quality compared to bulk starter. Little information exists in the literature today exploring the cause of these perceived differences by cheesemakers between traditional culture propagation technologies and modern direct to vat technologies.

Cheese Manufacture 2.2

Fox et al. (2017) have provided an extensive overview on the production of cheese from milk. Milk is first selected, standardized for protein and fat before pasteurization. The process of standardization enables the cheesemaker to have better control of the final composition of the cheese and produce a more consistent product. Acidification of the milk then occurs, typically from the in-situ fermentation of lactose to lactic acid from the addition of starter culture lactic acid bacteria preparations. Early in the fermentation, the milk is formed into a gel usually via enzymatic hydrolysis of the casein with the addition

of rennet, an aspartic protease enzyme. Syneresis of whey from the coagulum is then encouraged to express moisture from the cheese curd by heating, mechanical action, and acidification. The curds are then separated from the whey and further operations are done to form the curds into their characteristic shape. Most varieties would then require ripening of the curds and develop characteristic organoleptic properties such as flavor, aroma, and texture.

Cheddar cheese production follows this basic outline of cheese making with some special operations of curd handling. Milk is first selected and standardized for protein and fat. The protein to fat ratio is typically between 0.80 and 0.90. The milk is then pasteurized at a minimum of 72°C for 15 seconds. The pasteurized cheese milk is then delivered to the cheese vat, at the pre-established fermentation temperature, where filling begins. The temperature at which fermentation begins can depend on culture selection and individual cheese maker operations but generally is in the range of 31 °C to 33.5 °C. After the bottom of the cheese vat has filled with at least twelve inches of milk the starter culture consisting of homofermentative lactic acid bacteria (LAB), known as starter lactic acid bacteria (SLAB) is added. At this time, any adjunct non-starter (NSLAB) may also be added. The stirring tools in the vat may be gently mixing the milk to ensure even distribution of the bacterial cultures through the cheese vat.

Once the milk vat has reached capacity the rennet enzyme preparation is dosed into the cheese vat to begin coagulation of the milk. The enzyme is stirred into the milk until even distribution is achieved but before the enzymatic phase of coagulation has ended. When the milk has coagulated and set into a gel the cheesemaker then proceeds with cutting the coagulum in to roughly one-inch cubes to increase the surface area and enhance syneresis. When cutting is complete, the newly formed curd cubes would start to be gently stirred as indirect heating of the curds and whey begins. The increase in temperature continues to stimulate the fermentation of lactose to lactic acid via the SLAB and acts to enhance syneresis along with mechanical action during stirring, and thermal influence of the rising temperature. After reaching the final cooking temperature, the curds and whey are then held for a brief period of time while

stirring continues to promote syneresis. The whey and curds are then separated, and the curds are fused together to form a mat. The temperature of the curd cools slightly, but fermentation of lactose to lactic acid continues via SLAB metabolism. As the pH of the curd continues to drop the curd is then subjected to mechanical stress by cheddaring, a process of turning and flipping the curd mat. When the desired pH is reached, the curd is again subjected to mechanical stress by cutting the curd into small chips to prepare for salting. Salt is then applied to the curd chips enhancing syneresis, halting fermentation, and providing flavor potentiation. The salted curd is then formed into a block under pressure to further encourage syneresis. Once the block is formed it is packaged, cooled, and stored under selected conditions for ripening. During ripening biochemical changes such as carbohydrate metabolism, proteolysis, and lipolysis occur to contribute to flavor and texture development, and impact cheese quality.

Cheese Quality 2.3

There are several factors which can influence cheese quality and are extensively reviewed by Guinee and O'Callaghan (2010). Cheese quality may be defined as the degree of acceptability of the product to the end user, and thus may differ in definition among cheese types, but even within the same cheese technology. Cheddar cheese is characterized as a natural cheese possessing a smooth interior with no mechanical or gas openings, supple body that is easily machined, and a range of flavors are acceptable depending on the region. Cheddar cheese quality is often evaluated by sensory characteristics such as taste, aroma, texture, and appearance. Physical criteria such as sliceability, adhesiveness, hardness, and springiness often correlate to the ability of the cheddar block to be machined into the final form to be sold to the end-user: blocks, shreds, or slide for example. Composition characteristics such as protein, fat, moisture, salt content are important indicators of quality and legal status under the federal code. Chemical compositions such as intact casein, free amino acids, and free fatty acids can also be included in assessing cheddar quality. The presence of pathogens, toxic residues, biogenic amine, and foreign material can also be criteria to assess the safety of cheddar cheese. The chemical composition of milk and its pre-

treatments, manufacturing operations, acidification, culture selection, cheese composition, and cheese ripening all have a role to play in determining the quality of cheddar cheese.

Milk quality is arguably the most important factor in the quality of cheese and has been reviewed by Guinee and O'Callaghan (2010). Aspects such as milk composition, microbiology, chemical residues, and enzymatic activity are all important considerations for milk destined to be manufactured into cheddar cheese. The most crucial factor affecting cheese quality and yield is the concentration of protein and fat in the milk. These factors along with calcium content, its state, and pH have a major influence on the rennet coagulability and casein gel network organization and thus influence syneresis which in turn influences the cheese's final composition and yield. Factors such as species, breed, nutritional status, season, health, and lactation stage of the animal can all influence milk composition. Today most modern cheese processing includes techniques to limit these influences. The variability of milk composition is controlled through milk pre-treatments such as protein to fat standardization via membrane filtration of milk and addition of milk protein or micellar casein powders. However, it is not just the concentration of these individual components which can influence cheese quality and yield but also their intactness, the composition of individual caseins in the macromolecule, the integrity of the casein macromolecule and its ratio with colloidal calcium. Thus, not all variation in milk composition can be corrected or controlled by the cheesemaker.

There are a number of ways milk is treated prior to cheddar cheesemaking that are used by cheesemakers to influence cheese quality. Milk is generally stored cold on the farm, transported, and stored cold at the cheese plant until ready for use. During harvest, storage, and transport of milk a variety of changes can occur impacting milk quality for cheesemaking. It is generally accepted that the cold storage of milk impairs rennet coagulation properties, which in turn effects the organization of the rennet gel and hence syneresis and cheese composition and texture (Guinee & O'Callaghan, 2010). Some changes are reversible while other changes are irreversible. Chemical changes can occur in cold stored milk such

as the solubilization of micellar calcium which can negatively affect the ability of the milk to form a rennet induced gel and weaken the strength of the gel. In turn impacting the organization of the gel network and hence syneresis and cheese composition. Increases in serum casein can also occur after 24 hours of cold storage. These changes can largely be reversed with heat treatments such as pasteurization or even milder heat treatments of 49°C for five minutes, or by an increase in ionic calcium by 1mM with the addition of calcium chloride preparations.

In contrast, some changes occurring in cold stored milk cannot be reversed. Enzymatic hydrolysis of casein by proteinases from microbiological sources such as psychrotrophic bacteria or somatic cells increase soluble nitrogen in the milk, which is not captured as yield in the cheese. The enzymatic hydrolysis of milk fat by lipases from psychrotrophic bacteria can result in increased levels of free fatty acids. Free fatty acids often contribute to aroma and sensory defects. Damage to milk fat globule membranes can mean higher losses of fat during curd manufacture and ultimately lead to reduced yield. Improvements in dairy herd management, farm practices and sanitation, and more stringent standards for total bacterial counts are beneficial in limiting irreversible biochemical changes such as proteolysis and lipolysis during cold storage of milk.

Most industrial processes for the manufacture of cheddar cheese use pasteurization as a thermal heat treatment to inactivate pathogens, reduce the microbial load, and restore milk coagulability in cold storage milk. Pasteurization typically involves heating the milk to a temperature of 72°C to 75°C for 15 to 30 seconds in a continuous flow plate heat exchanger. This combination of temperature and time inactivates heat resistant human pathogens which are known to occur in milk making it safe for human consumption. This temperature also inactivates much of the natural non-pathogenic flora indigenous to milk which might otherwise contribute to the biochemical changes which take place during ripening of the cheese. The indigenous flora can sometimes positively promote desired flavor and texture development in some regional cheeses, but also have been well documented as causing off flavor, texture,

and other sensory defects such as gas formation, bloating, openness, and rancidity. Pasteurization can also inactivate Indigenous and microbial enzymes in milk which could otherwise contribute to proteolysis and lipolysis. The role pasteurization plays in controlling microbial quality of the milk is critical to cheddar cheese quality, though it may come at the cost of indigenous enzymes and non-pathogenic bacteria which may positively impact sensory quality through contribution to biochemical changes during ripening.

Pasteurization also contributes to the quality of cheddar cheese by restoring the coagulation properties of cold storage milk by reestablishing the ratio of colloidal to soluble calcium. Another consequence of pasteurization is the denaturing of whey proteins causing complexations between whey proteins and casein macromolecules. This complexation of whey proteins on to the casein macromolecule also has consequences on the structure and strength of the rennet gel. Whey proteins are otherwise stable to rennet and acidification, remaining soluble in the whey. Minimum pasteurization time and temperature treatments denature a low level of whey proteins which complex with the k-casein and are retained in the cheese and can improve yield. However, with increasing temperature there is an increasing level of whey protein denaturation and complexation with k-casein and in cheddar cheese this can lead to impairment of rennet gelation. Slower hydrolysis is a consequence of increased steric impedance and a lower hydrophobicity on the surface of the of the para-casein molecules. Since modern manufacture of cheddar cheese is time based, the rennet gel would not reach the proper cutting strength in the typical manufacturing time leading to a weak, brittle casein gel network which tends to shatter and break during mechanical stress leading poor retention of fat, casein, and therefore textural defects. Therefore, pasteurization influences cheddar cheese composition, texture, and yield. The effect of pasteurization is dependent on the temperature used to thermally treat the milk.

Milk components, protein, and casein are subject to seasonal variation. Standardization of the components in milk, in particular protein and fat, are common in modern industrial cheddar cheese manufacture and have a significant role to play in the cheddar yield, texture, and sensory quality. Cheddar

cheese is required to have a maximum moisture content of no more than 39% (*CFR - Code of Federal Regulations Title 21*, n.d.) and minimum fat in the dry matter (FDM) of 48%. Protein constitutes the majority of the dry matter recovered in cheese and therefore standardization of the fat in the dry matter is controlled by the protein to fat ratio in the cheese milk. Moisture composition in the cheese is mainly controlled by manufacturing protocols, and hence influences fat and protein composition. Cheddar manufacturers can use known compositional targets within the legal limit along with knowledge of protein and fat recovery factors for a particular process to then determine the optimal protein to fat ratio in the cheese milk. Recovery factors can be influenced by many variables such as milk composition, process technology, and practices. Increasing the protein to fat ratio increases the protein, moisture and minerals but reduces the fat in the dry matter and salt in the moisture phase. The reduction of fat in the protein matrix has a significant effect on the texture of the cheddar cheese(Rogers et al., 2009). While decreasing the protein to fat ratio increases fat in the dry matter it concomitantly decreases fat recovery in cheese from milk due to milk fat globules diluting the protein gel network. Moisture recovery from the milk increases, demonstrating the ability of fat globules to reduce the permeability of rennet milk gels. Cheese makers utilize altering the protein to fat ratio to control the composition of cheddar cheese and thus consistency and quality.

Protein, and casein content, standardization in cheddar cheese manufacturing is yet another tool for the cheesemaker to control quality and consistency. In large modern cheese plants coagulant and starter culture are added on the basis of activity and the time between rennet addition and cutting is done on the basis of time rather than gel firmness. The protein content of the cheese milk is a significant factor in determining the gelation time and firming rate of the gel, therefore variable protein levels in milk can produce varying rennet gel strength at the cutting step in a modern cheddar plant. If the rennet gel is cut when weak and underset the gel could shatter leading to decreased protein, fat, and moisture recovery.

As firmness of the gel increases the ability of the rennet gel to contract and synereses is reduced and can lead to higher moisture, lower pH, and lower salt in the moisture phase.

Increases in protein content is often done by utilizing membrane technology and low concentration ultra-filtration to increase the protein content from the typical 3.30% to no more than 5.00%. Protein content above 5.00% leads to curd firming rates which are too rapid to avoid overset gel in a modern cheese plant. When increasing the protein content of cheese milk using low concentration ultra-filtration and cutting the gel at the same gel strength leads to lower moisture in the cheddar cheese and can help avoid the elevated moisture defect. This practice also leads to significant increases in fat recovery as reported by (Guinee et al., 2006). It has also been observed that decreased proteolysis results when dosing the rennet at the same volume in low concentration ultra filtered (LCUF) milk, most likely attributed to the decrease in enzyme to substrate ratio in the cheese.

Increasing protein also concomitantly increases the buffering capacity of the milk through the addition of colloidal calcium phosphate associated with casein. This increase in buffer capacity depresses the ability of the starter culture to change the pH when added at the same per volume basis as whole milk. Higher pH in the milk at rennet addition and gelation can impact gelation time and firmness rate and lead to changes in the casein gel network organization. The use of LCUF milk as a technology to improve cheddar quality and consistency must be implemented with changes to the cheddar manufacturing protocol.

Once the milk has been standardized, pasteurized, and otherwise treated it is delivered to the cheese vat where the curd forming operations begin (Fox et al., 2017b). In cheddar cheese manufacture acidification is achieved by production of lactic acid by the fermentation of lactose to lactic acid by homofermentative LAB. A frequent practice in cheddar manufacture today includes the addition of starter culture of a standardized activity into a given volume of milk. Controlling the production of acid at the

appropriate time and rate is a key factor influencing the quality of cheddar cheese. Acidification plays a key role in influencing the organization of the gel network in rennet curd. Thus, effecting the gel strength at cutting influences yield and composition of cheddar cheese. Acidification can also influence the activity of the coagulant, retention of the coagulant in the curd, and gel strength. The retention rate of the coagulant in the curd in turn influences the rate of proteolysis during ripening and may affect cheese texture and sensory quality. Gel strength and organization at cutting can influence syneresis of the curd and in turn cheese yield and composition.

Acidification and shrinkage also promote the contraction of the curd via syneresis and influences cheese composition. Cheese composition especially moisture and salt regulate bacterial growth and enzyme activity and consequently biochemical changes that occur during ripening. In cheddar cheese the rate of acidification is characteristic of the manufacturing timing of generally three to four hours. The rate of acidification depends on the type of starter culture used, the amount of starter used, and the temperature profile of the manufacturing procedure. Acidification, its rate, and the extent have a significant effect on the texture of the cheese by influencing the solubilization of colloidal calcium phosphate from the casein micelle and modifies the susceptibility of caseins to proteolysis during ripening. Lastly, acidification and pH of the curd in cheddar controls the growth of many pathogenic and non-pathogenic bacteria and ensures the microbial quality and safety of the cheese.

Manufacture of Cheddar Cheese 2.4

Cheddar cheese is characterized as a rennet curd cheese in which limited acidification occurs before the gelation of the milk with an enzyme coagulant. A frequent practice of modern cheddar manufacturing includes the addition of a culture of standardized activity and rennet addition to a pre-determined volume of milk after the inoculation of the starter culture into the milk. However, unless milk composition is standardized and gel firmness at cutting is standardized then variation of firmness at

cutting can result in variability in syneresis and thus cheese composition. The inherent seasonality of milk can lead to fluctuations in protein, pH, and calcium. The protein content of milk directly influences the buffering capacity of milk and thus the starter culture's ability to change pH when producing lactic acid. The pH and extent of acidification at coagulation can influence the organization of the casein gel network, rennet activity, and the firming rate of the gel. These factors in turn influence the ability of the curd to retain coagulant, syneresis, and the ratio of soluble to insoluble calcium in the curd. Furthermore, reduction in rennet to casein ratio has been found to reduce the extent of proteolysis in cheddar cheese (Guinee et al., 1994). Modern cheddar manufacture operations are typically carried out on a basis of time rather than pH and thus variations in milk composition can lead to variation in acidification and gel organization at cutting. These variations would undoubtedly lead to variable cheddar quality in the final cheese (Lawrence et al., 1984).

In modern cheddar making operations, once the rennet gel is formed and hardened the mechanical cutting operations begin on the basis of time. Mechanical cutting involves the rotation of sharp cutting knives on a horizontal or vertical single axis at a defined number of rotations. Cutting the rennet gel has been described by Demjek and Walstra (2004) as disrupting the gel structure and creating cracks in the gel which initiate syneresis by forming new interaction in the para-casein network. The extent of cutting determines the size of the curd particle determines the surface area which is related to the velocity of whey release and the rate of syneresis. Typically, the number of rotations, speed of rotation, and overall cutting time are optimized for a particular recipe, vat design, and cheese composition. Johnston et al (1998) found that for a particular vat curd particle size distribution is influenced by the number of rotations. Therefore for each vat design there is an optimum. Below the optimum revolutions leads to larger curd particles remaining and these curd particles shattered or disintegrated upon mechanical stress leading to losses in moisture, fat, and yield. Too many revolutions above the optimum lead to smaller curd particles with greater surface area and more curd fines losses. As previously

discussed, the gel firmness at cutting has a considerable influence on the composition of the curd as well as casein and fat losses into the whey. Edvard et al. (2008) described If the gel is too weak the curd would crumble into fine particles which leave with the whey and release free fat. Conversely if the gel is too firm the gel would resist cutting and tearing. In commercial cheddar cheese operations, the parameters of the gelation of the milk and its firmness must be optimized along with the curd cutting program to ensure optimal cheese composition and yield.

In cheddar manufacture once the rennet gel is formed and cut, cooking, and stirring operations begin on a time basis. The cut curd particles release whey, contracting and firming and as they become heavier tend to sink. Stirring begins slowly to minimize shattering of the curd particle and must increase as curd particles contract and become denser to prevent curd particles from settling and fusing back together. The increase in stirring also produces mechanical stress which increases syneresis and enables the transfer of heat from the jacket of the vat to the curd particle. The temperature at which the curd is cooked has a direct impact on the final moisture content of the cheese. Final cooking temperature has an inverse relationship with the final moisture composition. Cheddar cheese curd temperature during manufacture is typically raised from 32°C to 39.5°C in modern operations. The rate of cooking also influences syneresis and therefore cheese composition. Generally, for cheddar manufacture a rate of two degrees F every 5 minutes allows for enhanced syneresis while preventing case hardening on the surface of the curd, which would inhibit syneresis and lead to excessive moisture entrapment in the curd. Excessive moisture trapped in the curd can lead to higher lactose levels within the curd and low pH.

Accompanying gelation, cutting, and cooking operations during cheddar manufacture is the decrease in curd pH from the fermentation of lactose to lactic acid by the starter culture LAB. The effect of acidification during cooking and stirring serves to enhance the overall level of syneresis (Rynne et al., 2008). The decrease in pH of the curd increases the negative charge on the casein and increases hydrophobicity of the para-casein through the solubilization of colloidal calcium phosphate (CCP).

Increases in CCP solubilization lead to increases in casein hydration and moisture content of the final cheese as well as influencing the textural characteristics and ability of the curd to melt and flow. Thus, cooking and stirring operations must be balanced with fermentation to reach target cheese composition and texture. The extent at which the pH decreases before the whey is removed from the curd determines the calcium to casein ratio of the curd, the degree of casein hydration and thus the cheese composition which has direct influence on cheese quality (Lawrence et al., 1987). Typical pH values at whey drainage for cheddar cheese manufacture are between pH 6.40 and 6.10. Lowering the pH of the curd at whey removal leads to more calcium solubilization, higher levels of moisture and lactose, which in turn lead to increased ratio of lactic acid to buffering capacity and thus lower cheese pH in the final cheese. Final moisture content, pH and CCP in the cheese influence proteolysis during ripening and therefore texture quality of the cheese.

Following gelation, cutting, cooking, and stirring operations is whey drainage and cheddar operations for modern cheddar manufacture. In commercial practice the whey and curd mixture are pumped out of the cheese vat on to perforated rotating curd belts to form a curd bed. The whey is drained off, collected and sent for further processing. On the belt the cheese curd particles begin to fuse together after whey separation to form a cohesive mass. The extent by which curd fusion occurs at this stage depends on curd temperature, pH, pressure, and time. Further expulsion of the whey continues via syneresis due to the continuing fermentation of lactose to lactic acid from the starter LAB, mechanical stress on the curd as it conveys on the belt, and pressure from the curd mass. As the pH decreases and whey syneresis continues so does the concomitant solubilization of CCP. The rate of acidification influences the extent of solubilization of calcium and thus influences the final composition of the cheese and hence its susceptibility to proteolysis during ripening and textural characteristics. In cheddar cheese manufacture the curd travels down a series of perforated belts dropping from one belt to another, enhancing mechanical stress on the para-casein gel networks to enhance syneresis and influence texture

through protein gel network interactions. After a pre-established period of time the curd mass is ready for salting, the curd mass must be milled into chips to increase the surface area and optimize salt uptake. Salting abruptly halts fermentation when an inhibitory concentration in the moisture phase is reached and consequently affects the final pH and composition of the cheese. The pH of the curd when salting occurs can influence the final moisture content and pH of the final cheese. The addition of salt itself enhances syneresis and is also one method of controlling the moisture content in the final cheese.

Curd temperature, moisture, surface area, and salting application all influence salt uptake into the curd. The level of salt uptake is positively correlated with moisture content of the curd at a constant salt addition rate. Salting operations in modern cheddar manufacture typically occur in three discrete applications with a portion of the salt applied at each salting step. The brief time gap in salt applications allows for better salt uptake into the curd and more efficient syneresis from salt addition to control the final composition of the cheese. Salt composition in the final cheese is a key compositional parameter affecting the quality of the cheese. The final salt composition can influence microbial activity, enzymatic activity of the residual coagulant, and therefore influence proteolysis and lipolysis during ripening affecting cheddar cheese quality. After the salt addition the curd is then formed into a block using block forming columns which use gravity, vacuum, and pressure gradients to expel the remaining whey and allow the curd to fuse into a homogenous mass and achieve the smooth interior texture free of mechanical opening between the junction or individual curd particles. This is a key quality characteristic of cheddar cheese.

The cheddar manufacturing operations of pasteurization, standardization, curd formation, cutting, heating, stirring, whey removal, cheddaring, salting, and block forming all have critical influence on controlling the final composition of the final cheese block. Composition of the cheddar has obvious influence on all aspects of quality characteristics including sensory, texture, and functionality (Guinee & O'Callaghan, 2010). Studies have investigated the influence of composition and quality of cheddar cheese

and agree that moisture content, salt in moisture (S/M) and pH are key factors in determining cheddar quality, however they do not agree on the relative importance of these factors (Fox et al., 2017b) . Lawrence and Giles (1973) proposed a grading scheme based on these compositional factors which is used commercially in New Zealand today. Lawrence and Giles also noted that quite wide ranges of FDM were acceptable and suggested that because little lipolysis occurs in cheddar that fat was of lesser importance but noted that when FDM dips below 48% the cheese is firmer with lower sensory scores.

Cheddar Cheese Chemistry 2.5

Pearce and Giles (Pearce & Gilles, 1979) found moisture was most highly correlated with acceptability in young cheeses and considered the optimum moisture fat free substances (MFFS) ranges to be 52% to 54%. Fox (Fox, 1975) reported salt concentration as the most important factor to influence cheese quality in Irish cheddar with the lowest percentage of downgraded cheeses containing between 4.0% and 4.9% S/M. Fox also reported a high percentage of those cheddar cheeses with compositional extremes of pH, moisture, and salt were downgraded. An extensive study by Lelievre and Giles (1982) examined the composition and quality of 10,000 New Zealand cheddars from five commercial factories and concluded that the composition and quality relationship varied among factories that certain generalizations did exist. The researchers concluded that as MFFS increased from 51 to 55% and S/M decreased from 6.00% to 4.50%, pH and FDM had no influence within the suggested ranges of Gilles and Lawrence (Gilles & Lawrence, 1973). Furthermore, the researchers suggested that within the compositional ranges for premium cheese, composition does not have a conclusive effect on the quality. This suggests other factors such as mineralization of the protein, culture selection, coagulant, selection, and others may also play a key role in cheese quality.

The role of calcium concentration in cheddar quality received little attention in the aforementioned studies though the work of Gilles and Lawrence (1980) pointed out that it determines

the cheese matrix organization, structure, and along with pH can determine if proper operations were carried out to produce the cheddar. In cheddar manufacture the majority of the whey is removed from the curd during cooking and stirring operations and it is in the whey that colloidal calcium phosphate is readily solubilized as pH decreases. “The whey removed after cooking comprises 90-95% of the total whey lost during cheesemaking, under normal conditions, and 85% and 90% of the phosphate lost from the cheese curd” (Fox et al., 2017b). The pH of the curd at whey removal and the calcium content are strongly correlated (Lawrence et al., 1984). Insoluble calcium acts as a buffer and therefore the whey pH at drainage also correlates to the final pH in the cheese. As previously mentioned, the extent of de-mineralization of the casein before gelation and whey removal also influences moisture content of the final cheese, and susceptibility to proteolysis during ripening. Calcium solubilization can be summarized to influence composition, pH, protein structure, and ripening in cheddar. Calcium concentration is then a good record of the history of the curd during manufacture and a key indicator of characteristic quality of cheddar cheese.

The process of ripening involves biochemical and microbiological changes which catalyze physical, structural, and sensory changes during storage of fresh curd blocks into cheese. For cheddar ripening is a critical process that varies in time from 4 weeks to 5 years, depending on the target age, flavor, and texture properties of the cheese. Typical industrial cheddar cheese is aged anywhere from two months to six months to develop its characteristic texture and sensory properties. Biochemical changes such as glycolysis, proteolysis, and lipolysis are associated with changes in pH, protein hydration, and fat coalescence. These physico-chemical changes in turn influence sensory quality such as aroma and texture, as well as functional properties like the machineability of the cheese block.

Proteolysis during ripening in cheese has been extensively reviewed by Fox et al. (2017a). Proteolysis is the hydrolysis of the casein to peptides and free amino acids by the activity of the residual coagulant in the cheese, starter culture and non-starter lactic acid bacteria (NSLAB) proteinases and

peptidases, and indigenous enzymes found in milk (Guinee & O'Callaghan, 2010). Proteolysis is an important biochemical change that occurs during the ripening of cheddar cheese that influences the texture, and therefore machinability of the cheese, and sensory quality. The residual activity of the coagulant is a primary contributor to primary proteolysis in cheddar. The α_{s1}- casein is cleaved at the Phe_{23}—Phe_{24} bond by the residual coagulant and is associated with a weakening in the cheese matrix which results in a reduced stress to fracture the cheese and lower firmness (de Jong, 1976) Cleavage of the residues of 14-24 α_{s1}- casein are generally associated with the decrease in rubberiness of young cheddar cheeses to a smoother bodied cheese (Guinee & O'Callaghan, 2010) .

Sources of proteolytic proteinases not only come from the residual coagulant but also indigenous enzymes such as plasmin, which has been implicated in the hydrolysis of β-casein (Fox et al., 2017b). Starter culture proteolytic enzymes are generally responsible for the formation of small peptides and amino acids, often from the larger peptides hydrolyzed by chymosin from caseins. Lactococci are commonly used species in cheddar starter culture and their proteolytic systems have been studied extensively by Kunji *et al* (1996). The proteinases associated with LAB are cell envelope proteinases (CEPs). *Lactococcus* CEPs are classified into seven groups depending on their specificity. These proteinases are associated to the outside of the cell wall and can participate in primary proteolysis. Intracellular proteolytic enzymes of *Lactococcus* that contribute to ripening are primarily peptidases and amino peptidases. These enzymes are generally responsible for taking large peptide sequences and hydrolyzing them to smaller peptides or liberating free amino acids. In contrast the *Streptococcus thermophilus* species, also used extensively in starter cultures for cheddar manufacture, are often characterized as having low expression of CEP, or called PrtS proteinase. However, some strains have been characterized as having fast acidification due to proteinase activity at a level close to that of *lactococci* (Fernandez-Espia et al, 2000). These species probably contribute less to the proteolysis during ripening of cheddar than Lactococcus when PrtS is expressed at low concentration on the cell wall. *Lactobacillus* species are also

often used in the production of cheddar cheese and the proteolytic systems of these species can vary but generally are found to be similar to *Lactococcus* as they contain CEPs and intricate intracellular proteolytic system. The Indigenous NSLAB population in cheddar cheese also represent a significant contribution to proteolysis during ripening. The NSLAB population is thought to contribute significantly to the release of free amino acids, but very little to primary proteolysis in cheddar (Fox et al., 1998, 2004; Guinee et al., 2008). Some NSLAB are indigenous to the milk while others are intentionally added as adjuncts with the starter culture to promote positive flavor contribution.

The fate of amino acids produced via proteolysis has been extensively studied. Amino acids in themselves do contribute to cheddar flavor, though no single amino acid has been characterized as cheesy, they are believed to contribute to savory taste of cheese. Amino acid catabolism is a significant contributor to cheddar flavor and aroma. The catabolism of amino acids in cheese has been reviewed by Curtin and McSweeney (Curtin & McSweeney, 2004). The catabolism of amino acids involves reactions such as deamination, decarboxylation, transamination, desulphuration, and hydrolysis of side chains. This leads to the genesis of a multitude of compounds that have been shown to significantly contribute to the complex flavor in cheddar cheese. The catabolism of amino acids seems to be initiated by the action of aminotransferase, which moves the NH_2 group to α-ketoglutarate. The acids produced from this reaction are unstable and are converted to a range of other compounds such that one amino acid can generate several volatile compounds which are often found to be significant in cheese flavor (Fox et al., 2017b).

Lipolysis is yet another important biochemical change occurring during cheese ripening that involves the hydrolysis of triacylglycerols to mono and di-glycerides by lipases and esterases from sources such as the milk, starter cultures, NSLAB, secondary starters, and added pre-gastric esterase. Fat is a significant component in cheddar cheese and contributes to texture, sensory, and functional properties of the cheeses. Lipolysis in cheddar cheese is considered to be low to moderate. Cheddar cheeses with a high degree of lipolysis can often be rejected for atypical rancidity. Guinee *et al*.(2008) surveyed matured

cheddar cheeses and showed that the level of free fatty acids as a percentage of milk fat was lower than the threshold of two per cent considered necessary to induce rancid off flavors in cheddar cheese.

Starter Culture Biotechnology 2.6

Undoubtedly the most crucial step in cheese production, and therefore cheddar production, is the conversion of lactose to lactic acid by LAB. In modern cheddar manufacture the intentional addition of starter LAB to the cheese milk promotes rennet activity, aids in syneresis of whey from the curd, thus influencing the moisture composition and preventing the growth of undesirable organisms in the cheese. Cheddar starter cultures are typically characterized as homofermentative cultures, or those bacteria that ferment lactose primarily to lactic acid, due to the expectation that high-quality cheddar have a smooth closed knit interior body. The use of defined strains in cheddar cheese production was first developed in the 1930's in New Zealand to combat open texture development in the cheese from CO_2 production present in the undefined mesophilic mixed strain starters (Law & Tamine, 2011). Since then, the use of defined strains starters is used almost exclusively for modern cheddar production. Cheese starter cultures are commonly divided into categories based on their optimum temperature range for fermentation.

Mesophilic cultures are mainly comprised of strains of *Lactococcus lactis* and *Lactococcus cremoris* and have an optimum fermentation temperature of 30°C. Thermophilic starter cultures are primarily comprised of strains from *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii ssp. lactis* and have an optimum fermentation temperature of 42°C. In modern cheddar manufacture it is common that cheese makers use mesophilic starter cultures or increasingly more common today is a combination of mesophilic and thermophilic strain in the starter culture composed of *Lactococcus lactis*, *Lactococcus cremoris*, and *Streptococcus thermophilus* strains.

The cultures for cheddar production are generally sourced from specialized biotechnology manufacturers. Cheesemakers in modern cheddar factories have the choice between two different formats of starter culture and add it to the cheese milk. Production of bulk cultures for cheddar making is characterized as a process in which the inoculum is obtained from the specialized biotechnology manufacturer and the cheesemaker then takes the inoculum and grows to a larger volume in a specialized media at the cheese plant to be used as the starter culture added to the cheese milk in the vat. The pH of the medium is typically controlled during growth using internal buffers in the medium or the external addition of a neutralizer such as ammonium hydroxide. This pH control of the growth medium during bulk starter propagation allows for higher cell density of the bulk starter and lower rate of usage in the cheese vat. The pH is usually regulated between a pH of 5.80 and 6.30 for *Lactococcus* and *Streptococcus* species, and thus the bulk starter has an appreciable level of acidity even when pH controlled.

Increasingly more common today is the use of frozen concentrated starter cultures often referred to as DVI or DVS cultures. They are manufactured by specialized biotechnology production sites by propagating the strains in a specialized medium under pH control. When fully grown the medium is neutralized and the cells harvested using centrifugation or membrane filtration to concentrate the cells and then by dropping the liquid in to liquid nitrogen, they are frozen into pellets. The pellets are then either packaged and stored frozen or lyophilized. The pH control, neutralization, and removal of the supernatant through concentration means that the DVI or DVS cultures have a low concentration of acid in the culture.

Reliable and predictable acidification via the starter culture is a prerequisite in modern cheddar manufacturing where the process is timing based and pH during the process has critical influence on the quality of cheese. Both bulk starter propagation and DVI have the ability to deliver reliable and consistent acidification through advances in microbial genetics, defined strain starter cultures, and sanitation practices. However, key differences do exist between bulk starter and DVI preparations including volume

of culture added to the vat, culture activity including the lag phase, acidity added to the vat, and enzymatic activity of the culture (Salji & Kroger, 1981b). Little research has investigated the influence of these differences between starter culture products on the time-based cheddar manufacturing process and cheddar quality. Yet, it is generally believed by cheesemakers that cheddar meant for long ripening, and thus considered to be of higher quality, is produced using bulk starter culture preparations in lieu of DVI. A general perception of DVI or DVS cultures by cheesemakers is that these starter cultures produce cheddar of lesser quality with particular attention to texture and flavor development during ripening. Again, little research exists to elucidate the mechanisms which could explain such differences in texture and sensory quality. Bulk starter production at the cheese plant requires specialized knowledge, training, and equipment. Over the past several years there has been a loss of knowledge in the art and science of bulk starter production at the cheese plant due to previous generations of workers aging out of the workforce, automation in modernized cheese production, and high turnover in employment. Research on the topic would be useful to inform cheesemakers and cheese producers when choosing which culture technology is most suitable for their operation for delivering consistent quality cheddar cheese, while protecting against the threat of knowledge loss in cheese production.

CHAPTER 3 METHODS

Culture Preparation 3.1

A single strain *Lactococcus cremoris* was provided by Danisco USA Inc. (New Century, KS, USA) as concentrated frozen pellets in a direct to vat (DVI) format, and this material was used as DVI treatment. This material was also used to propagate the strain in a bulk starter media. The media, Magnum PH II is an internally buffered media and was sourced from Danisco USA Inc. (New Century, KS, USA). The culture was inoculated in the sterile media and fermented at a temperature of 90°F. The resulting cultured media would be used as bulk starter treatment.

Bulk Starter Preparation 3.2

The bulk starter was prepared using a commercial bulk starter medium, Magnum PH™ II (Danisco USA Inc, New Century, KS, USA), which is an internally buffered starter medium designed for the growth of mesophilic LAB, including *Lactococcus cremoris*. The starter medium powder was rehydrated in deionized water at a rate of 5.80% solids (w/w) under constant agitation and rapidly heated to a temperature of 195°F and held for 45 minutes. The bulk starter medium was then rapidly cooled to the setting temperature of 80.5°F. The bulk starter liquid media was then aseptically transferred to a benchtop fermentation reactor New Brunswick™ BioFlo®115 (Eppendorf NA, CT, USA). The temperature was maintained at a set point of 80.5°F, and the DVI material for the *Lactococcus cremoris* strain was aseptically inoculated at a rate of 0.05% (w/w). Agitation was maintained throughout the fermentation at the lowest setting of 35 rpm to keep the insoluble buffer suspended in the liquid starter medium. The pH was monitored and recorded every hour until the pH reached 5.80 or below, at which time the bulk starter was then cooled to 45°F, agitation was stopped, and the ripened bulk starer was held until its use.

Starter Culture Activity Test 3.3

Starter culture activity testing was completed on site at IFF's state of the art Cheese Application Laboratory in New Century Kansas. The activity of the DVI material and the ripened bulk starter were evaluated in an activity test to determine the inoculation rate of the culture materials to reach the pH targets of the cheddar production recipe. The DVI culture inoculum was aseptically prepared by 10-fold serial dilution in commercial whole milk at or below 45°F until a 1:100 dilution was obtained. A sterile 10,000 ppm solution of sodium formate was aseptically mixed into refrigerated bulk fresh commercial milk (Alma Dairy, Alma Kansas, USA) standardized with cream (Hiland Dairy, Kansas City, Kansas, USA) and ultrafiltered skim milk (Kansas Dairy Ingredients, Hugoton, Kansas, USA) at 5 ppm. A sterile 10,000 ppm solution of inosine-monophosphate was aseptically mixed into refrigerated bulk fresh commercial milk at a rate of 10 ppm. The standardized cheese milk, held at or below 45°F, was than aseptically dispensed in 250 ml glass bottles in 200-gram aliquots.

The 1:100 serial dilution of the DVI frozen pellet culture sample was then aseptically inoculated into the bottles containing 45°F or below milk at rates of 0.0142%, 0.0222%, and 0.0284%, respectively. The ripened bulk starter media was gently agitated in the bioreactor vessel before aseptically harvested and inoculated into the bottles containing the milk at a rate of 0.350%, 0.650% and 1.00%, respectively. The bottles were then placed in a 90°F water bath regulated by a programmable immersion circulator. The immersion circulator was programmed to ramp the temperature to simulate the time and temperature conditions of the cheddar production procedure. The temperature held at 90°F for 75 minutes and then ramped up to 101.5F over a period of 30 minutes where it was held constant at 101.5°F for 45 minutes, and then ramped back down to 90°F over a period of 80 minutes. The temperature was then held at 90°F for the remainder of the test. The pH of the milk was continuously recorded from when the milk reached incubation temperature until the activity test ended using the iCinac (KPM Analytics USA, MA, USA).

All samples were tested in triplicate and the data were calculated for the mean. Using the pH targets at specific timing in the cheddar production recipe, the data obtained from the activity test was used to construct dose response curves by plotting the responses of the overall change in pH at 135 minutes and 220 minutes after inoculation, respectively. The resulting linear equation was used to predict the dosage necessary to reach the respective pH target in the cheddar production recipe. The predicted inoculation dosages for the overall change in pH at 135 minutes and 220 minutes after inoculation was averaged, and this was the established culture dosage for cheddar production.

Cheddar Production 3.4

Cheddar was produced on a one of a kind six vat cheese making system at the state-of-the-art Cheese Applications pilot plant in New Century, Kansas. Fresh pasteurized cheese milk was sourced from a local cheesemaker (Alma Dairy, Alma, KS, USA) for each week cheese production took place. Two consecutive days of cheesemaking occurred each week of cheese production. The order of production by variable was randomized using statistical software (Minitab®, LLC 2021) Once the milk arrived it was standardized by the addition of cream and ultra filtered skim milk to obtain a protein and fat target of 0.85 +/- 0.03. The milk was then stored and refrigerated until use. Cheddar was produced in a six-liter vat system and each variable was produced in three vats simultaneously, and two variables were used to produce cheddar each production run for a total of six vats per day. The milk was heated to 88°F in the vat, and culture was then added at the dosage concluded from the activity test and dose response curve and allowed to stir continuously under gentle agitation to prevent cream separation. The rennet was then prepared using a commercial fermentation produced chymosin (FPC), Chymostar (Danisco USA inc., New Century, Kansas, USA), by diluting 0.36 mL of enzyme 20-fold in deionized water. Once prepared the enzyme was stirred into the milk until evenly distributed to deliver the equivalent of 47 international milk clotting units (IMCU) per 100 liters of milk. Once the rennet was stirred agitation was stopped and Relco® Optiset coagulation detection probes (Relco, Willmar, MN, USA) were placed in the milk then removed

after coagulation was detected. Cutting time was calculated as a factor of 1.67 multiplied by the coagulation time detected by the probe. Following cutting of the coagulum occurred, the curds were allowed to heal for 5 minutes before gentle stirring occurred. The curd and whey were then heated from 88°F to 102°F for over 30 minutes. After the temperature reached the final cooking temperature the curd continued to be stirred in whey for an additional 45 minutes. The whey and curd were then drained from the vat into a strainer which released the whey and captured the curd in a heat jacketed draining vat. The curd was allowed to form a cohesive matt and was flipped every 15 minutes for 80 minutes. The curd from three vats containing the same variable was then milled using a wire grid, combined, and salted at a rate of 2.75% (w/w). The salted curd was then filled into small round molds, pressed in a pneumatic press for 60 minutes at 20 PSI and then removed from the mold, flipped, placed back in the mold, and pressed again for 60 minutes at 40 PSI. Six wheels approximately 300 g each were obtained from three combined vats of curd. Cheeses were then removed from the pressing molds and sprayed with a 10-ppm solution of Natamycin (Danisco USA Inc, New Century Kansas, USA), allowed to dry for 30 minutes, vacuum packaged, and placed in a ripening chamber at 50°F for 60 days.

Biochemical Analysis 3.5

The determination of milk components fat, protein, and acidity was done using spectrophotometric method on a Foss MilkoScan™ 2 instrument (FOSS Analytics, Hillerod, DE). The pH of the cheeses was measured using a pH electrode (Mettler-Toledo, LLC, Columbus Ohio, USA). Moisture content in the cheese was analyzed using a vacuum oven method (*AOAC 926.08-1927, Loss on Drying (Moisture) in Cheese. Method I*, n.d.). Fat content in the cheese was determined using the Mojonnier method (*AOAC 989.05-1992, Fat in Milk - Modified Mojonnier Ether Extract*, n.d.) . Total protein was established using the Kjeldahl method (Lynch et al., 2002). Non-casein nitrogen was analyzed using AOAC method 998.05 (*AOAC 998.05-2001, Noncasein Nitrogen Content of Milk. Kjeldahl Method*, n.d.). Non-protein nitrogen was ascertained using the Kjeldahl method (*AOAC 991.21-1994(1996), Nonprotein*

Nitrogen in Whole Milk. Kjeldahl Method, n.d.). Salt was analyzed using a direct titration of chloride method (Wehr & Frank, 2004). Free amino acids were determined using High Performance Liquid Chromatography (Rachamin, n.d.). Total free fatty acids were analyzed using the AOCS method CA 5a-40 (Gordon I. et al., 2021). Total calcium content in the cheese was measured using spectroscopy (*AOAC 984.27-1986, Calcium, Copper, Iron, Magnesium, Manganese, Phosphosphorus, Potassium, Sodium, and Zinc in Infant Formula. Inductively Coupled Plasma Emission Spectroscopic Method*, n.d.).

Sensory Analysis of Cheeses 3.6

Sensory analysis of the cheeses was supported and completed at the Center for Dairy Research at the University of Wisconsin and was led by Sensory Coordinator Brandon Prochaska. The cheddar samples were evaluated by a minimum of five trained descriptive panelists in duplicate on the same day for each shipment. Cheeses were cubed into uniform size (1/2") and tempered to 52°F for evaluation. Samples were presented in 2-ounce cups labeled with random three-digit codes. Panelists were provided filtered water and unsalted crackers as palate cleansers between each sample and each replication. Thirty second breaks were enforced between each sample and each replication. All attributes were assessed using a 15-point scale (see score card in appendix). Texture and basic taste attributes were evaluated using Spectrum® methodology and flavor attributes were evaluated using Quantitative Descriptive Analysis (QDA) methodology (Meilgaard et al., 1999). Data collection was performed using Compusense® Cloud (version 21.0.7773.19239, Compusense Inc., Ontario, Canada).

Statistical Analysis 3.7

To detect if there were differences in process, biochemical, or sensory characteristics among the three treatments, while controlling for batch of milk, mixed models were fit separately for each response variable. Each mixed model, treatment was included as a fixed effect and batch (of milk) was included as a random effect. The decision to add batch as a random effect was made to account for variability among

different batches of milk and cheese production dates. Due to multiple testing, FDR (False Discovery Rate) adjustments were made after fitting the mixed models. The FDR adjustments were made separately for each response variable grouping (Process, Biochemical, and Sensory). Diagnostic plots were investigated to check model assumptions, and no major violations appeared. Pairwise comparisons were analyzed for response variables that were found to have significant differences among treatments before multiple testing adjustments.

Additionally, MANOVA (multivariate analysis of variance) models were fit separately for each grouping of response variables. Before the model was fit, response variables that were highly correlated (correlation >0.7) with other response variables in the same grouping were removed for all groupings. This decision was made in order to fit the MANOVA models, which could not be fit with more than ten response variables due to the small number of observations. Diagnostics were performed to check for homogeneity of variance among groups using Levene's test, and no major violations were found. Statistical analysis was completed using R software (2023).

Chapter 4 Results

Bulk Starter Production 4.1

For each week, or run, that cheeses were produced over two production days a bulk starter culture was prepared from the frozen pellet material containing pure single strain DGCC 5927. The results of the fermentation time and cooling pH are presented in Table 4.1. The first bulk starter preparation total fermentation time was six hours and the pH reached 5.28 in that time, and then was cooled. The second run of bulk starter preparation was observed to reach a pH of 5.60 in eight hours before cooling was initiated. The third run was then observed to reach a pH of 5.75 in six hours, and then was cooled before use. The final and fourth run reached a pH of 5.70 in seven hours and then was cooled before use in activity test to determine bulk starter dosage to reach the cheddar recipe pH targets.

Table 4.1 Cooling pH and hours of growth in bulk starter made with DGCC 5927.

Run	Cooling pH	Hours of Growth
1	5.28	6.00
2	5.60	8.00
3	5.75	6.00
4	5.70	7.00

Activity Testing and Culture Dosage Determination 4.2

Dose response studies were conducted in the cheese milk for each run before cheesemaking to determine the dosage of the bulk starter and the DVI culture in the cheese milk to attain the target pH's at critical steps in the cheese making process. A control culture, CHOOZIT® MC 70 FRO 1000 DCU, was also used in the test at a dosage known to reach the target pH at critical steps in the cheese production process and used in industrial cheddar production successfully. The results of the delta pH from 0 to 135 minutes (DpH135min) and the delta pH from 0 to 225 minutes (DpH220min) for each activity test run of the control

is displayed in Table 4.2. In the first run the control was observed to reach a delta pH of 0.375 135 minutes into the activity test, and 1.007 220 minutes into the activity test. In the second run of activity testing the control was also observed to change the pH by 0.375 pH units after 135 minutes, and 1.007 pH units after 220 minutes of fermentation. In the third activity test run the control was observed to change the pH of the milk by 0.394 and 1.038 pH units after 135 and 220 minutes, respectively. In the fourth and final production run the control was observed to change the pH of the milk by 0.373 and 1.008 pH units after 135 minutes and 220 minutes, respectively.

Table 4.2: Extent of acidification during activity testing of CHOOZIT® MC 70 FRO 1000 DCU after 135 minutes and 220 minutes

Run	DpH135 (min)	DpH220 (min)
1	0.375	1.007
2	0.375	1.007
3	0.394	1.038
4	0.373	1.008

The resulting linear equations and r^2 values of the dose response study with the bulk starter preparation and DVI preparation of the DGCC 5927 culture are displayed in Table 4.3. The linear equations for the delta pH after 135 minutes of fermentation for the DVI preparation was observed to have a positive slope for each run, indicating that as the dosage increased the change in pH increased. The same was observed for the delta pH after 220 minutes of fermentation for the DVI preparation culture. The bulk starter preparation was also observed to have a positive slope in the linear equation for the change in pH after 135 and 220 minutes. Again, indicating that as the culture dosage was increased the extent of fermentation and therefore the change in pH increased.

The R^2 values for the DVI prepared starter culture were observed to be in a range of 0.960 to 0.999 for the linear regression equations for delta pH after 135 minutes of fermentation, indicating that the

dose of the culture and the corresponding change in pH were highly correlated and the linear regression has a high goodness of fit. The R^2 values for the DVI prepared starter culture were observed to be in the range of 0.938 to 0.999 for the linear regression equations for the delta pH after 220 minutes, also indicating that change in pH at this time point was highly correlated to the dosage of the culture. The R^2 values for the bulk starter culture preparation were in a range of 0.996 to 0.999 and 0.962 to 0.976 for the delta pH after 135 minutes and 220 minutes, respectively. A high degree of correlation was also found between the dosage of the bulk starter and the change in pH at these time points in the fermentation.

Table 4.3: Linear equations and R^2 for dose response curves for the extent of acidification after 135 minutes and 220 minutes for DVI and Bulk Starter treatments

	DVI				Bulk Starter			
	DpH135min		DpH220min		DpH135min		DpH220min	
Run	Linear Equation	R^2	Linear Equation	R^2	Linear Equation	R^2	Linear Equation	R^2
1	y = 12.26x + 0.070	0.961	y = 37.20x + 0.26	0.996	y = 46.77x + 0.12	0.996	y = 74.13x + 0.58	0.974
2	y = 11.73x + 0.088	0.978	y = 26.28x + 0.28	0.956	y = 0.46x + 0.064	0.997	y = 0.83x + 0.32	0.974
3	y = 11.56x + 0.12	0.960	y = 22.63x + 0.42	0.938	y = 0.51x + 0.067	0.999	y = 0.83x + 0.38	0.962
4	y = 10.44x + 0.056	0.999	y = 25.02x + 0.20	0.999	y = 0.48x + 0.065	0.999	y = 0.85x + 0.37	0.976

Table 4.4 displays the inoculation rate calculated from linear regression equations to change the pH to the control culture targets displayed in Table 2.0 at the specified time points. The dosages represent the mean of the inoculation rates calculated from both linear equations for each culture preparation. These calculated inoculation rates were used to inoculate the cheese milk on both cheesemaking days in each production run. In run one the dosage established for the first cheese production run was 0.0222% and 0.750%, for DVI and bulk starter, respectively. Production run two had calculated dosage rates of 0.313% and 0.900% for DVI and bulk starter preparations, respectively. Production run three used

calculated culture dosage rates or 0.266% and 0.750% for DVI and bulk starter culture preparations, respectively. The fourth production run used 0.408% and 0.916% dosage rates for DVI and bulk starter culture preparations, respectively.

Table 4.4: Inoculation rates of DVI and Bulk Starter culture treatments for cheddar production

Run	DVI (%)	BS (%)
1	0.0222	0.750
2	0.0313	0.900
3	0.0266	0.750
4	0.0408	0.916

Cheese Production 4.3

The composition of the milk used for cheese production in each run is displayed in Table 4.5. In the first run the protein, fat, and casein content was measured at 3.65%, 4.34% and 2.63%, respectively. This gave a protein to fat (P:F) ratio and casein to fat (C:F) of 0.84 and 0.60, respectively. The acidity of the milk was .1425% lactic acid. The milk protein, fat, and casein content in the milk of the second run was 3.87%, 4.61%, and 3.13%, respectively. The P:F and C:F ratios were then measured as 0.84 and 0.68, respectively. The acidity was .1715% lactic acid. The milk composition in the third run for protein, fat, and casein was observed to be 3.36%, 4.08%, and 2.63%, respectively. The P:F and C:F ratios were calculated as 0.82 and 0.64, respectively. The acidity of the milk was observed to be .1470% lactic acid. In the fourth run, the milk composition for protein, fat, and casein, was observed to be 3.87%, 4.70%, and 2.95%, respectively. The P:F and C:F ratios were calculated as 0.82 and 0.63, respectively. The acidity was measured as .1670% lactic acid.

Table 4.5: Milk composition for cheddar production

Production Run	Protein (%)	Fat (%)	Casein (%)	P:F	C:F	Acidity (% la)
1	3.65	4.34	2.63	0.84	0.60	.1425
2	3.87	4.61	3.13	0.84	0.68	.1715
3	3.36	4.08	2.63	0.82	0.64	.1470
4	3.87	4.70	2.95	0.82	0.63	.1670

Protein to fat ratio (P:F), casein to fat ratio (C:F), lactic acid (la)

The results of the in-process cheese making at the critical steps in the cheese making process are displayed in Table 4.6 for each of the treatments. The bulk starter with no pre- acidification (BS NO) treatment coagulation pH, coagulation time and coagulation factor on average was observed to be 6.55, 23.9 minutes, and 1.66, respectively. The time at whey removal and the whey removal pH were observed to be 139.13 minutes and 6.26, respectively. The mean milling time and milling pH were observed to be 221.88 minutes and 5.42, respectively. The DVI with no pre-acidification (DVI NO) treatment was observed to have a mean coagulation pH, coagulation time, and cutting factor of 6.61, 31.46 minutes, and 1.70, respectively. The whey removal time and whey removal pH were observed to be 136.50 minutes, and 6.25 on average, respectively. The mean milling time and milling pH were observed to be 222.00 minutes and 5.41, respectively. The treatment with DVI and pre-acidification (DVI YES) was observed to have a mean coagulation pH, coagulation time, and cutting factor of 6.41, 21.87 minutes, and 1.67, respectively. The mean whey removal time and pH were observed to be 134.25 minutes and 6.18, respectively. The milling time and pH were observed to be 217.50 minutes and 5.40, respectively.

Table 4.6: Mean cheddar production data for each treatment

	BS NO	DVI NO	DVI YES
Coagulation pH	6.55 ± 0.09	6.61 ± 0.04	6.51 ± 0.08
Coagulation Time (min.)	23.56 ± 4.83	31.46 ± 6.19	21.87 ± 3.86
Cutting Factor	1.66 ± 0.01	1.70 ± 0.05	1.67 ± 0.01
Whey Removal Time (min.)	139.13 ± 6.85	136.50 ± 5.97	134.25 ± 6.50
Whey Removal pH	6.26 ± 0.05	6.25 ± 0.10	6.18 ± 0.07
Milling Time (min.)	221.88 ± 5.94	222.00 ± 9.63	217.50 ± 8.66
Milling pH	5.42 ± 0.04	5.41 ± 0.06	5.40 ± 0.04

BS NO n =8 , DVI NO n=4, DVI YES n=4 1

Cheese Composition and Biochemistry 4.4

The results of the mean cheese composition for each of the treatments is displayed in Table 4.7. The BS NO treatment cheeses had a mean moisture, fat, and protein content of 35.35, 33.48, and 25.71%, respectively. Salt in moisture (S/M), fat on a dry basis (FDB), and MFFS were calculated as 5.08%, 51.79%, and 53.15%, on average. The mean calcium concentration was 0.790% , and the mean pH was 5.18. The DVI NO treatment had an average moisture, fat, and protein content of 35.29%, 33.19%, and 26.01%, respectively. The S/M, FDB, and MFFS were calculated and reported as a mean of 5.07%, 51.30%, and 52.82%, respectively. The calcium composition was 0.784% on average, and the mean pH as 5.13. The DVI YES treatment cheeses had a mean moisture, fat, and protein composition of 35.51%, 33.43%, and 25.54%, respectively. The S/M, FDB, and MFFS in the DVI YES treated cheeses were calculated and reported as 4.88%, 51.83%, and 53.34% on average, respectively. The mean composition of calcium was 0.764%, and the pH was 5.10 on average for DVI YES treated cheeses.

Table 4.7: Mean Cheddar Composition

	BS NO	DVI NO	DVI YES
Moisture(%)	35.35 ± 0.70	35.29 ± 1.19	35.51 ± 0.24
Fat(%)	33.48 ± 0.71	33.19 ± 0.49	33.43 ± 0.53
Protein(%)	25.71 ± 0.75	26.01 ± 0.58	25.54 ± 0.58
S/M(%)	5.08 ± 0.49	5.07 ± 0.30	4.88 ± 0.12
FDB(%)	51.79 ± 1.40	51.30 ± 0.42	51.83 ± 0.78
MFFS(%)	53.15 ± 1.37	52.82 ± 1.45	53.34 ± 0.46
Calcium(%)	0.790 ± 0.053	0.784 ± 0.074	0.764 ± 0.051
pH	5.18 ± 0.09	5.13 ± 0.05	5.10 ± 0.10

BS NO n =8 , DVI NO n=4, DVI YES n=4 2, Salt in moisture (S/M), Fat on a dry basis (FDB), Moisture fat free substances (MFFS)

Primary proteolysis was measured by determining the pH 4.60 soluble nitrogen content in the cheese and dividing it by the total nitrogen content and expressing it as a per cent in the cheese after 90 days of ripening, Figure 4.1 displays the results for primary proteolysis measurements in the cheeses. The BS NO treatment cheeses were observed to be 14.75% pH 4.60 soluble nitrogen from total nitrogen content on average with a standard deviation of 0.96%. The DVI NO treatment cheeses had a mean of 15.00% pH 4.60 soluble nitrogen from the total nitrogen content with a standard deviation of 1.11%. The DVI YES treatment cheeses had a 15.59% of pH 4.60 soluble nitrogen from total nitrogen in the cheese on average, and a standard deviation of 1.49%. Secondary proteolysis was measured and expressed as the per cent of 12% TCA soluble nitrogen from total nitrogen in the cheese, and the results for the cheeses after 90 days of ripening are reported in Figure 4.2. The secondary proteolysis for the BS NO, DVI NO, DVI YES cheeses were 9.37%, 9.48%, and 9.84% on average, respectively. The standard deviations were 1.17%, 1.04%, and 0.91% for the BS NO, DVI NO, DVI YES cheeses, respectively.

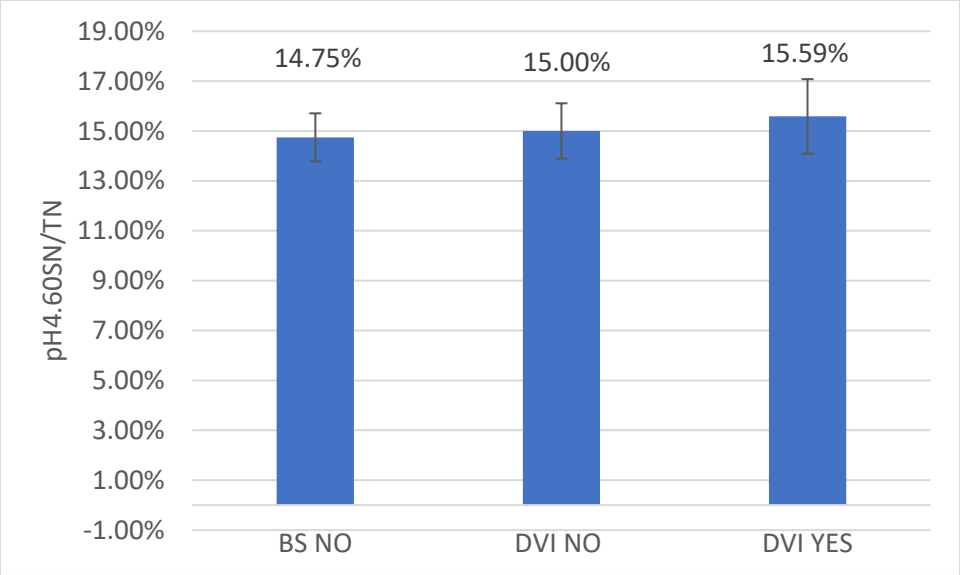

Figure 4.1: Primary proteolysis in cheddar cheeses expressed as % pH 4.60 soluble nitrogen of total nitrogen. BS NO n =8 , DVI NO n=4, DVI YES n=4

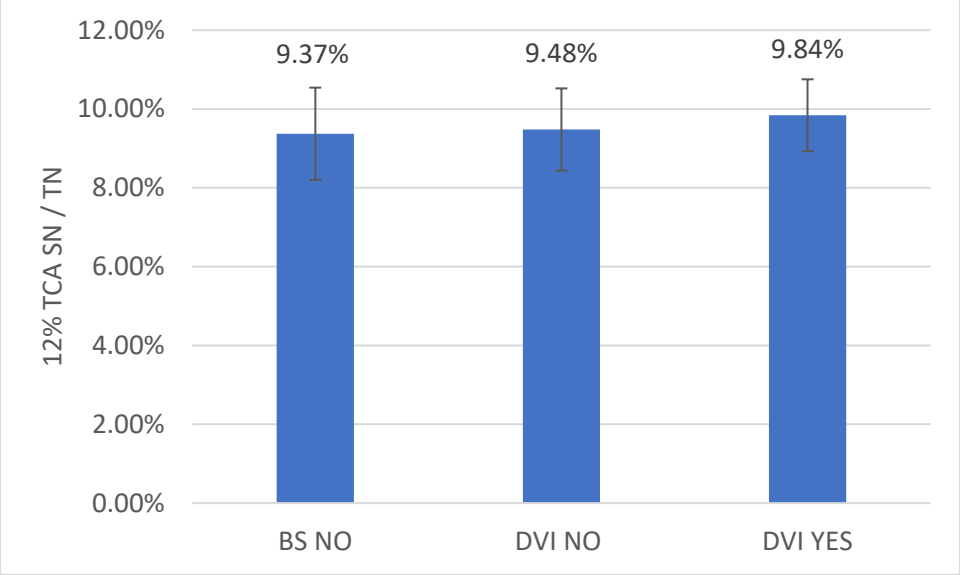

Figure 4.2: Secondary proteolysis in cheeses expressed as % of 12% TCA soluble nitrogen of total nitrogen. BS NO n =8 , DVI NO n=4, DVI YES n=4

Free amino acids were measured in the cheeses after 90 days. The results of the free amino acids in the cheeses are summarized in Figure 4.3. Free amino acids glutamic acid, threonine, proline,

asparagine, methionine, leucine, alanine, phenylalanine, tyrosine, serine, glycine, and valine were detected in all cheeses. Some free amino acids were not detected in any of the cheeses after 90 days such as cysteine, tryptophan, and arginine. Histidine was detected the BS NO and DVI YES cheeses but not in DVI NO cheeses after 90 days of ripening. Glutamine was detected in BS NO and DVI NO cheeses, but not in DVI yes cheeses after 90 days. Isoleucine was only detected in DVI YES cheeses after 90 days. The in key amino acids such as glutamic acid, asparagine, aspartic acid, leucine, phenylalanine, lysine, and valine that on average DVI YES cheeses had slightly higher concentrations than DVI NO cheeses, which in turn had slightly higher concentration of these free amino acids than the BS NO cheeses. These same amino acids were found in the highest concentrations for all cheeses compared to the others.

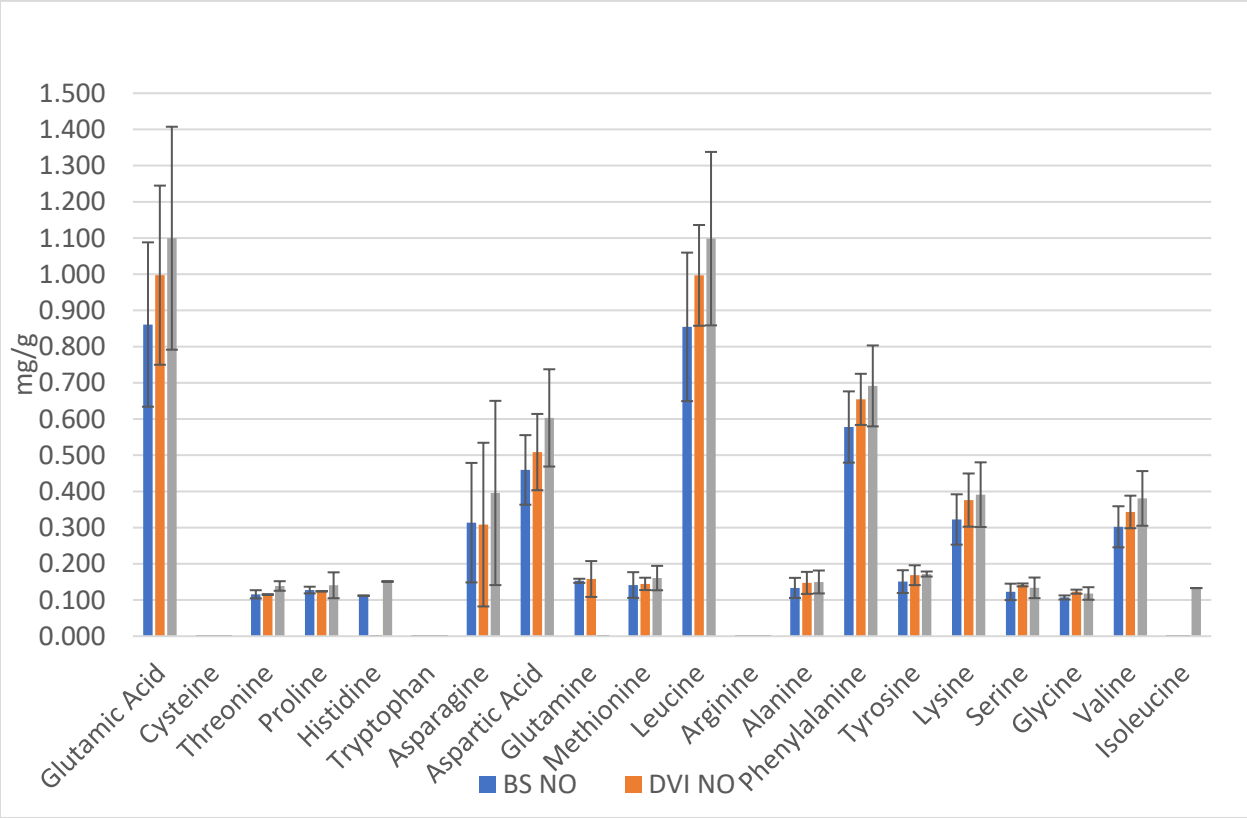

Figure 4.3: Free amino acids in cheddar cheese. BS NO n =8 , DVI NO n=4, DVI YES n=4

Figure 4.4 summarizes the total free amino acids and the aliphatic to acidic free amino acid ration mean concentrations in the cheeses after 90 days for each treatment Similar to the observation of the highest concentration free amino acids, the level of free amino acids increased from BS NO to DVI NO and DVI NO to DVI YES cheeses. the BS NO, DVI NO, and DVI YES cheeses had on average 4.018, 4.844, and 5.136 mg/g total free amino acids after 90 days of ripening, respectively. It was also observed that the means for the BS NO, DVI NO, and DVI YES cheeses had a ratio of aliphatic to acidic free amino acids of 6.946, 5.115, and 4.018 , respectively.

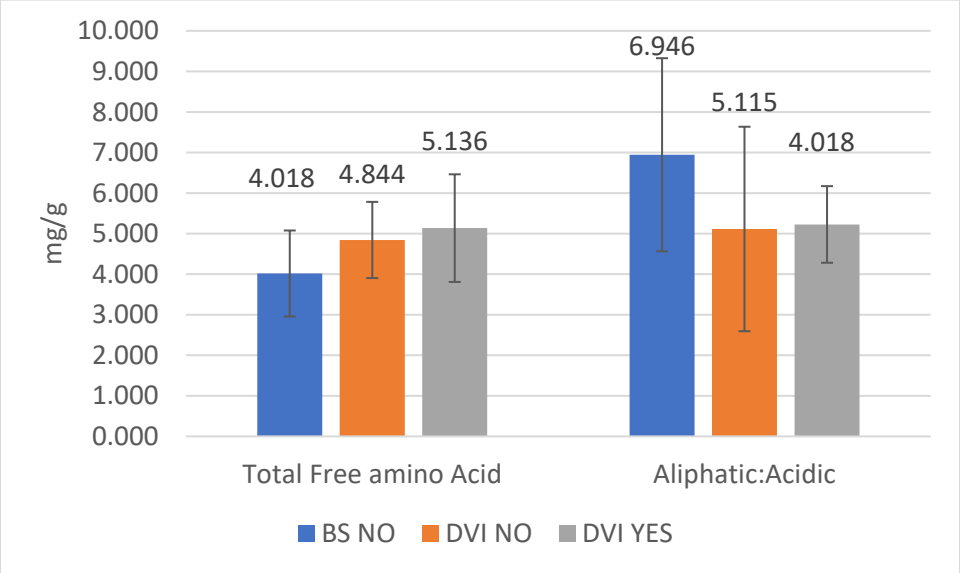

Figure 4.4: Total Free amino acids and aliphatic to acid amino acid ratio for cheddar cheese. BS NO n =8 , DVI NO n=4, DVI YES n=4

Free fatty acids were measured in the cheeses after 90 days of ripening and the results are summarized in Figure 4.5. The DVI NO cheeses had the lowest total free fatty acids on average with 0.0044%. The BS NO treated cheeses had 0.0069% of total free fatty acids. The BS NO cheeses had a slightly higher concentration of total free fatty acids compared to the BS NO treatment but lower than the DVI YES treatment with a mean of 0.0069%. The DVI YES treated cheeses had the highest mean concentration of free fatty acids with a reported concentration of 0.00985%.

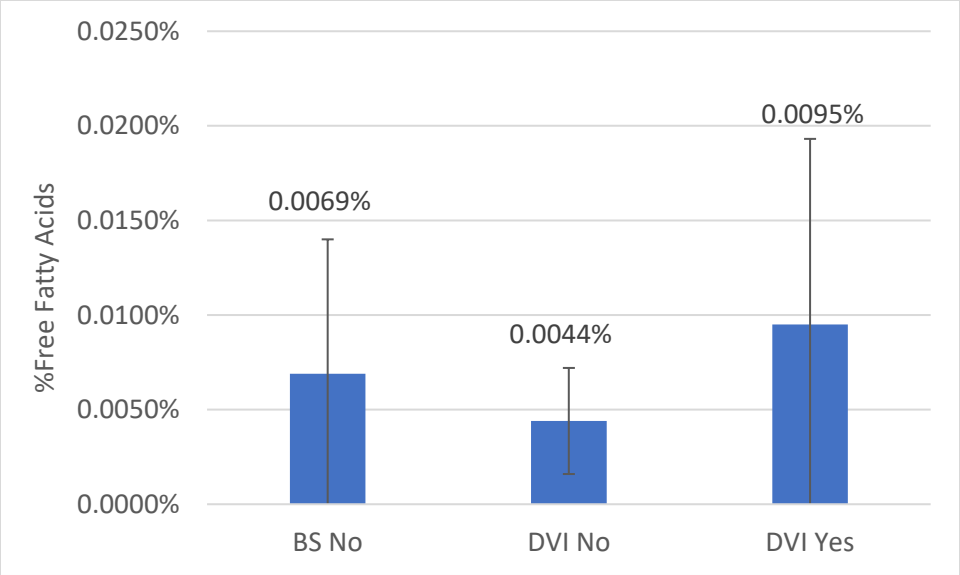

Figure 4.5: Total free fatty acids in cheddar cheese. BS NO n =8 , DVI NO n=4, DVI YES n=4.

Sensory Quantitative Descriptive Analysis 4.5

After 90 days of ripening the cheeses were analyzed for texture, taste, and aroma sensory attributes using quantitative descriptive analysis. In Figure 4.6 the results of the quantitative descriptive analysis are summarized for the textural attributes of the cheese. The adhesiveness of the cheese was the degree to which the chewed mass (not unbroken down individual particulate) sticks to teeth and mouth surfaces. The mean of the cheeses was 9.65, 9.08, and 9.77 for the BS NO, DVI NO, DVI YES cheese, respectively. Cohesiveness of the cheese was defined as the degree to which the chewed mass holds together. Average cohesiveness of the cheeses was reported as 11.87,11.71, and 12.15 for BS NO, DVI NO, and DVI YES cheeses, respectively.

Hand firmness was characterized as the amount of force required to compress the sample. Hand Firmness means scored of the cheese was 12.07, 12.34, and 12.22 for the BS NO, DVI NO, and DVI YES treated cheeses, respectively. Chewiness was the degree of chewing needed to break up the cheese

pending swallowing. Chewiness was 6.08,5.88, and 5.96 on average for the BS NO, DVI NO, and DVI YES cheese, respectively. Particle size was the size of the particles in the chewed mass. Particle size of the cheese when chewed was 12.30, 12.34, and 12.22 on average for the BS NO, DVI NO, DVI YES treatments, respectively.

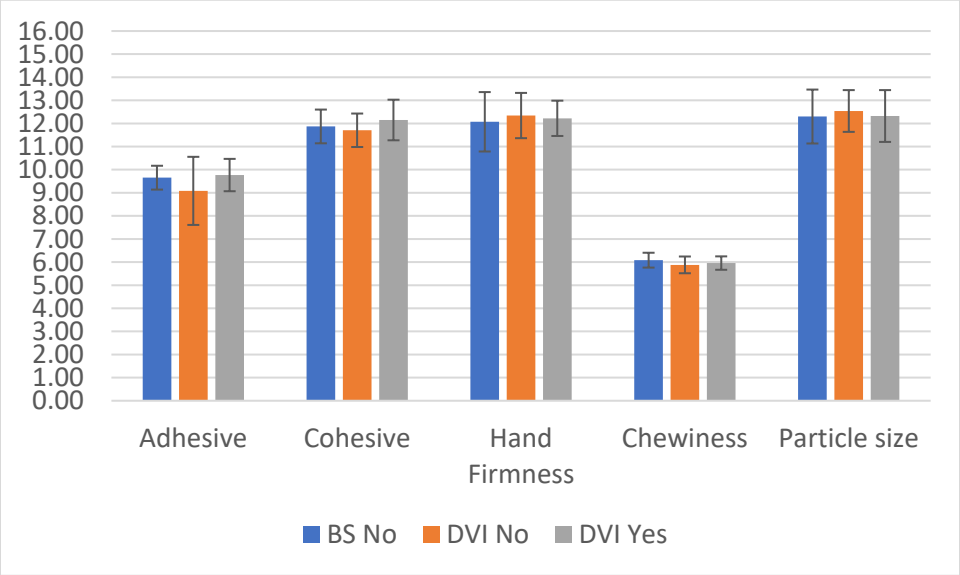

Figure 4.6: Mean scores for quantitative descriptive analysis of textural attributes for cheddar cheeses. BS NO n =8 , DVI NO n=4, DVI YES n=4. Absent = 0, Slight = 5, Definite = 10, Pronounced = 15

The mean scores from the quantitative descriptive analysis of the taste and flavor sensory attributes for the cheese are summarized in Figure 4.7. The attribute scored the highest intensity for all treatments was salt with an average score of 5.75, 5.81, and 5.70 for BS NO, DVI NO, and DVI YES treated cheese, respectively. The acid taste attribute was 1.84, 1.71, and 1.82 for the BS NO, DVI NO, and DVI YES treated cheeses, respectively. The astringent attribute for the BS NO treated cheese was 1.69, and 1.54 for DVI NO treated cheese, and 1.99 for DVI YES treated cheese, on average. Butter flavor means in the BS NO, DVI NO, and DVI YES cheese was 0.74, 0.86, and 0.84, respectively. Mean bitterness scores in the cheeses were 1.08 for BS NO treated cheese, 0.66 for DVI NO treated cheese, and 1.23 for DVI YES treated

cheese. Mean brothy flavor levels were observed as 1.55, 1.51, and 1.47 for BS NO, DVI NO, and DVI YES treated cheese, respectively.

Fruity flavor is aromatics associated with cooked fruits and ester compounds. Fruity flavor in the cheeses was scored relatively low in intensity for all cheeses. The scores for BS NO, DVI NO, and DVI YES cheese was 0.16, 0.28, and 0.18, respectively. Rancid flavor was aroma associated with butyric acid, piquant note. The rancid flavor attribute was also relatively low intensity across all three treatments. The BS NO treatment cheese had a mean rancid flavor intensity of 0.34, the DVI NO cheese a mean score of 0.22, and the DVI YES treated cheese a mean score of 0.54. Savory taste attribute was scored relatively high for all treatments. The mean score for savory attribute was 1.40, 1.50, and 1.45 for the BS NO, DVI NO, and DVI YES treatments, respectively. Sweet taste was the basic taste sensation elicited by sugars. Sweet taste was scored relatively low for all treatments, and the BS NO cheese had a mean score of 0.07, the DVI NO treated cheese had a mean score of 0.16, and the DVI YES treated cheese a mean score of 0.18.

Sulfur flavor is a sulfurous aroma typically associated with aged cheddar and boiled egg. Sulfur flavor was a characteristic that all treatments scored relatively high. The BS NO, DVI NO, and DVI YES treated cheeses were reported to have a sulfur attribute mean score of 1.0.3, 0.92, and 0.94, respectively. Sour is the aroma associated with vinegar or citrus notes. Sour flavor was evaluated in the sensory analysis and the BS NO, DVI NO, and BS NO treatments on mean were scored 0.93, 0.80, and 1.14, respectively. Whey taint is the flavor of entrapped whey within the cheese matrix, entrapped whey becomes fermented as cheese ages. Whey taint is another flavor attribute analyzed in the descriptive analysis with a mean score of 0.41, 0.59, and 0.79, for BS NO, DVI NO, and DVI YES treated cheese, respectively. The last flavor characteristic analyzed is Other, a catch all for any off flavors in the cheese not described by other terms. The BS NO, DVI NO, and DVI YES treated cheese had Other attribute mean scores of 0.12, 0.08, and 0.013, respectively.

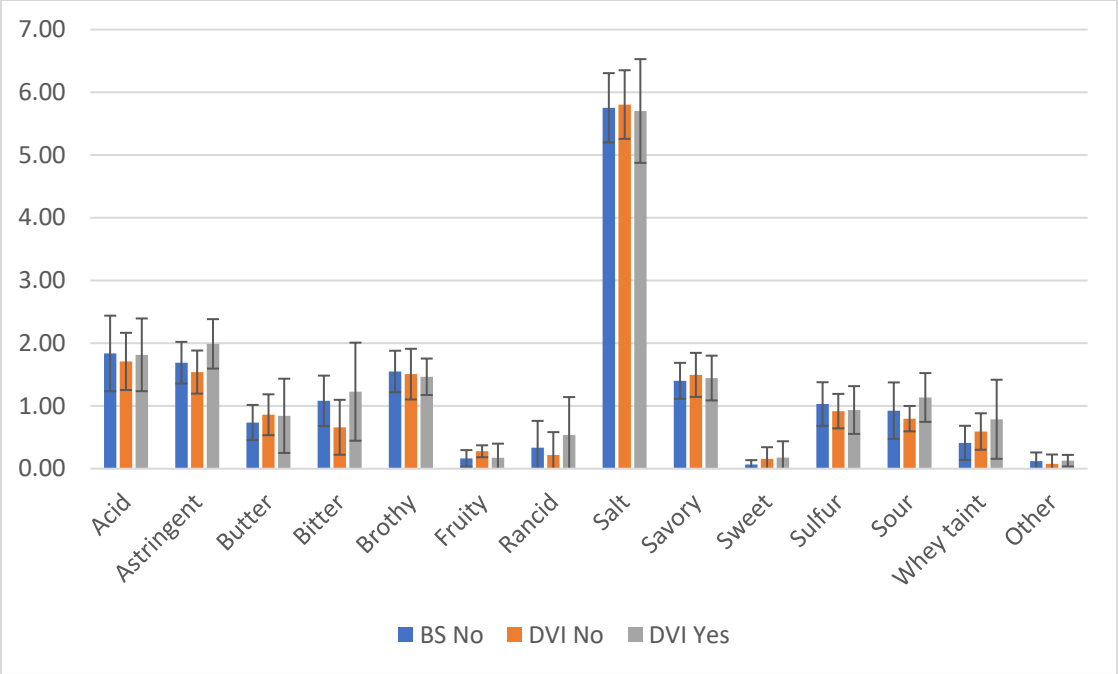

Figure 4.7: Mean scores for quantitative descriptive analysis of taste and flavor attributes for cheddar cheeses. BS NO n =8 , DVI NO n=4, DVI YES n=4. Absent = 0, Slight = 5, Definite = 10, Pronounced = 15.

Statistical Analysis 4.6

Mixed models were fit separately for each response variable while controlling for milk batch or production run to detect differences in production responses, biochemical responses, and sensory responses**.** To limit the number of responses in the model not all free amino acids were fit in to the model. Glutamic acid, leucine and lysine were chosen for statistical analysis. Both raw and adjusted p-values were produced for each response variable. Six response variables were found to have p-values less than 0.05 before adjustments, which are displayed in Table 4.8. After adjusting the p-values using the FDR adjustment for each grouping of response variables, we see that only Coagulation time had a p-value less than 0.05. Multiple adjustments were made separately for each class of responses, production, biochemical, and sensory. The results of the mixed model statistical analysis are summarized in Table 4.8, and only those responses with potential statistical significance are reported (α=0.05). Leucine, lysine, and

protein concentrations in cheeses had p-values less than 0.05 before adjustments for false discovery rate. Leucine had a F-value of 4.15409 and p- value of 0.04862. Lysine concentration in the cheeses had a F-value and p value of 4.42496 and 0.4202, respectively. Protein concentration in the cheeses had a F-value and p-value of 4.98678 and 0.03146, respectively. After adjustments for false discovery rate all three responses had a p-value of 0.27549, indicating they were not significantly different across treatments (α=0.05).

Process data responses coagulation time and whey removal pH were potentially significantly different across the treatments before adjustments were made for false discovery rate. The F-value and p-value reported for coagulation time was 24.71195 and 0.00013, respectively. After testing adjustments for false discovery rate the p-value was 0.00081 for coagulation time, indicating the treatments had a significant effect on this response (α=0.05). Whey removal pH F-value and p-value was 5.545413 and 0.02397, respectively before adjustments were made for false discovery rate. After adjustments were made the p-value was 0.07190, indicating the treatments did not have a statistically significant effect on whey removal pH (α=0.05). The sensory characteristic astringency was the only sensory response variable that was potentially statistically significant in the mixed model analysis (α=0.05). The F-value and p-value were 7.67091 and 0.00957, respectively, before testing adjustments were made for false discovery rate. After the adjustments, the p-value was 0.1879, indicating the treatments did not have a statistically significant effect on this response.

Table 4.8: Mixed model results with significant fixed effects before adjustments

Response	Sum sq	Mean sq	NumDF	DenDF	F value	Raw Pr(>F)	FDR Pr(>F)
Biochemical Data							
Leucine	0.17728	0.08864	2	10	4.15409	0.04862	0.27549
Lysine	0.01512	0.00756	2	10	4.42496	0.04202	0.27549
Protein(%)	0.46214	0.23107	2	10	4.98678	0.03146	0.27549
Process Data							
Coagulation time (minutes)	227.46634	113.73317	2	10	24.71195	0.00013	0.00081
Whey Removal pH	0.01551	0.00776	2	10	5.545413	0.02397	0.07190
Sensory Data							
Astringent	0.42750	0.21375	2	10	7.67091	0.00957	0.18179

Table 4.9 summarizes the pairwise comparisons for the response variables found to have significant differences among treatments before multiple testing adjustments. For coagulation time only BS NO comparison with DVI YES treatments had a p-value above 0.05. The p-values for the comparison of BS NO to DVI NO and DVI NO to DVI YES treatments were 0.00028 and 0.00023, respectively. Only the whey removal pH response for the BS NO comparison to DVI YES treatments had a p-value smaller than 0.05, with a p-value of 0.2278. The pairwise comparison of DVI NO and DVI YES treated cheeses was the only comparison for the protein concentration to report a p-value less than 0.05, with a value of 0.02756. For the leucine concentration response only one pairwise comparison, BS NO compared to DVI YES, had a p-value less than 0.05, and a value of 0.04326. Similarly, lysine concentration in the cheeses also was the only pairwise comparison with a p-value less than 0.05 with the comparison of BS NO and DVI YES cheeses having a p-value of 0.04806. Lastly, the astringent sensory attribute response also had one comparison of treatments with a p-value less than 0.05. BS NO and DI YES comparison had a p-value of 0.03641.

Table 4.9: Pairwise comparisons of treatments

Contrast	Estimate	SE	Df	t ratio	p value
Coagulation Time (min)					
BS NO – DVI NO	-8.09625	1.31373	10	-6.16280	0.00028
BS NO – DVI YES	1.49125	1.31373	10	1.13513	0.51556
DVI NO – DVI YES	9.58750	1.51696	10	6.32019	0.00023
Whey removal pH					
BS NO – DVI NO	0.00625	0.02290	10	0.27289	0.95994
BS NO – DVI YES	0.07375	0.02290	10	3.22015	0.02278
DVI NO – DVI YES	0.06750	0.02645	10	2.55240	0.06802
Protein(%)					
BS NO – DVI NO	-0.29875	0.13182	10	-2.26638	0.10757
BS NO – DVI YES	0.17375	0.13182	10	1.31810	0.41750
DVI NO – DVI YES	0.47252	0.15221	10	3.10425	0.02756
Leucine					
BS NO – DVI NO	-0.13062	0.08945	10	-1.46029	0.34929
BS NO – DVI YES	-0.25312	0.08945	10	-2.82974	0.04326
DVI NO – DVI YES	-0.12250	0.10329	10	-1.18598	0.48730
Lysine					
BS NO – DVI NO	-0.04950	0.02531	10	-1.95557	0.17385
BS NO – DVI YES	-0.07000	0.02531	10	-2.76545	0.04806
DVI NO – DVI YES	-0.02050	0.02923	10	-0.70138	0.76812
Astringent					
BS NO – DVI NO	0.15000	0.10222	10	1.46739	0.34609
BS NO – DVI YES	-0.30000	0.10222	10	-2.93478	0.03641
DVI NO – DVI YES	-0.45000	0.11804	10	-3.81240	0.00871

The results of the MANOVA models, fit separately for each grouping of response variables, is in Table 4.10. Treatment and make data were evaluated for their effect on the process data responses, biochemical data responses, and sensory data responses for all cheeses. Both treatment and make date effects had p-values below 0.05, and were 0.00318, and 0.00036, respectively. The effect of the treatment on biochemical data responses had a p-value of 0.52109, and the effect of make date had a p-value of 0.04391. The effect of the treatment on the sensory response variables had a p-value of 0.80890, and the effect of the make data had a p-value of 0.00002.

Table 4.10: MANOVA model results

Term	df	Pillai	Statistic	Num. df	Den. Df	p value
Process Data						
Treatment	2	1.67863	5.22345	12	12	0.00381
Make Date	3	2.42536	4.92411	18	21	0.00036
Biochemical Data						
Treatment	2	1.51516	1.04170	18	6	0.52109
Make Date	3	2.55838	2.57473	27	12	0.04391
Sensory Data						
Treatment	2	1.09997	0.61108	16	8	0.80890
Make Date	3	2.81340	9.42331	24	15	0.00002

The correlations among process responses and cheese biochemistry responses are displayed in Figure 4.8. For the production responses there was a moderately strong positive correlation observed between the coagulation time and the whey removal pH. There was also a moderately negative correlation observed between the coagulation pH and mill pH in the process. In the cheese biochemical responses there was a strong positive correlation observed between protein per cent and free amino acids glutamine, leucine, lysine, and total free amino acids. As well, strong positive correlations between the free amino acid species leucine, lysine, glutamine, and total free amino acids. A strong negative correlation was observed among those free amino acids, and the ratio of aliphatic and acidic free amino acids. Moisture was positively correlated with moisture in the fat free substances and negatively correlated with protein, fat, salt in moisture, and calcium content in the cheese.

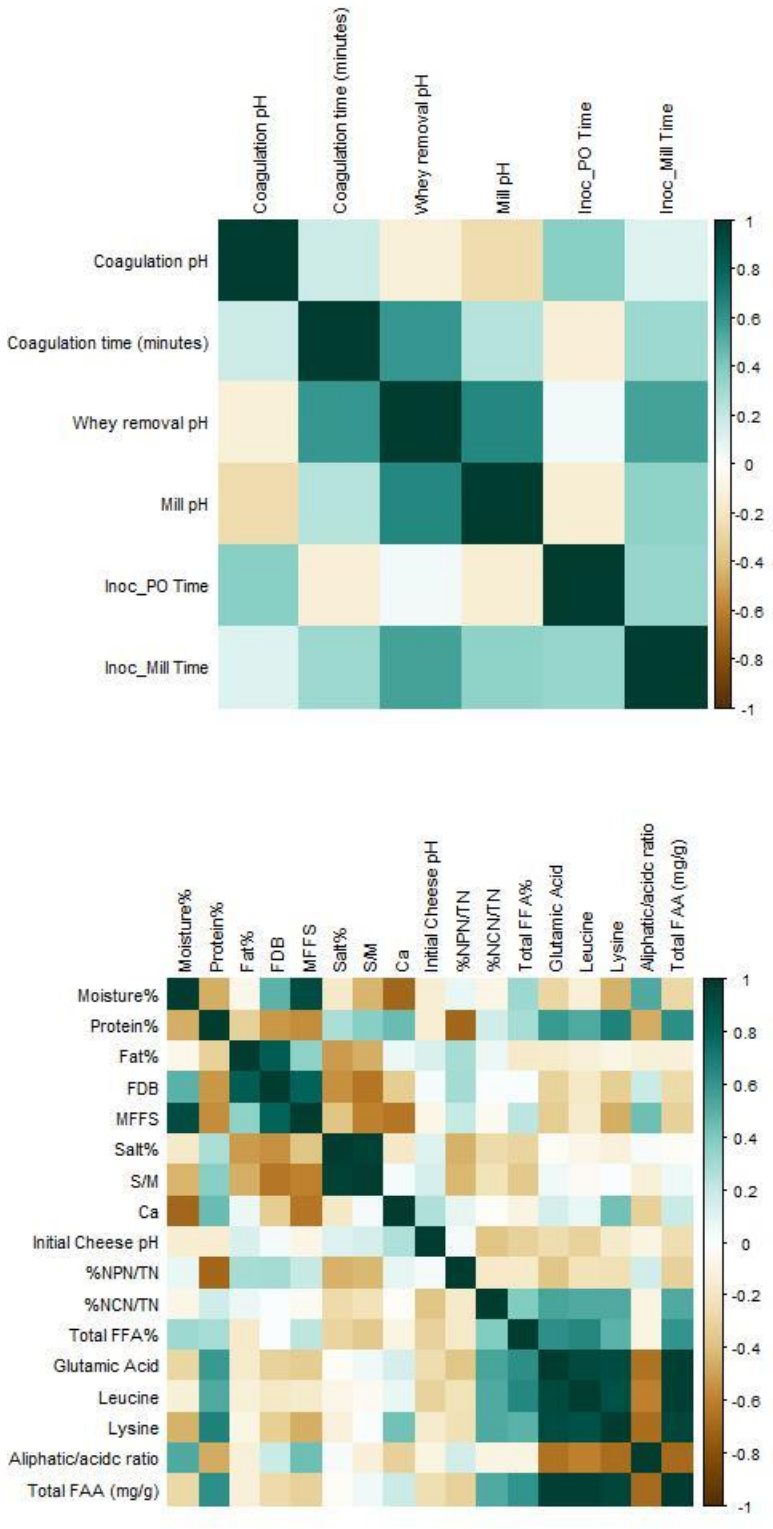

Figure 4.8: Correlation plots of process responses and biochemical responses

Chapter 5: Discussion

Culture Dosage Determination 5.1

Due to variability in the activity of the ripened bulk starter, batch of DVI culture pellets, and milk composition, in particular casein content, the dosage of each culture preparation was established in an activity test that simulated the cheddar time and temperature process parameters by inoculating the culture at three dosages and measuring the overall change in pH after 135 minutes and 220 minutes. This was performed to ensure that the activity of the culture preparation treatments would have the same extent and rate of fermentation at critical time points in the process, giving curd and ultimately cheese of a similar composition. These time points, or feature points, represent the time at which the whey was removed from the curds and the curd was milled and salted in the process, respectively.

The responses were then plotted in a scatterplot and a line fit to allow for a prediction of the dosage of each culture preparation to reach the targeted change in pH for each feature point. The target was established by using a DVI starter culture, CHOOZIT® MC 70 1000 DCU, known to be used in cheddar production in the selected manufacturing process, each time the activity test was run. Using the linear regression equations and the results of the CHOOZIT® MC 70 reference, the dosage of each culture preparation was established to reach the same change in pH at the selected feature points. However, the predicted dosages varied slightly for each feature point, and therefore an average of the dosage needed to reach the target at each time point was calculated and used.

A strong correlation was observed for each of the starter culture preparations for both feature points, in each run of the activity test. This indicates that the dosage was related to the overall change in pH observed in the activity test. The DVI preparation culture was observed to be lower in inoculation rate volume than the bulk starter. This was to be expected because the process of preparing the DVI culture

includes concentration of the cell mass by removal of supernatant in a centrifuge. The first run of the activity test predicted that the DVI dosage rate would be 0.0222% which was lower than the rate predicted in the second run. This can be explained by the increase in protein and casein content in the milk for the second run of cheese production. The casein protein contains calcium phosphate bridges which cross link the different casein proteins together. When acid is produced the hydrogen ion replaces this calcium phosphate in the cross linking, effectively buffering the pH. Therefore, the casein acts a buffer to the acid produced in the milk and curd, and an increase in casein requires more acid to change the pH the same absolute value. The results suggest that it requires more culture to do this in the same production process time.

The third run returned to a similar inoculation rate as the first run, which was expected considering the casein content was similar. The fourth run had the highest rate of inoculation, and this could be explained by the increased casein content in the milk and for this run a different batch of DVI culture preparation was used, which had less activity per gram of culture. The variability in the bulk starter preparation inoculation rate could be explained by the casein content in the milk. The inoculation rate was similar for the first and third runs, which had similar casein content in the milk, and the second and fourth runs, which also had similar casein content in the milk.

Cheese Production 5.2

The rate and extent of acidification in the cheesemaking process indirectly determines the composition of the cheese (Lawrence et al., 1984). The pH of the milk at coagulation was a principal factor affecting the activity of the coagulant and can influence the onset or flocculation of the proteins and therefore the firmness of the coagulum when cut at a standardized time. Cutting of the coagulum was standardized in this study by measuring the onset of flocculation and applying a factor to determine the approximate cutting time, and physically checking the firmness of the coagulum before cutting. This would

ensure that cutting of the coagulum was done at approximately the same firmness across all treatments. The mixed model statistical analysis did not indicate that this response was significantly different across the treatments (p=0.3696) . This could be explained by the high variability in the pH of the milk, which was evidenced by the standard deviations of the coagulation pH, and the number of replicates in the study not being large enough to detect a statistical difference.

The total coagulation time, or the flocculation and the hardening time, was a response that was statistically significant in the mixed model with a p-value of 0.00081 after multiple testing adjustments. This was expected because the rennet activity was influenced by the pH of the milk when the rennet was added to initiate the coagulation step, and cutting was decided by the cutting factor after flocculation was detected. When the BS culture preparation was added, the acid from the bulk starter immediately drops the pH in the milk. In contrast when the DVI culture preparation was added no acid comes with the culture treatment and the milk pH remains the same. This was evidenced by the mean coagulation pH of 6.61 of the DVI NO treatment compared to the BS NO treatment mean coagulation pH of 6.55. This difference in pH explains the difference in coagulation time between the BS NO and DVI NO treatments, 23.56 and 31.46 minutes, respectively. The pairwise comparisons of the treatments for this response showed a p-value of 0.00028, indicating a significant difference between the two treatments. If the DVI NO treatment coagulum was cut at the same time as the BS NO, without any adjustment to coagulant dosage or other factors, the gel firmness would have been less organized and the kinetics of syneresis affected, resulting in compositional differences in the cheese.

The DVI YES treatment cheese production included a pre-acidification treatment of the milk with lactic acid before rennet addition to simulate the acid inherently added with the BS NO treatment. The mean pH at coagulation was 6.51, remarkably similar to the mean pH of the BS NO treatment, 6.55 (p=0.36960). It was also observed that the mean coagulation time of the DVI YES treatment was also similar to the BS NO treatment and was observed to be 21.87 minutes. The pairwise comparison of these

treatments had a p-value of 0.51556 and was found not to be significantly different. The mean DVI YES and DVI NO treatments had significantly different coagulation times with a p-value of 0.00023. This was expected and explained by the addition of the lactic acid to the milk to lower the pH at renneting. The mean cutting factors were 1.66, 1.70, and 1.67 for the BS NO, DVI NO, and DVI YES treatments to control for the firmness of the coagulum at cutting, and ultimately control for syneresis kinetics of the curd and final cheese composition.

The difference in coagulation time (p=0.00081) would have led to differences in timing of the critical steps in cheese production if no adjustment were made. In the case of the DVI NO treatment production the 10-minute curd healing step was removed and stirring and cooking was commenced immediately after cutting, whereas the BS NO and DVI YES treatments had a ten-minute curd healing between the cutting and cooking and stirring steps. This adjustment explains why the whey removal times are similar across all treatments on average and the mixed model analysis did not indicate a statistically significant difference across all treatments for this response (p=0.58932). The whey removal pH was a critical pH in the process of cheese production. The extent of acidification influences the solubility of calcium in the casein which influences cheese texture, and water retention of the curd particles. The mean whey removal pH's of all the treatments were 6.26, 6.25, and 6.18 for the BS NO, DVI NO, and DVI YES treatments, respectively. The mixed model statistical analysis did not report a statistically significant difference in whey removal pH across the treatments (p=0.071897). The mean pH's at this critical step were similar because of the dosage determination activity testing to ensure the extent of fermentation was the same across the treatments. The mean DVI YES was observed to have a slightly lower whey removal pH than the other treatments and could be due to the modestly lower coagulation pH on average for this treatment.

The milling of the curd after matting and draining was also a critical step as it was accompanied by salting, and the abrupt halting of the fermentation. The mean time at which milling occurred was

similar across all treatments and was not found to be statistically significant across all treatments in the mixed model analysis (p=0.58932). This was expected and can be explained by the process in which timing of steps was controlled solely on time, and the determination of the starter culture dosage for each treatment to have the same extent and rate of acid production in the process. The mean pH of the curd for each treatment at milling was also observed to be similar and no statistical differences were observed across all treatments (p=0.87178). This was expected because the activity testing ensured each treatment was dosed at a rate which would give the same extent of fermentation at each step in the process, and that the timing of each step in the process was standardized. The milling and salting pH have an important influence on the moisture content of the final cheese and the pH of the final cheese in the block as well.

Lawrence et al. (1984) suggested compositional ranges for premium quality cheddar cheese in New Zealand. A pH range of 4.95 to 5.1, FDB from 52% to 55%, MFFS 52% to 56%, and salt in moisture 4.0% to 6.0%. The mean composition for the BS NO treated cheeses met all this criterion except the FDB, which was observed to be slightly lower at 51.79%. The DVI NO treated cheeses had a composition which fell in the premium grading for pH, salt in moisture, MFFS, but FDB on average was 51.30%, slightly lower than the suggested range. The mean for the DVI YES treated cheeses compositions were observed to fall in the suggested range except for FDB. Which was 51.83% and was only slightly below the target range. This indicates that composition of the cheeses would be acceptable.

The composition of the cheese from each treatment was not found to be significantly different among any of the treatments as indicated from the MANOVA model results for the biochemical data (p=0.52108). This indicated many of the factors that influence cheese composition such as acidification rate and extent in the process, firmness of the gel at cutting, cooking, stirring, and heating, were all controlled for during the production of the cheese across all treatments. This was also evidenced by the results of the mixed model analysis on showing none of the compositional responses were found to be statistically significant across all treatments after multiple testing adjustments.

The longer time of coagulation with the DVI NO treatment would have led to a ten-minute delay to whey removal and milling times if no other adjustments were made in the production, thus, this ten minutes was removed from the healing step after cutting to ensure timing at these critical steps was controlled for across all treatments in the experiment. If this did not occur it could be expected that the ten-minute delay would have led to deeper extent of acidification at these critical steps, and therefore lower pH. Since acidification increases syneresis of whey from the curd and further demineralizes the casein of calcium phosphate it might have been expected that these differences would lead to compositional differences in the cheese, lower initial pH in the cheese, and potentially textural differences in the sensory analysis of the cheese for this treatment.

Cheese Biochemistry 5.3

The milk quality, composition of the cheese, choice of rennet, lipase enzymes addition, and culture selection directly influence the hydrolysis of protein and fat in the cheese matrix during ripening, and therefore influence the sensory characteristics of the cheese (Lawrence et al., 1984). No lipase enzymes were used in the production of any of the treated cheeses. The rennet, and culture selection was constant for all treatments and each production run used the same batch of milk. As mentioned previously the extent and rate of acidification was controlled to give similar composition of cheese for all treatments. Primary proteolysis was defined as the breakdown of casein into peptide chains, the action of which was mostly influenced by the coagulant (Upadhyay et al., 2004). Primary proteolysis can have a direct influence on textural attributes of the cheese (Fox et al., 2017b). In this study primary proteolysis was measured by quantifying the amount of nitrogen soluble at pH 4.60 and expressing it as a per cent of the total nitrogen in the cheese. The mixed model analysis found no statistical differences among the treatments for primary proteolysis ($p=0.79992$). This was expected considering the rennet was the same across all treatments.

Secondary proteolysis was defined as the further break down of proteins and large peptides into smaller fragments and amino acids that are soluble in 12% trichloroacetic acid and was expressed as a per cent of the total nitrogen content in the cheese. Both the action of the rennet and the proteolytic and peptidolytic capacity of the starter contribute to secondary proteolysis (Rank et al., 1985). Because each production of cheese for each treatment included the same rennet and dose, the same batch of milk, and the same starter strain, but different preparations of starter, any differences in secondary proteolysis might be contributed to the starter culture preparation. However, in the mixed model analysis no statistically significant differences were found for any other the treatments for secondary proteolysis response ($p=0.63520$). Thus, indicating that the treatments did not influence the proteolysis of the cheese nor the proteolytic activity of the strain in the culture.

Lipolysis was defined as the hydrolysis and liberation of fatty acids from the triacyl glycerides was expressed as the per cent of the total fat in the cheese, and comes from the action of lipases, either indigenous to the milk, from the starter or other indigenous bacteria in the milk (Fox et al., 2017a). Lactococci have a relatively small capacity for lipolysis during ripening and cheddar cheese was not typically characterized as having extensive lipolysis (Fox et al., 2017b). The total free fatty acids in the cheese was not observed to be significantly different across all treatments in the mixed model ($p=0.79992$). However, some variability was observed in the mean concentration of free fatty acids and the standard deviations quite large compared to the means.

The DVI YES cheeses were observed to have the highest free fatty acid concentration on average with a value of 0.0095% and a standard deviation of 0.0098%. The BS NO treatment had the next highest mean free fatty acid concentration of 0.0069% with a standard deviation of 0.0071%. The mean of the DVI NO cheeses had the lowest free fatty acid concentration mean of 0.0044% and a standard deviation of 0.0028%. This was most likely due to variable milk quality from the farm or milk processor rather than the treatments of the cheese. Free fatty acids in cheese contribute to the rancid flavor attribute in the

cheeses. It was also observed that the rancid flavor attribute correlated to the free fatty acid concentration. The DVI YES cheeses had the highest mean score of 0.54 ± 0.60, the BS NO had the second highest mean score with 0.34 ± 0.43, and the DVI NO cheeses had the lowest score of 0.22 ± 0.37. This high degree of variability was not expected nor was the rancid flavor in the cheese without the use of lipase, and most likely was linked to milk quality.

The liberation of free amino acids was attributed to the action of amino peptidase enzymes from the starter culture and non-starter lactic acids bacteria in the cheese. Some of the most important amino acids in cheddar flavor are leucine , glutamic acid, and lysine, which are liberated by aminopeptidases PepL, PepA, PepC, and PepN (Upadhyay et al., 2004). Glutamic acid contributes to a savory umami flavor which was a key characteristic of cheddar cheese, and leucine further participates in amino acid catabolism to produce sulfur containing compounds which are known to create the characteristic cheddar note. Lysine is considered one of the most abundant amino acids in cheeses during ripening and its concentration in cheese has been proposed as a measure of ripening time (Ciriello et al., 2015). Because of their relative importance in cheddar and cheese ripening and the need to limit the number of responses in the mixed model only these three amino acids were analyzed in the mixed model for statical significance across the treatments.

Glutamic acid was not statistically significant in the mixed model before multiple testing adjustments (p=0.41062). However, leucine and lysine were statistically significant before multiple testing adjustments with p-values of 0.04862 and 0.4202, respectively. After multiple testing adjustments the p-value for both was 0.27549 and not concluded to be statistically significant. Multiple testing adjustments are used to protect against false conclusions of significance or the error rate. This was especially important when the number of replications of the experiment was low, and the number of responses was high. The likelihood of finding statistical significance can be higher but certainty of the conclusion was lower.

the means of BS NO, DVI NO, and DVI YES cheeses had sequentially increased concentrations of the most abundant free amino acids. This trend could be explained by the number of cells of starter culture inoculated into the cheese milk. Because the cells in the bulk starter are in a metabolically active state compared to those cells in the DVI preparation, which are frozen, it could be that a higher number of cells was needed in the DVI treated cheeses to reach the same extent and rate of acidification in the process. This higher concentration of cells would lead to modestly higher concentrations of amino peptidase enzymes in the cheese and could explain the trend. The amount of dead damaged or lysed cells in the direct to vat culture preparation could also be higher due to the stress of processing and deep freezing, allowing intracellular enzymes to potentially be released into the milk and trapped in the curd. However, these enzymes and the resulting amino acids formed from protein catabolism are mostly water soluble and would be presumed to go out with the whey.

Quantitative Descriptive Analysis 5.4

The cheeses were evaluated by a trained panel to quantitatively describe the sensory characteristics on a scale of one to twelve. Tactile descriptors are used to describe the texture sensation of touch of the cheese when manipulated between the fingers and when chewing. Texture of cheese is influenced by several factors including the composition of fat, protein, and water in the cheese, the extent of demineralization of the casein and therefore the rate and extent of acidification during production, the extent of proteolysis in the cheese, and the final pH of the cheese. No significant differences were detected for adhesive ($p=0.56203$), cohesive ($p=0.81979$), firmness ($p=0.81979$), chewiness ($p=0.81979$), and particle size ($p=0.81979$) in the mixed model results. This could be explained by control of the rate and extent of acidification for each treatment so that the acidification profile of each of the cheeses in production was similar from one treatment to the next. This was supported by the results of the mixed model reporting no significant differences for any production responses except the coagulation time ($p=0.00081$). No significant differences were detected in the mixed model for composition of the cheeses

across all treatments, including for pH (p= 0.63520), and calcium content (p=0.41062). Further supporting the findings of the descriptive analysis was the extent of primary proteolysis (p=0.79920) and secondary proteolysis (p=0.63520) for each of the cheeses, of which the mixed model reported no significant differences among treatments.

Taste and flavor characteristics typical to cheddar cheese were also evaluated by the trained panel in the quantitative descriptive analysis. Many flavor compounds are derived from volatile organic compounds which are formed by the catabolism of free amino acids, often by chemical reactions (Fox et al., 2017a). Cheddar is typically characterized by a prominent acid and salt taste with savory, brothy, sulfur, umami flavors, and sometimes with a slight fruity or sweet note. All treatments reported the highest scores for salt, acid, brothy, savory and astringent characteristics. Astringent was reported in the mixed model as statistically significant before multiple testing adjustments with a p-value of 0.0095. After multiple testing adjustments the p-value was 0.18179, which cannot be concluded as statistically significant. The lack of effect of the treatments on the taste and flavor sensory attributes could be explained by the observation that the chemistry of the cheeses was similar, the milk used to produce the cheeses was from the same batch each production run, and that the same strain was used as the starter, albeit delivered to the vat in different treatments. Since the same strain was used for all treatments the enzymology of the starter culture did not differ among treatments and therefore a similar pattern of proteolysis and peptidolysis could be assumed to have occurred. If any differences were expected to be detected among treatments it might be that some taste and flavor attributes would be more intense, however that was not the case in this study.

Statistical Analysis 5.5

A mixed model statistical analysis was used to incorporate fixed effects and random effects of the study design to determine if responses were significant across treatments. The responses were categorized in to process responses, biochemical responses, and sensory responses. Some of the responses were limited to avoid overfitting of the data and ensure statistical validity. There were forty-three responses included in the mixed model. Each mixed model treatment was included as a fixed effect and the batch of milk was included as a random effect. The decision to include the batch of milk was made to account for the variability among different batches of milk and production dates. Due to multiple testing, FDR (False Discovery Rate) adjustments were made after fitting the mixed models. The FDR adjustments were made separately for each response variable grouping (Process, Biochemical, and Sensory). Diagnostic plots were investigated to check model assumptions, and no major violations appeared.

Six response variables were found to have p-values less than 0.05 before adjustments, which are displayed in the Table 4.8. After adjusting the p-values using the FDR adjustment for each grouping of response variables, we see that only coagulation time had a p-value less than 0.05 ($p=0.00081$). The coagulation time was expected to be different between BS NO and DVI NO, and DVI YES and DVI NO treatments because of the effect of the BS treatment on the pH of the milk, and pH was a significant factor affecting the activity of rennet in the cheesemaking process. This agrees with the pairwise comparisons reported. However, the coagulation pH was reported as having a p-value greater than 0.05 before the multiple testing adjustments ($p=0.36960$). This could be due to the small number of replicates and the high variability in milk pH and quality.

The MANOVA model results for the process data evaluated the effect of treatment and make date . The treatment was found to have a significant effect on the process data with a reported p-value of

0.00381. This was expected because of the effect of the acid coming with the bulk starter, and the DVI with pre-acidification treatment towards the chemical composition of the milk. This change in pH of the milk affects the activity of the rennet and therefore the coagulation time. The addition of acid into the milk before renneting also demineralizes the protein, removing buffering capacity and allowing the pH to change more quickly given the same amount of acid production from the culture. All of this considered in a timing-based production means we could expect differences in pH at the whey removal and milling steps in production. Make date or milk batch was also reported to have a significant effect on the process data ($p=0.00036$). Milk composition, especially casein content and acidity, can influence the buffering capacity of the milk and therefore the pH at renneting and the ability of the culture to change the pH during production steps.

The treatments were not reported to have a significant effect on the biochemical data of the cheeses with a reported p-value of 0.52109. This was not surprising because the composition of the cheese is largely influenced by the manufacturing procedure as well as the rate and extent of acid production during the process. The dosage determination experiments controlled for rate and extent of acid production, and the process was standardized across all treatments. The results of the composition responses in the mixed model are also in agreement with this conclusion. The concentration of free amino acids was most likely linked to the concentration of bacterial cells carrying the intracellular peptidases, and therefore if there was a significantly higher concentration of starter culture cells in any given treatment it could be expected that concentration of any given free amino acid would be higher.

The DVI treatments did have higher concentrations of total free amino acids than the BS treatment, but this was not reported as significant in the mixed model ($p=0.38461$). This does not agree with the results of Salji and Kroger (1981), who reported higher level of free amino acids in conventional bulk starter cheeses compared to concentrated DVI culture cheeses. While the researchers did use multi strain cultures containing the same strains in both the culture treatments, the growth of bulk starter could

have changed the ratios of the strain composition in this treatment compared to the DVI treatment, and therefore the differences could possibly be attributed to differences in enzymatic activities of the strains composing the cultures. However, in the current study the lack of significant effect of the treatments on the total free amino acids (p=0.384661) and biochemical composition (p=0.52108) of the cheese could be due to too few replications of the experiment. Though the starter culture treatments in the present study were dosed on activity rather than cell count it was reasonable to expect that the DVI treatments were inoculated at a higher cell concentration per volume of milk due to the different state of metabolism of the culture treatments when added to the milk. As well, when acid came with the culture treatment this changed the pH of the milk and lowered the buffer capacity of the milk, which means less acid production, and therefore bacterial growth was needed to reach the pH targets and endpoint.

The make date or batch of milk had a significant effect on the biochemical data with a reported p-value of 0.04391. This was expected because the chemical composition of the milk can play a significant role in the final composition of the cheese, especially the concentration of fat and protein, as well as the pH and acidity of the milk. Even when the protein to fat ratio was standardized, varying protein and fat concentrations can influence the casein gel network and therefore the kinetics of syneresis during production. These findings are also in agreement with Salji and Kroger (1981a) who reported variation in individual cheese composition from batch to batch.

The sensory data were also evaluated for significant effects of treatment and make date or milk batch. The treatment effect p-value of 0.80890 was not found to be significant. This was also in agreement with Salji and Kroger (1981a). However, the milk batch was found to have a significant effect on the sensory attributes of the cheeses when evaluated by a trained panel for quantitative descriptive analysis (p=0.00002). This was most likely to due to the differences in chemical and microbial composition of the milk. Though the composition of the milk was not evaluated for significant differences from batch to batch it was clearly seen that differences did exist in protein and fat content. Microbial quality was no assessed

in the milk, but titratable acidity could be used as an indicator of microbial quality in the milk and considerable differences were observed in titratable acidity from batch to batch of milk. When acidity was higher the rancidity was detected in cheeses made from these batches of milk and could also indicate lesser microbial quality milk.

A moderately strong correlation was detected between coagulation time and whey removal pH. This was expected because rennet activity was significantly influenced by pH, and as pH decreases rennet activity increases resulting in faster coagulation time, or vice versa (Fox et al., 2017b). A moderately strong correlation was also reported between the responses for whey removal pH and milling pH. This also was expected in a timing-based cheese production process and can be explained by the cultures ability to produce acid at a constant rate under consistent conditions, and therefore the overall change in pH remains constant but as the whey removal pH increases so does the milling pH. A moderate negative correlation was observed between coagulation pH and milling pH, which can be explained by the demineralization of the casein by acid production which concomitantly reduces the pH. This lowers the buffer capacity of the curd and therefore with the same amount of acid production a lower pH would be obtained at the milling step in production.

Moisture was positively correlated with moisture in the fat free substances and negatively correlated with protein, fat, salt in moisture, and calcium content in the cheese. This can be explained because the calculation for moisture in fat free substances is dependent on the moisture content of the cheese. As well, the ratio of the moisture fat, and protein in the cheese are all dependent on the concentration of one another. As moisture increases the other components must decrease. In this study it was reported that protein was more significantly negatively correlated to moisture concentration than fat concentration. As moisture increases salt in the moisture phase decreases since salt was added at a constant rate of curd mass obtained from the cheesemaking process. Calcium content also decreases as

moisture content increases because most of the calcium is found in the casein protein, which also decreases as moisture increases.

Strong positive correlations were found among the free amino acid species leucine, lysine, glutamic acid, and total free amino acids. This would be expected since the peptidase activity of LAB can have wide specificity, especially aminopeptidase N which is well documented in lactococci, for various amino acids, including leucine, lysine and glutamic acid (Pederson et al., 1999), thus it can be expected that as some of these free amino acids increase concomitantly so would other species and the total amount of free amino acids. A strong negative correlation was observed among those free amino acids, and the ratio of aliphatic and acidic free amino acids. That can be explained because glutamic acid was the most concentrated free amino acid reported in the cheeses and is an acidic amino acid, so as its concentration increase the ratio would be expected to decrease. However, leucine is an aliphatic free amino acid, and it would be expected as its concentration increases in the cheese the ratio would also increase, but since a strong correlation was observed between the concentration of leucine and glutamic acid it could be that the peptidase activity of the culture was more specific to glutamic acid and increased the concentration of this free amino acid at a higher rate than leucine.

Chapter 6: Conclusion

In this research, it was hypothesized , there would be no difference in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and DVI culture preparations without recipe adjustment for expected differences in coagulation. It was also hypothesized that no difference would be observed in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and DVI culture preparations with recipe adjustment for the coagulation step using pre-acidification. Rate and extent of acidification were controlled by using a dose response study to predict the dosage of each of the culture preparations to reach the desired pH at critical steps in the cheesemaking process. The timing and mechanical conditions of the cheesemaking process were standardized for all treatments. The thermal conditions of the cheese process were also standardized for all treatments in the cheesemaking process.

Based on the in-process results of the pH at the critical stages of cheesemaking, between whey removal ($p=0.71897$) milling ($p=0.87178$) and the timing of the cheesemaking there were no statistical differences between the BS NO and DVI YES treatments in the production of the cheddar cheeses. However, the BS NO and DVI NO cheeses did have one statistically significant difference in the production of the cheeses, the total coagulation time ($p=0.00028$) to cut the coagulum at the same firmness. This was anticipated because of the influence of pH on coagulant activity, and the influence of the bulk starter and pre-acidification preparations on the coagulation pH. Practically, this means if a cheesemaker were to consider using a direct to vat prepared culture and did not want to change the timing of the coagulation step, pre-acidification would be needed or an increase in coagulant dosage. Otherwise, more time would be required for the coagulation step for the cheesemaker to cut the coagulum at the same firmness.

The rate and extent of acidification, timing of the process, thermal and mechanical processes in cheesemaking, and gel organization or firmness at cutting all influence cheese composition. The statistical

analysis of the composition of the cheeses reported no statistical differences among any of the treatments (p=0.52109). This result was most likely influenced by the decision to cut the coagulum at the same firmness and therefore gel organization, rather than at the same time for the DVI NO treatment. In turn the similar compositions of the cheeses then influence the biochemical changes that occur during ripening. No statistical differences were detected in the cheddar cheeses for primary proteolysis (p=0.79992), secondary proteolysis (p=0.63520), total free amino acids (0.38461), or free fatty acid content (0.79992) for any of the treatments.

The catabolism of carbohydrates, protein, and fats significantly influences the sensory characteristics of cheese. Since no differences were observed in the composition of the cheeses it would be rational to assume any differences in cheese sensory properties would be due to differences in enzymatic activities of the treatments. However, no differences were detected in the extent of protein or fat catabolism in the cheese as reported earlier by the content of free amino acids and free fatty acids. In turn no statical differences were observed in the cheeses for any of the sensory characteristics assessed in the quantitative descriptive analysis for any of the treatments. However, from the results of the MANOVA statistical analysis it can be concluded that the treatment did have a statically significant effect on the process of cheesemaking (p=0.00036). The mixed model results showing that the coagulation time was significantly different among the BS NO and DVI NO treatments (p=0.00028) further support this conclusion. It can also be concluded from the mixed model results that the make date or milk batch, had a significant effect on the process (p=0.00036), biochemistry (p=0.04391), and sensory characteristics (p=0.00002) of the cheeses made with the treatments. We can conclude that the majority of the variability among the treatments for the process, composition, ripening biochemistry, and sensory characteristics was due to the make date or milk batch.

It can then be concluded that we fail to reject the null hypothesis that there was no difference in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and

DVI culture preparations without recipe adjustment for coagulation. This is supported by the statistical analysis of the process data, cheese composition and biochemistry, and cheese sensory characteristics. It can also be concluded that we fail to reject the null hypothesis there was no difference in cheddar proteolysis, lipolysis, and sensory characteristics in cheeses manufactured with bulk starter and DVI culture preparations with recipe adjustment for coagulation. This also is supported by the statistical analysis of the process data, cheese composition and biochemistry, and cheese sensory characteristics.

Further research into this topic is still needed. In this study the number of replicates for each treatment had to be limited due to resource constraints in the pilot cheese making facility. It was therefore not possible to replicate the experiment more than the number of times in the current study due to these limitations. The number of replicates limited the power of the study and therefore follow up studies should include a higher number of replicates to further investigate if differences in cheese sensory characteristics exist between cheeses using DVI or BS technology to deliver the culture to the vat. In a future study the starter culture preparations should be further investigated for cell count, proteolytic, peptidase, and lipolytic activity.

This was not included in the current study due to resource constraints. It could however provide insight into the number of cells inoculated into the milk to provide the needed acidification activity and the metabolic state at inoculation might be inferred. However, the measurement of proteolysis also infers cell count at inoculation and the end of fermentation if we consider the starter cultures are the primary source of secondary proteolysis, it is reasonable to assume a higher number of cells in the cheese at end of fermentation would lead to more extensive proteolysis. In the current research the trend was toward more extensive proteolysis and higher concentrations of free amino acids in the DVI treatments compared to the BS treatment. However, the differences were not statistically significant with the number of replicates.

A future study might also consider starter culture treatments composed of multiple strains, which more closely resembles current starter cultures used in industry. The current research did not use multiple strains because of the difficulty controlling for strain ratios in the bulk starter preparations compared to DVI, and the intent was to isolate the variable of culture preparation and mode of inoculation to the greatest extent possible. If a direct comparison of the impact of starter culture preparation would be made in a future study with multiple strains this would be an important consideration to control for, or another treatment could include a bulk starter without controlling for strain ratios for comparison. This could answer questions about potential cheese quality impacts when cheesemaker expertise is lost from the industry.

As well, a further study should also investigate if cutting the coagulum based on timing rather than a cutting factor determined by flocculation would lead to compositional and sensory characteristic differences among cheeses produced with the same culture preparation technology and different culture preparation technology. In future studies the quantitative descriptive analysis of the cheeses should include more trained panelists to increase the likelihood of finding significant differences in the cheese sensory characteristics. Lastly, a consumer sensory test could also be conducted to ascertain if consumers can detect differences in cheddar cheese produced with bulk starter of direct to vat starter culture preparation technologies. These future study considerations could be carried out in a production facility rather than pilot to further relate to real world application of starter cultures.

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APPENDICES

APPENDIX 1

CONFIDENTIAL DISCLOSURE AGREEMENT

THIS CONFIDENTIAL DISCLOSURE AGREEMENT ("Agreement") effective **August 11, 2020** ("Effective Date") is entered into by and between **Danisco US Inc.**, a Delaware corporation with offices located at DuPont ExperimentalStation, Building E353, 200 Powder Mill Road, Wilmington, DE 19803 U.S.A. and operating as part of the Nutrition &Biosciences business (together with all Affiliates (as defined herein) collectively herein referred to as "Discloser") and **The Board of Governors of the Colorado State University System, acting by and through Colorado State University**, an institution of higher education of the State of Colorado, for the benefit of the Department of Food Science and Human Nutrition located at 234 Gifford Building, Fort Collins, Colorado 80523 (herein referred to as "Receiver").

The Agreement is written to facilitate discussions and the exchange of "Confidential Information" between or on behalf of, **Discloser and Receiver** (individually a "party" and collectively the "parties" and their respective "Representatives" (as defined below)) concerning: James Musetti's (hereinafter "Student") thesis work, as a student of Receiver and employee of Discloser, directed to evaluation of differences of using bulk starters versus direct vat inoculation in industrial cheese making in cheese quality, functionality and production reproducibility, which student will undertakepursuant to his employment with Discloser (herein collectively referred to as the "Purpose"). In order to undertake the thesis work and disclosure of information related thereto, each party agrees that the information developed and furnished by Student in the course of his employment, shall be held and used by Receiver, in accordance with the terms of this Agreement.

The parties agree that the following conditions shall govern such disclosures:

1. **Information**. Information is "Confidential Information" if it relates to the Purpose and is disclosed by the Discloser, its Affiliates, or any of its of its respective Representatives, including Student, to the Receiver. Confidential Information includes, but is not limited to, information disclosed in writing, orally, visually, electronicor other media, or in other tangible form and information acquired by observation or otherwise during any site visit at the other party's facilities. Confidential Information includes, but is not limited to, all proprietary technologies, know-how, trade secrets and any other intellectual property (whether or not patented), analyses, compilations, business or technical information or other materials prepared by the Receiver, its Affiliates or any of their respective Representatives, containing or based in whole or in part on any Confidential Information of the Discloser. Confidential Information also includes the existence of this Agreement, and its terms and the fact that the Receiver is evaluating the Discloser's Confidential Information. Confidential Information will not, however, include any information which:

    (a) is or becomes publicly known through no fault of the Receiver or in breach of this Agreement;

    (b) is lawfully received by the Receiver from a third party that has no obligation of confidentiality to theDiscloser regarding such information;

    (c) is already known by the Receiver before receipt hereunder, as shown by its prior written records; or

    (d) was or is independently developed by or for the Receiver without use of the other party's ConfidentialInformation, as evidenced by Receiver's written records.

Each party shall bear the burden of proving by clear and convincing evidence the existence of the preceding exceptions to its obligations of confidentiality and restrictions on use under this Agreement. If a party, at any time, elects to rely on any of the exceptions set forth above, then, before making any disclosure or use of Confidential Information of the other party for any purpose other than in performance of this Agreement, the partyshall give prior written notice to the other party of its intent to so disclose or use and shall supply the other partywith: (i) an identification of such Confidential Information and the exception relied upon, (ii) the basis for its reliance upon such exception, and (iii) the nature of the intended disclosure or use.

2. **Explanation of Exceptions**. Information disclosed hereunder shall not be deemed to be within the foregoing exceptions merely because such information is embraced by more general knowledge in the public domain or inthe Receiver's possession. In addition, no combination of features shall be deemed to be within the foregoing exceptions merely because individual features are in the public domain or in the Receiver's possession, unless the combination itself and its principle of operations are in the public domain or in the Receiver's possession.

3. **Obligations Concerning Confidential Information**. From the Effective Date and until five (5) years after the expiration or termination of the "Disclosure Period" (as defined herein), the Receiver shall not disclose the Confidential Information to any third party (including, without limitation, any patent office) and shall use the Confidential Information only for the Purpose; provided, however, notwithstanding the foregoing, nothing herein shall in any way restrict Discloser from disclosing its Confidential Information to any third party or from using Confidential Information in any manner for any purpose at its sole discretion. Any Confidential Information that is a formula, trade secret, know-how and/or regulatory information, shall remain Confidential Information under applicable law, or until they are no longer considered confidential by virtue of the fact that it falls under an exception to confidentiality as set forth in Section 1 above. Notwithstanding the foregoing, if the Receiver is required by law, rule or regulation to disclose Confidential Information, it shall give the Discloser prompt written notice of such requirement for and contents of such disclosure, as is practicable under the circumstances, prior to such legally required disclosure, however, shall not relieve the Receiver of its obligations contained in this Agreement.

4. **Duty of Care**. The Receiver shall use the same degree of care with respect to its obligations under this Agreement as the Receiver employs with its own information of a similar nature, but no less than a reasonable degree of care. Neither party shall disclose Confidential Information received hereunder or generated using Confidential Information received hereunder to anyone other than their Affiliates, or any of their respective officers, directors, agents, accountants, attorneys, consultants or other professional advisors (collectively "Representatives"), who need to know the Confidential Information other than for the Purpose, are advised of the contents of this Agreement, and are similarly obligated to maintain the Confidential Information as confidential byobligations of confidentiality at least as restrictive as the terms of this Agreement. Each party will observe that its Affiliates, or any of their respective Representatives use of the Confidential Information only for the Purpose stated above and shall be responsible for any breach of this Agreement by its Affiliates or their respective Representatives.

5. **No License**. Except for the limited right to use Confidential Information for the Purpose, no right, conveyance orlicense, express or implied, is granted hereunder to any intellectual property right of either party. All work performed by Student shall be solely owned by Discloser and all Confidential Information received by any party and any developments materially derived therefrom are and shall remain the exclusive property of the DiscloserAll results and any intellectual property developed by Student shall be the sole and exclusive property of Discloser.

6. **Affiliates**. A party may directly involve one or more of their Affiliates in the discussions under this Agreement, which has agreed to be bound by this Agreement, and which shall be considered to be a "party" for purposes ofthis Agreement. "Affiliate(s)" shall mean any with respect to any individual, corporation, partnership, limited liability company, association, trust, unincorporated entity or other legal entity (each a "Person"), any other Person which directly or indirectly through one or more intermediaries, controls, is

controlled by, or is under common control with such Person. “Control’ (including, with correlative meanings, “controlled by” and “under common control with”) shall mean possession, directly or indirectly, of the power to direct the management and policies ofa Person, whether through the ownership of at least 50% or more of voting interests of such Person, through contract, or otherwise.

7. **Return of Information**. Upon written request of the Discloser, the Receiver shall return or, at the option of the Discloser, destroy (and confirm in writing - email being sufficient - to the Discloser that it has destroyed) all written, tangible and electronic forms of the Confidential Information it has received from the Discloser; provided, however, that each party understands that the other party may not be in a position to destroy all computer recordsor files that have been created pursuant to Receiver’s automatic archiving and back-up procedures. However, both parties acknowledge and agree that any such computer records or files created pursuant to Receiver’s automatic archiving and back-up procedures will be deleted in accordance with the Receiver’s standard retentionpolicies. Notwithstanding the foregoing, Receiver may retain one copy of such information for the sole purpose of monitoring its obligations under this Agreement.

8. **Disclosure Period, Termination and Continuing Obligations**. The period for disclosing information will begin on the Effective Date and end on **August 11, 2021** unless earlier terminated by either party upon thirty (30) days’written notice or extended by mutual written consent (“Disclosure Period”). Termination or expiration of the Disclosure Period shall not relieve either party of any obligations of confidentiality and non-use as imposed underthis Agreement, which shall continue for a period of five (5) from such termination or expiration of the DisclosurePeriod in accordance with Section 3 above.

9. **Publications.** Receiver and Student may wish to seek publication of information related to the Purpose in accordance with normal academic practice, and in pursuance of the Receiver's academic functions. However, the Receiver will give Discloser thirty (30) days’ prior written notice of any publications proposed by Receiver thatincludes publication of Discloser Confidential Information, including publication of thesis documents in Receiverlibrary or websites. Discloser will have the right to comment, edit and remove proprietary information. Any publication of Discloser information will require express written permission which will not be unreasonably withheld.

10. **Confidentiality of Agreement**. The existence and terms of this Agreement shall be considered Confidential Information subject to this Agreement. Further, no party shall use the name of another party in any publicity or advertising without that party’s prior written approval.

11. **No Warranty**. Neither party makes any representation or warranty concerning the accuracy or completeness ofany information disclosed hereunder. The Discloser shall have no liability to the Receiver in connection with thisReceiver’s use of the Discloser’s Confidential Information.

12. **Choice of Law**. This Agreement shall be construed in accordance with the laws of the State of Colorado, U.S.A.,but excluding the conflict-of-laws principles of Colorado and all other jurisdictions. The sole and exclusive venueto bring any action under this Agreement shall be the state and federal courts in Fort Collins, Colorado U.S.A. The parties hereto submit to the jurisdiction of such courts. This Agreement does not prevent either party from seeking injunctive relief as a remedy to protect against any breach or threatened breach of this Agreement.

13. **Export Control; Compliance with Law**. Use and disclosure of information acquired hereunder shall be subjectto all pertinent laws and regulations of the country of the party disclosing the information, including export control laws and regulations of the U.S.A. and any other country applicable to the export or re-export of information, equipment, and/or products produced by use of such information provided hereunder.

14. **Assignment**. This Agreement is not assignable or otherwise transferable by either party without the prior writtenconsent of the other party; provided, however, notwithstanding the foregoing, Discloser may, without Receiver’sprior written consent, assign or otherwise transfer this Agreement, or delegate its rights

or obligations hereunder,in whole or in part, to: (i) any current or future Affiliate, and/or (ii) any purchaser, assignee, successor or other transferee (in whole or in part) of the business to which this Agreement relates, and/or (iii) any entity that is formed to conduct all or a portion of the business currently conducted by Discloser's Nutrition & Biosciences business.

15. **Expenses**. Each party shall bear its own expenses under this Agreement and Student's work shall be at Discloser's discretion and expense.

16. **Contacts for Agreement**: The contacts for this Agreement shall be:

For Discloser:	**For Receiver:**
Name: Cindy Stewart	Name: Martha Stone
E-mail: cindy.stewart@dupont.com	E-mail: Martha.Stone@ColoState.EDU
Phone: 302-685-3772	Phone: 970-491-6772

17. **Entireties**. This Agreement contains the entire agreement and understanding of the parties pertaining to the Purpose and shall be amended, modified and waiver only in writing agreed to by both parties. Further, nothing contained in this Agreement shall be construed by any party as an obligation to enter into any further agreementconcerning the Purpose or Confidential Information.

18. **Other Projects**. Notwithstanding anything contained herein, the parties acknowledge that each has and will haveunder development, both internally and through third parties, various projects relating to the general subject matter of the discussions and disclosures contemplated under this Agreement, including other product developments and services. For the sake of clarity, so long as a party does not breach its obligations under thisAgreement, nothing in this Agreement shall be deemed to limit or restrict either party from pursuing, alone or in conjunction with others, any business or activity, whether similar to or competitive with the Purpose.

19. **Existing Agreements**. This Agreement does not supersede or replace previous confidentiality agreements between the parties concerning the Purpose and this Agreement will not affect any rights or obligations of the parties which are accrued or outstanding prior to the Effective Date pursuant to any prior agreement between theparties.

20. **Original Version**. This Agreement may be executed in counterparts, or facsimile versions, each of which shall be deemed to be an original, and both of which together shall be deemed to be one and the same agreement. Should this Agreement be translated into a language other than English, the English version of this Agreement shall control. Fax and/or (*.pdf) signatures shall be treated as original signatures for purposes of this Agreement.

21. **Severability**. The invalidity or unenforceability of any provisions of this Agreement shall not affect the validity orenforceability of any other provision of this Agreement, which shall remain in full force and effect.

IN WITNESS WHEREOF, the parties have caused the Agreement to be executed by their duly authorizedrepresentatives.

DANISCO US INC.

Casper Vroemen (for Cindy Stewart)
Casper Vroemen (for Cindy Stewart) (Sep 11, 2020 08:17 PDT)

(Signature)

Cindy Stewart
(Printed Name)

VP Research
(Title)

Sep 11, 2020

THE BOARD OF GOVERNORS OF THE COLORADO STATE UNIVERSITY SYSTEM, ACTING BY AND THROUGH COLORADO STATE UNIVERSITY

Martha Stone
Martha Stone (Sep 4, 2020 12:42 MDT)

(Signature)

Martha Stone
(Printed Name)

Professor, Food Science and Human Nutrition
(Title)

James Musetti (Sep 2, 2020 12:25 MDT)

(Signature)

James Musetti
(Printed Name)

Student
(Title)

APPROVED AS TO FORM

Brian Anderson
Brian Anderson (Sep 2, 2020 12:18 MDT)

Brian Anderson,
Esq. Assistant
Legal Counsel
Office of the
General
Counsel

COLORADO STATE UNIVERSITY

03/21/2023

Dear Participant,

My name is James Musetti and I am a researcher from Colorado State University in the Food Science and Human Nutrition department. We are conducting a research study on the impact of starter culture technology on the sensory and biochemical characteristics of cheddar cheese. The title of our project is Biochemical and Sensory Characteristics of Cheddar Cheeses made with Bulk Starter and Direct to Vat Inoculant. The Principal Investigator is Marisa Bunning and the Co-Principal Investigator is James Musetti.

We would like you to perform qualitative sensory analysis at the Center for Dairy Research at University of Wisconsin. Participation will take approximately 1 hour. Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participation at any time without penalty.

The researchers will not collect or receive any data specific to the identity of the human participants and all data will be collected anonymously While there are no direct benefits to you, we hope to gain more knowledge on the impact of the starter culture technology on the sensory characteristics of cheddar cheese.

There are no known risks associated with participation.

If you would like to participants or have any questions, please contact James Musetti or Marisa Bunning at Marisa.Bunning@Colostate.edu. If you have any questions about your rights as a volunteer in this research, contact the CSU IRB at: CSU_IRB@colostate.edu; 970-491-1553.

Sincerely,

Marisa Bunning
Professor and Extension Specialist

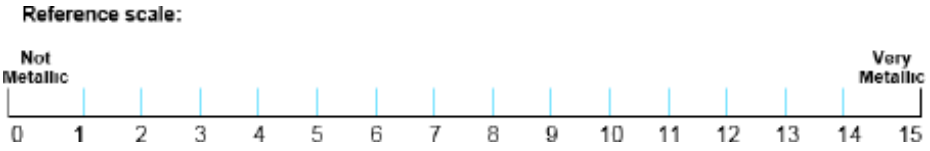
James Musetti
Ph.D. Candidate

APPENDIX 3

	Term	Definition
TEXTURAL ATTRIBUTES	Hand firmness	Amount of force required to compress the sample.
	Cohesiveness	Degree to which the chewed mass holds together.
	Chewiness	Degree of chewing needed to break up the cheese pendingswallowing.
	Adhesiveness	The degree to which the chewed mass (not unbroken downindividual particulate) sticks to teeth and mouth surfaces.
	Particle Size	Size of the particles in the chewed mass.
TASTE & FLAVOR ATTRIBUTES	Sweet	Basic taste sensation elicited by sugars
	Salt	Basic taste sensation elicited by salt
	Acid	Basic taste sensation elicited by acids
	Bitter	Basic taste sensation elicited by bitter compounds
	Butter (Diacetyl)	Aromatics commonly associated with natural, fresh butter
	Sulfur	Sulfurous aroma typically associated with agedcheddar/boiled egg
	Cardboard/Oxidized	Aroma associated with wet cardboard and lipid oxidation
	Brothy	Aromatics associated with boiled meat or vegetable soupstock
	Astringent	Harsh, drying, puckering sensation on the surfaces of themouth.
	Whey Taint	Flavor of entrapped whey within the cheese matrix, can becomefermented as cheese is aged.
	Fruity	Aromatics associated with cooked fruits & estercompounds
	Rancid	Aroma associated with butyric acid; piquant note
	Sour	Aroma associated with vinegar or citrus notes

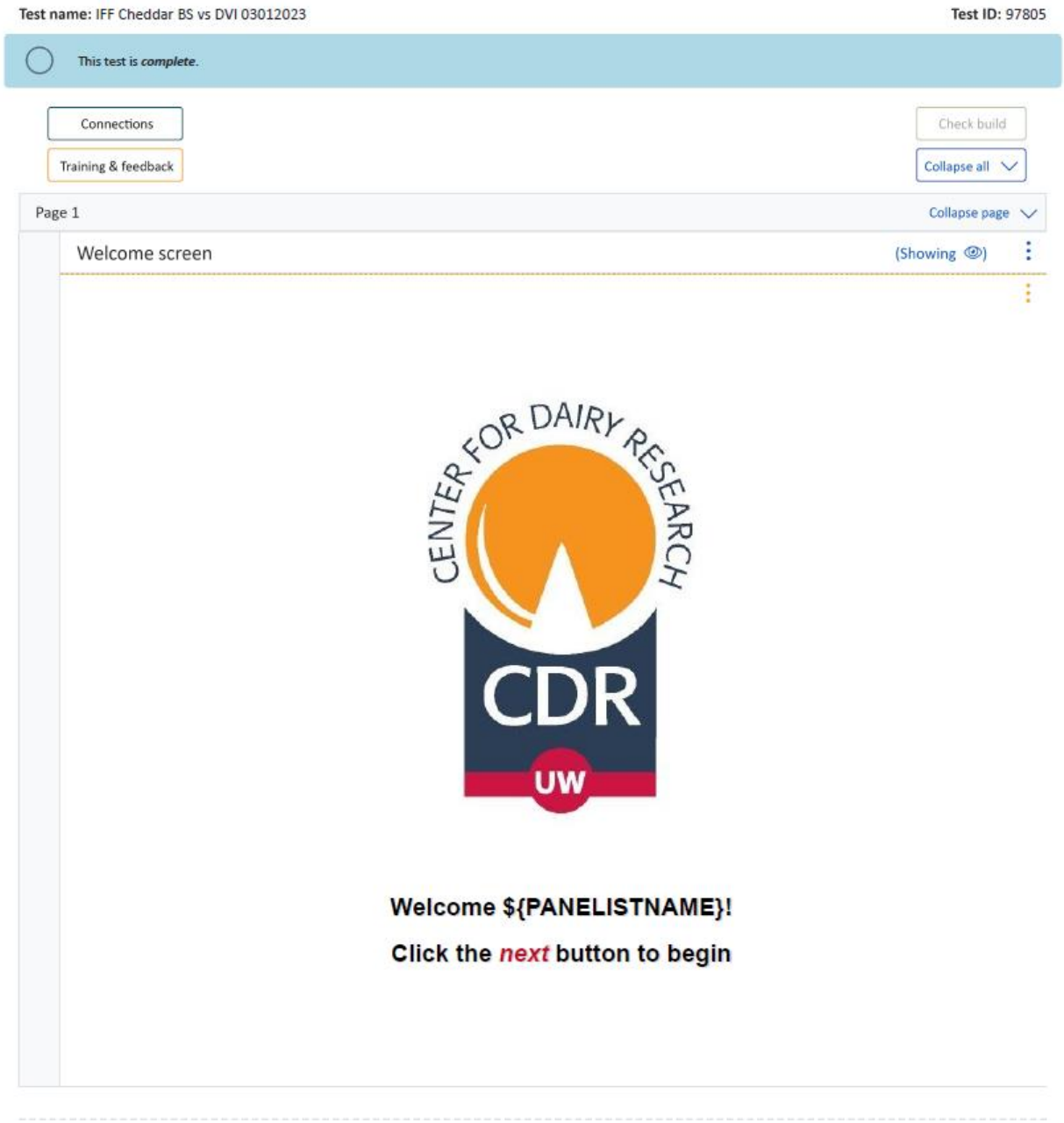
Scales

Our descriptive panel uses a universal 15-point scale (0-15). Some of the scales have anchorpoints (Spectrum®), some do not (QDA). An example of such a scale is given below.



When using QDA-based scales, our panel follows as USDA-style procedure. Absent = 0, Slight = 5, Definite = 10, Pronounced = 15. During discussion and training, we often discussattribute intensities to the nearest 0.5.

APPENDIX 4


Test name: IFF Cheddar BS vs DVI 03012023
Test ID: 97805
This test is complete.
Connections
Training & feedback
Check build
Collapse all
Page 1
Collapse page
Welcome screen
(Showing
CENTER FOR DAIRY RESEARCH
CDR
UW
Welcome ${PANELISTNAME}!
Click the next button to begin

Welcome to the Cheddar descriptive panel.

In this session you will evaluate the following attributes:

Texture:

- *Hand Firmness*
- *Cohesiveness of mass*
- *Particle Size*
- *Adhesiveness*
- *Chewiness*

Flavors:

- *Basic tastes*
- *Milkfat / Lactone*
- *Buttery*
- *Brothy / Meaty*
- *Sour (citrusy / vinegary)*
- *Sulfur*
- *Butyric/Rancid*
- *Oxidized*
- *Fruity*
- *Astringent*

Page 3 Collapse page

Next Sample

Appears in reps: All reps

Appears in cycle: All cycles

Please have a sip of water and some cracker before moving to the next sample.

Your next sample is

${SAMPLEBC}

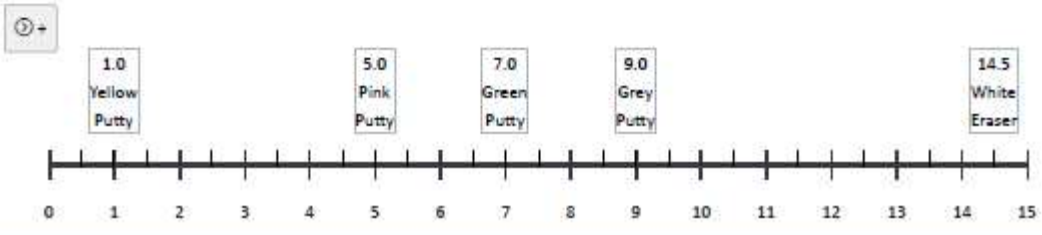
0:30

Remove page break

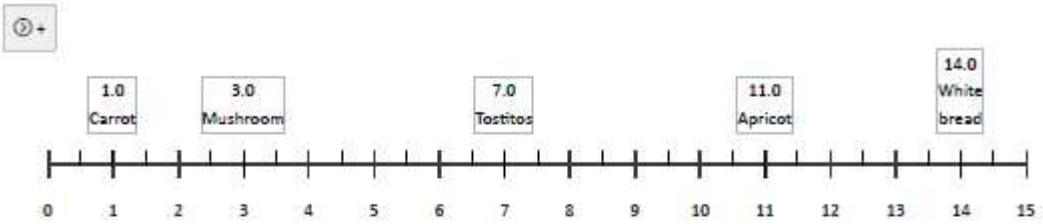
Page 4 Collapse page

1. Texture (Question name)

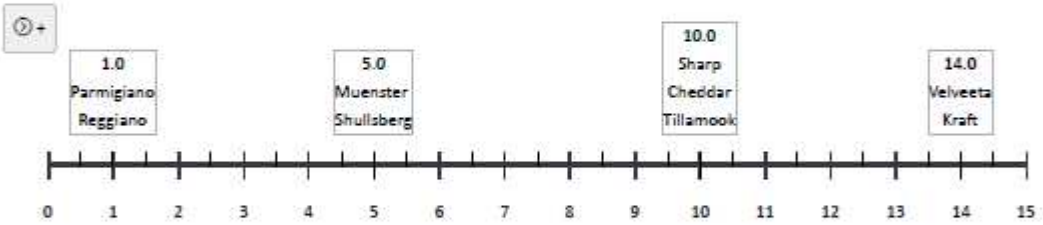
1.1. Hand Firmness



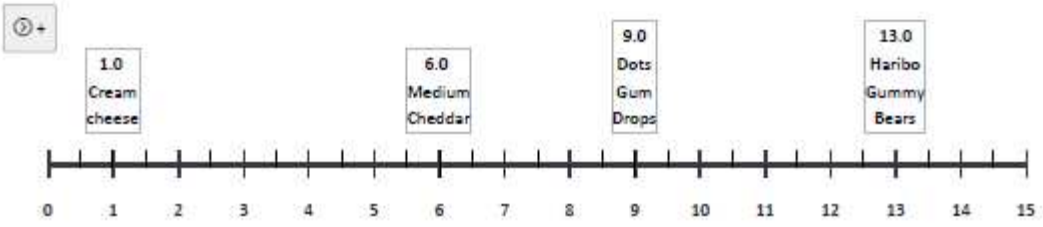
1.2. Cohesiveness of Mass



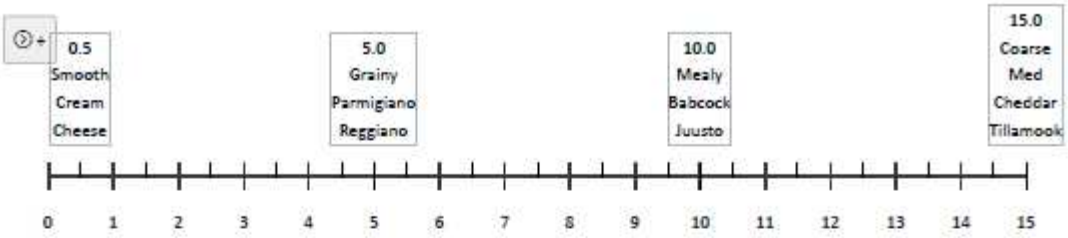
1.3. Adhesiveness



The total amount of energy required to masticate the sample to a state pending swallowing. Chewiness is a product of cohesiveness, hardness, and springiness. The longer time required to chew, the chewier the sample. First chew (molars).



1.5. Particle Size



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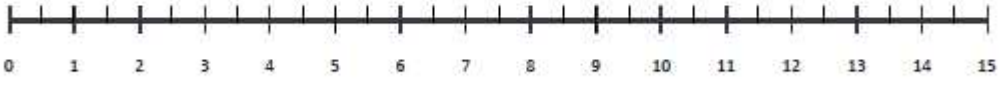
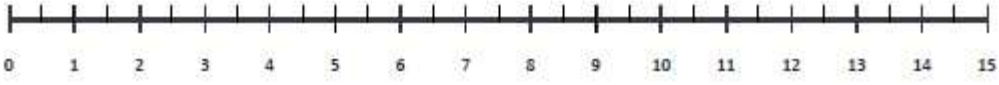
2. Flavor (Question name)

2.1. Bitter

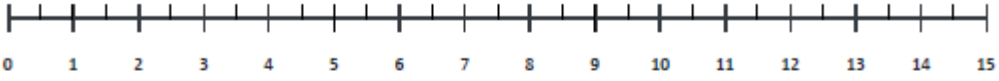


2.2. Acid

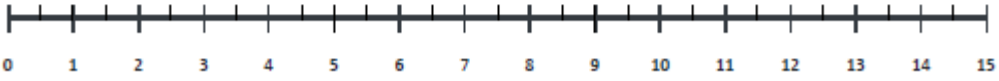


2.3. Salt

2.4. Sweet



2.5. Savory



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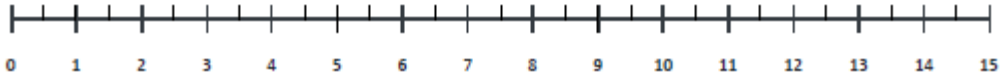
3. Flavor (Question name)

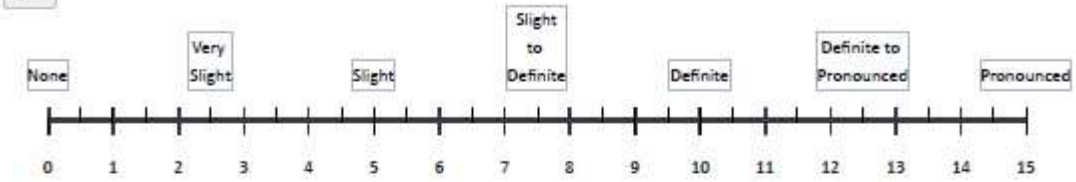
3.1. Butter

3.2. Brothy

3.3. Sour



3.4. Sulfur



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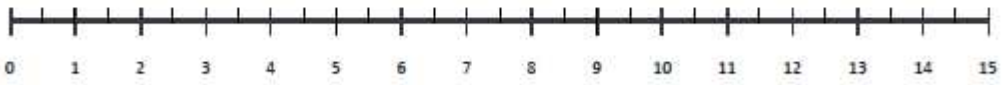
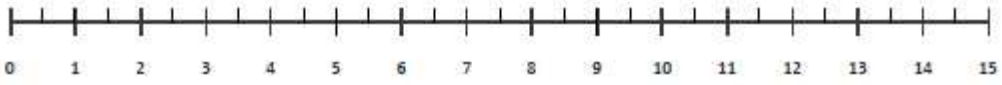
4. Flavor (Question name)

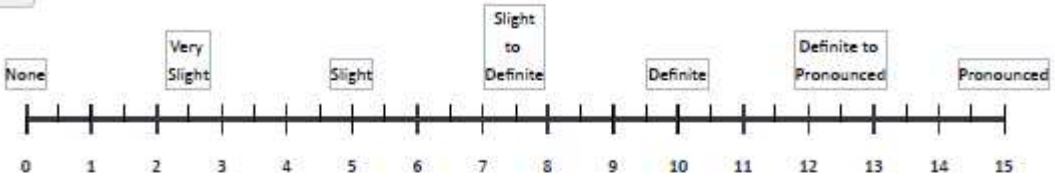
4.1. Rancid



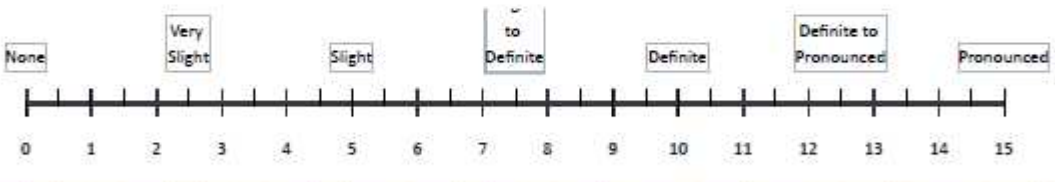
4.2. Fruity



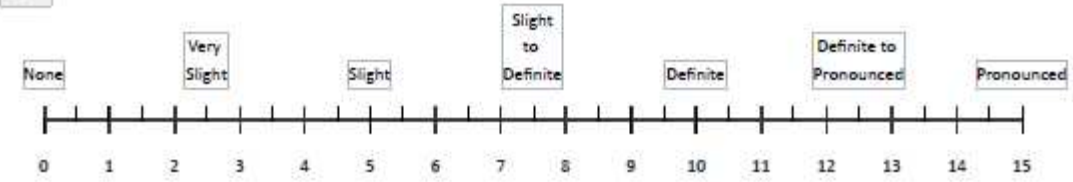
4.3. Whey Taint



4.4. Astringent



4.5. Other (List in Comments)



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5. Comment (Question name)

Comments:

Please comment on any flavor/texture attributes*

Comment field

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Thank You screen

Thanks for completing this test!