Sodium Reduction in Blue Cheese With and Without Replacement by KCl

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Dedication

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Abstract

Recent initiatives encourage the reduction of sodium in foods to improve consumer health. Blue cheese contains approximately 370 mg sodium per 28 g serving, 16% of the daily recommended value of sodium and twice the amount found in a serving of Cheddar (21 CFR 101.9 (c)(9)). However, salt is essential for control of blue cheese enzymatic and microbial activity during ripening (Morris, 1981). One way to maintain this control in cheese is to reduce sodium and implement a molar replacement of NaCl with KCl. Because blue cheese is often surface-salted, the effects of sodium reduction may be more noticeable in the center of the cheese wheel as the salt and moisture equilibrate from the surface to the center during aging (Cantor et al., 2004). The purpose of this study was to evaluate the effects of 25% sodium reduction on blue cheese composition, flavor, sensory, and proteolytic properties with and without the use of potassium chloride (KCl) at two locations in the cheese wheel.

Three-kg wheels of pasteurized milk blue cheese were produced from 2,100 kg of milk (in duplicate), and three salting treatments were applied to an equal number of randomly selected wheels. Salt was applied by % weight of the wheel in the following treatments: Control (3.5 wt% NaCl), reduced sodium (2.63 wt% NaCl), and reduced sodium with KCl (2.63 wt% NaCl, 1.17wt%KCl). Wheels were evaluated monthly during 5 months of aging, sampling both inner and outer portions of the cheese wheel. Sodium and potassium concentrations, fat, moisture, pH, a_w, volatile free fatty acids, and extent of proteolysis (as measured by free amino acids) were measured. Sensory attributes and volatile flavor chemicals were measured at months 3 and 5. Salt reductions of 23% and

21% in reduced with KCl and reduced treatments, respectively, were achieved. The water activity of control and reduced with KCl treatments was the same during aging, while the reduced treatment had higher a_w. A greater extent of proteolysis was observed in inner samples as compared to outer wheel samples, with reduced treatments trending (though not significantly) higher than control or reduced with KCl. A descriptive sensory panel found higher overall flavor intensity and 12 contributing aroma or flavors which were more intense in the inner portion of the cheese compared to outer portions ($P<0.01$). Many sensory attributes were also different between salting treatments as evaluated by the descriptive panel. Concentrations of free fatty acids varied between location and treatment for some acids, though medium chain free fatty acids were unaffected by salting treatment. Flavor volatiles associated with blue cheese were found in higher concentrations in reduced treatment and inner wheel samples, specifically 2-heptanone, 2-pentanone, ethyl hexanoate, and methyl propanoate. A consumer panel (n=95) ranked overall liking for all treatments similarly, and higher texture liking for reduced with KCl.

These data suggest sodium reduction in blue cheese (up to 25%) with and without molar replacement by KCl can result in a blue cheese which is equally acceptable to the consumer, despite some compositional and sensorial differences. These results hold true for full sodium blue cheese with approximately 1200mg Na/100g cheese. Because of the extensive differences noted between inner and outer wheel, where sodium concentrations differed for the first 61 days of aging, and because reduced sodium treatment cheeses were often distinguishable from control and reduced with KCl, reducing the sodium of blue cheese to a larger extent than 25% may result in loss of product quality or safety.

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Chapter 1: Introduction

Sodium chloride (salt) is essential to the production of cheese. Salt imparts a desired salty flavor, controls microbial growth via osmotic pressure and influence on a_w, participates curd formation, and inhibits excessive growth of *Penicillium roqueforti* (Fox et al., 2004). The over-consumption of sodium in the American diet has lead to the demand for reduced and low sodium products (Sacks et al., 2001; Krol, 2013).

Blue cheese contains approximately 1300mg Na/100g cheese, or 370mg/28g serving, about 16% of the daily recommended value of sodium in a single serving. This is nearly twice the amount of sodium in Cheddar (21 CFR 101.9 (c)(9)). This high level of salt makes blue cheese a strong candidate for sodium reduction, but is in no way arbitrary. Blue cheese is characterized by the mold *Penicillium roqueforti* which is capable of growing at up to 13% NaCl (Cantor et al., 2004). However, growth and sporulation are ideal around 3.5% NaCl and water activity of 0.98-0.96, depending on strain (Godinho and Fox, 1981; Lopez-Diaz et al., 1996; Cantor et al., 2004). The high level of salt in blue cheese controls the growth and sporulation of *P. roqueforti* while inhibiting the growth of unwanted spoilage bacteria.

One mechanism for sodium reduction in cheese is the use of replacement mineral salts such as KCl, $MgCl_2$, and $CaCl_2$ to obtain similar water activities while reducing the level of Na (Lindsay et al. 1982, Grummer et al., 2013). The results of these studies confirm that KCl can produce a reduced or low sodium Cheddar cheese with similar compositional and sensory attributes. The strategy for sodium reduction in this study will include a molar replacement of sodium chloride by potassium chloride.

Blue cheese is also characteristically different from Cheddar in its shape and salting

application. Blue cheese is formed into wheels and salt is applied (either brined or dry-rubbed) onto the outer surface of the wheel (Morris, 1981). Because the majority of the salt is applied to the surface of the wheel, a salt, a_w, and pH gradient are present until at least 5 weeks of aging (Cantor et al.,2004; Morris, 1985).

Salt can directly affect the a_w and pH of cheese via solute concentration and lactic acid inhibition, respectively (Labuza, 1984; Fox et al., 2004). Further, the pH, a_w, and salt concentration can affect the lipolytic and enzymatic activities within the cheese, which are produced by either the coagulum, LAB, NSLAB, or *P.roqueforti* itself (Modler, et al. 1974; Gripon1993).

Lipolysis within the cheese wheel is responsible for the formation of free fatty acids which have distinct aromas and flavors and can be oxidized to methyl ketones, many of which are distinctly characteristic of blue cheese, such as 2-heptanone (Tomasini et al. 1993, Kilcawley et al. 1998, McGorrin 2007; Cadwallader et al., 2007).

Proteolysis in blue cheese is often more extensive than other cheeses because of the endo and exo peptidases secreted by *P.roqueforti* (Marcos et al. 1979, Larsen et al. 1998, Upadhyay et al. 2004). Proteolysis of casein proteins leads to the formation of free amino acids, which also contribute to the flavor of blue cheese and can be further broken down to acids, alcohols, and sulfur compounds with more complex flavors and aromas of their own (Kinsella & Hwang, 1976; Cadwallader et al., 2007).

Thus, to fully understand the possible changes in blue cheese with sodium reduction, it was important to consider all of the aforementioned constituents and their changing gradient between the center and surface of the wheel.

In the current study, 6-lb surface-salted blue cheese wheels were produced from the curd of the same cheese make and given one of three salting treatments (Control, 25% Reduced NaCl, and 25% Reduced NaCl with KCl added at a molar replacement to the NaCl reduced). Cheese samples for all salting treatments were tested at inner and outer wheel locations for fat, moisture, water activity, pH, sodium concentration, potassium concentration, proteolysis, production of free fatty acids, and production of flavor volatiles. A trained descriptive sensory panel analyzed the cheeses at months 3 and 5. A consumer panel (n=95) evaluated the cheeses at the completion of aging. Cheese production and testing was completed in duplicate, one month apart, in order to account for variation in initial milk composition.

1.2 Hypotheses

It was hypothesized that 25% sodium reduction in blue cheese would affect the composition and sensory aspects of the cheese in the following manner as compared to the control: a_w would increase, % moisture would increase, salt concentration would decrease, pH would increase, proteolysis would increase, lipolysis would increase, total fat and protein on a dry basis would remain the same, free fatty acid, free amino acid, and percent soluble peptides would increase, flavor volatiles would increase, and sensory attributes would be distinctly different.

It was further hypothesized that 25% reduced sodium blue cheese with a molar replacement of KCl would not vary from the control cheese in composition, but may have different sensory attributes, flavor volatiles, and free fatty acid profiles.

Finally, it was hypothesized that inner wheel samples compared to outer wheel samples would have lower sodium concentrations at the beginning of aging, a larger extent of proteolysis, lower pH, larger concentrations of FFA and flavor volatiles, and distinctly different sensory profiles.

Chapter 2: A review of sodium reduction and impact on blue cheese.

2.1 Sodium Reduction

Sodium chloride (salt) is an essential mineral, food preservative, and food flavor enhancer. In cheese, salt dissociates and increases the osmotic pressure, reducing the water activity (a_w) and controlling microbial growth while adding a salty flavor (Fox et al., 2004). In 2012, the CDC reported that cheese was one of the top 10 sources of sodium in the American diet (CDC 2012). Sodium is required in the human body for muscle function, blood fluidity, nerve function, and nutrient uptake. The recommended dietary allowance of sodium is 1,500mg Na/day for people ages 9-50 years old (USDA, 2013). However, it is estimated that the average American consumes more than 3,400mg/day (Sacks et al., 2001, CDC 2012). Excess sodium intake has been linked to cardiovascular disease; the number one leading cause of death in the United States as reported by the CDC. Reducing the intake of sodium has been shown to reduce systolic blood pressure in patients with and without preexisting hypertension with a larger impact on those individuals with preexisting conditions (Sacks et al. 2001). Reduction of sodium with potassium salt replacements was shown to have a similarly positive impact on blood pressure (Geleijnse, JM, 2003).

The state of New York created the National Sodium Reduction Initiative (NSRI) in an effort to positively impact the cardiovascular health of the general public by reducing average sodium consumption 20% by the year 2014. The NRSI set a 2014 sodium content target of 600g Na /100g cheese in Cheddar, Colby, Jack, mozzarella, Muenster, Provolone and Swiss (firm and semi hard). The initiative also set cream cheese targets for 2014 at 350g Na /100g cheese. A specific target was not set for blue cheese,

which has one of the highest relative sodium contents. Blue cheese varieties contain approximately 800-1300mg sodium/100g cheese.

2.2 Consumer acceptability

Consumers are becoming increasingly aware of the potential consequences of excess sodium consumption and are beginning to make choices based on this awareness. Mintel reports that 58% of consumers aged 18 and older are conscious of their sodium intake (Krol, 2013). Natural cheese sales have steadily increased over the past 4 years and approximately one quarter of the UK cheese market is dominated by blue cheese purchase (Frank J.N., 2012, Price A., 2012). In addition, 42% of consumers believe that "reduced" sodium foods will taste better than "low" sodium foods (Krol, 2013).

Because cheese is one of the main contributors to dietary sodium intake, it is a good candidate for sodium reduction research. One challenge of sodium reduction in cheese is the variability of salt concentration used between cheese manufacturers. Agarwal et al. (2011) analyzed the sodium content of over 600 types of Cheddar, Mozerella, and processed cheese and found the variation between cheeses in each variety to be 300mg Na/serving or more. There is no federal standard of identity for the required sodium content of any cheese type. Instead, the cheese maker targets certain sodium levels based on experience, tradition, consumer expectations, and functionality (like in the case of blue, where salt acts to control the growth of *P.roqueforti*) (Johnson & Paulus, 2008). The Code of Federal Regulations (21 CFR 101.61) does require the term "reduced sodium" may only be used for food products (excluding meals) which contain at least 25% less sodium per reference amount customarily consumed (RACC) than an appropriate reference food. With the observed variance in cheese production, however,

the levels of sodium within "reduced sodium" cheeses could vary greatly and cheese makers must take this into consideration when applying reduction strategies to their manufacturing process.

2.3 Sodium reduction strategies

Because of the functional properties of sodium chloride in cheese (such as microbial control, influence on texture, ripening, and overall flavor) reducing the sodium levels in cheese can have a direct effect on cheese composition. Replacement salts have been employed to mimic the ionic activities of sodium chloride. The main strategy behind these replacement salts is to obtain a similar water activity.

The water activity (a_w) of a food can be determined by the ratio of the vapor pressure of the headspace immediately above the food, to that of pure water at the same environmental conditions (Labuza, 1984). A major effect that can lower the water activity of a food is the colligative effect. That is, the interactions of dissolved solutes with water molecules in a food, which have an affect on the properties of water in the food based on the number of solute particles present. The influence of solute content on water activity can be estimated by Raoult's Law:

$$a_w = \gamma_s X_{water} = \gamma_s \frac{\eta_{water}}{\eta_{water} + \eta_{solutes}}$$

where a_w=water activity, γs=the activity coefficient, η_{water} = moles of water in solution, and η_{solute}= moles of dissolved solute in a solution of η_{water} moles of water. Thus, as you reduce sodium, η_{solute} decreases and the resultant water activity increases. By the same logic, replacing the NaCl with KCl on a molar basis (that is, the number of dissociated

moles of solute will not change) should result in the same water activity and a lower concentration of sodium.

Commonly used replacement salts for sodium reduction in cheese include magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$) and potassium chloride (KCl). Fitzgerald and Buckley (1985) produced Cheddar cheese that completely replaced the NaCl with either $MgCl_2$, $CaCl_2$, KCl, or a mixture of these salts with NaCl. They found that $MgCl_2$ and $CaCl_2$ salted cheeses (alone or in blends with NaCl) were unaccepted by the consumer (largely due to flavor) and had greater extents of proteolysis and lipolysis. However, Cheddar salted with a KCl had similar sensory scores as the control (NaCl) cheese as well as similar levels of lipolysis and proteolysis. Grummer et al. (2013) tested $MgCl_2$, $CaCl_2$ and KCl salting blends on Cheddar cheese but in ratios that targeted similar water activities. Their results also confirmed the unacceptable flavor profile of magnesium and calcium chloride.

Sodium reduction studies, which employ the use of KCl replacement salts, have been carried out for Cheddar, mozzarella, Minas fresh cheese, Feta, and Halloumi (Lindsay et al. 1982, Grummer et al., 2013, Katsiari et al. 1997, Aly, 1995, Gomes et al., 2011, Ayyash et al. 2011,Kamleh et al. 2012, Kumar S., 2012). Some studies report equivalent consumer acceptance of reduced sodium cheeses with KCl replacement as compared to control cheeses (Grummer et al. 2013, Gomes et al. 2011) while others observed consumer preference or detection of difference in salting treatments (Kumar S., 2012, Lindsay et al., 1982, Kamleh et al. 2012).

Schroeder et al. (1988) compared 1.44%, 1.12% and 0.73% NaCl Cheddar cheese (with no KCl replacement) and noted that sensory differences were only detected

between 1.12% and 0.73% but not between 1.44% and 1.12%. Similarly, Rulikowska et al. (2013) noted that reduced NaCl Cheddar cheeses were associated with more negative sensory descriptors such as “bitterness” and “sulfur” as compared to higher NaCl Cheddar cheese.

These studies are a guide for the potential consumer response to reduced sodium blue cheese, with or without KCl. Sodium reduction will also impact the overall composition of the cheese, which will be discussed at length in the following sections.

2.4 Blue Cheese

The history of Blue Cheese

It is estimated that mold ripened cheese has existed for thousands of years and was enjoyed by historic figures such as Charlemagne and Casanova (Toussaint-Samat, 2009). Mold ripened cheese has a rich French history. The people of Roquefort-sur-Soulzon were granted monopoly of mold-veined cheese ripening in 1411 and in 1925, France awarded the Appellation d’Origine Contrôlée to sheep milk Roquefort cheese, defining the location of its production as Roquefort-sur-Soulzon (AOC, INAO). Today there are many varieties of mold ripened, blue cheese produced all over the world.

In the United States, studies on blue cheese mold species were first published around 1906 lead by researchers such as Charles Thom (Raper, 1965). In the following years, the USDA supported many studies on the production and manufacture of blue cheese in the United States. Coulter and Combs first investigated blue cheese in 1933 at the University of Minnesota, using methods developed by Charles Thom at the Storrs Agricultural Experimental Station in Connecticut (Raper, 1965). Combs aged the cheese in sandstone caves along the Mississippi river in St. Paul, Minnesota, simulating the

renowned Roquefort production methods (Morris, 1981, Brick 2003). The production of this blue veined cheese quickly spread to researchers at Iowa State University. In 1936, Felix Frederickson began the Treasure Cave Company in Faribault, MN, manufacturing blue cheese based on methods developed at the University of Minnesota and Iowa State. This is believed to be the first commercial producer of American Blue Cheese (Morris, 1981).

Standard of Identity

The standard of identity for Blue cheese is listed in the Code of Federal Regulations, title 21, section 133.106. It requires that blue cheese, which is specifically characterized by the mold *Penicillium roqueforti*, have no greater than 46% moisture and a minimum of 50% milkfat on a solids basis. Regulations also require that blue cheese be made from cow's milk and aged for a minimum 60 days. The milk may or may not be pasteurized. The CFR further states that ingredients such as clotting enzymes, calcium chloride, benzoyl peroxide, and surface antimycotics may be used as optional ingredients (CFR 21 133.106).

Processing and Manufacture

Blue cheese can be made from either pasteurized or raw milk. Many artisan cheese makers choose not to pasteurize their milk, claiming it helps the cheese develop a unique flavor profile. However, several studies have shown that the FDA 60-day aging requirement for raw milk cheese is likely insufficient to eliminate potential pathogenic contaminants in the cheese (DeValk, et al., 2000; Bachmann et al., 1995; D'amico et al., 2010). Pasteurization condition requirements are for Grade B milk, as the milk is not directly consumed but made into cheese first. Grade B milk standards can be found in the

Wisconsin administrative code for Agriculture, Trade, and Consumer Protection, Chapter ATCP 60. The cream portion of the milk is often homogenized when making blue cheese. The homogenization reduces fat globule size, disrupts the milk fat globule membrane, and is thought to make the fat more readily acted upon by indigenous and mold derived lipases (Morris, 1981). Homogenization is thought to contribute to the flavor profile of cheese as the disrupted globules are more readily acted on, generating free fatty acids.

After pasteurization and homogenization, the milk is warmed to approximately 30°C. At milk storage temperatures (4°C) calcium is more soluble and will leach out of the casein micelle (Fox et al., 2004). The warming of milk before cheese making facilitates the migration of calcium back into the micelle, improving rennet coagulation and cheese yield. Warming the milk is also essential for the activity of the starter culture. Starter culture bacteria or "primary cultures" are species of lactic acid bacteria (LAB) added to milk either as bulk starter or as concentrated direct vat starter (DVS) (Fox, et. al, 2004). The starter culture ferments lactose naturally present in the milk, producing lactic acid and reducing the pH. This reduction in pH is essential to rennet activity, syneresis, texture, and flavor development of the cheese (Fox, et al., 2004). Starter cultures can be chosen for consistency of acid production, tolerance to additional ingredients, and flavor production. Common mesophilic varieties of starter culture include *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris, Lactobacillus bulgaricus,* and *Streptococcus thermophilus.* (Kindstedt, 2005). In addition, secondary heterofermentative cultures, such as *Leuconostoc mesenteroides* ssp. *cremoris,* are capable of fermenting citrate in milk to produce CO_2 and diacetyl. The production of CO_2 and diacetyl contribute to the open texture and buttery flavor typical of blue cheese, Gouda, and Havarti (Kindstedt, 2005).

In the current study, the secondary cultures are *Lactobacillus lactis,* spp*. lactis* biovar *diaccetylactis* and *Leuconostoc mesenteroides* ssp*. cremoris*. A bulk set solution of *P. roqueforti* is also added with the starter culture. Alternatively, spores of P. roqueforti may be "dusted" onto drained curd later in cheese making, but this is typically discouraged as it may result in less homogenous distribution of the mold (Morris, 1981).

Once the starter culture has been added and lowers the pH slightly, rennet is added. Rennet is a blend of digestive enzymes that are extracted from the abomasums of young ruminants (typically calves). The major clotting enzyme in calf rennet is chymosin. Modern cheese making uses recombinant chymosin produced by genetically modified yeast or bacteria cells. These alternative sources of chymosin have proven to produce equivalent cheese yields as calf extracted rennet, although the overall proteolysis may differ somewhat as it is a not a blend of enzymes, like the natural product is (Green et. al, 1985; Ustunol et al., 1990; Mandy, 2011). The chymosin enzyme (or rennet) hydrolyses κ-casein, at a bond between phenyalanine and methionine releasing a portion of the protein that contains a hydrophilic functional group (oligosaccharides) into the whey. This fraction is called the glycomacropeptide and is lost to the whey stream. The loss of this portion of κ-casein causes the destabilization of the casein micelle due to the reduction of the steric hindrance provided by the carbohydrate (Fox, et. al 2004). The exposed hydrophobic portions of the casein micelle interact with one another and positively charged calcium ions, creating a gel coagulum that entraps the milk fat. The formation of this gel is known as rennet coagulation. Gel formation can be influenced by acidity, temperature, and rennet activity. Once formed to the desired degree of firmness, the gel is cut into curds by the cheesemaker. Immediately after cutting and, the *para-*

casein micelles contract slightly and rearrange themselves into a thicker film, while the more hydrophilic portions of the micelle are expelled as cheese whey (Fox, et. al 2004). This process is referred to as syneresis. The expelled whey contains water-soluble proteins, lactose, minerals, and some trace amounts of fat (Hwang, et. al 1995). In general, syneresis increases with decreasing pH and increasing temperature.

The curd is gently stirred and cooked at a constant temperature of 96°F for 30 minutes during which time, the starter culture continues to produce lactic acid and lower the pH. Some salt is added to the curd at this time to aid in curd syneresis and texture, but it is relatively small in comparison to the percentage of salt applied to the blue cheese wheel at the completion of manufacture. At a target pH near 6.0, the whey is drained. A small amount of sodium chloride and calcium salts are added to the drained curd to further the extent of syneresis and improve curd texture (Fox et al., 2004).

The drained curds are loaded into open ended, cylindrical, stainless steel hoops on a draining table (to continue the expelling of whey as the curds set). Unlike pressed cheeses like Cheddar, blue cheese curds set naturally under the force of gravity. The filled hoops are inverted 5 to 10 times and then once every 4 hours for the next 12 hours at 72-78°F in order to allow the cheese wheel to form and to prevent the curd from sticking to the hoops (Morris, 1981). As they set, the cheese wheels continue to drive off more moisture and increase in acidity. At a titratable acidity of approximately 1.10, the wheels are removed from the hoops, weighed, and salted (Morris, 1981).

The majority of the sodium chloride (salt) found in blue cheese is applied directly to the surface using either a dry rub or a brine technique. Cheese texture could be negatively affected if the final desired percentage of salt were added all at once as this

could create a thick, tough rind. To avoid this when surface salting, salt is applied to the surface of the cheese wheel over the course of 4 days. For example, the 1982 "Minnesota" method of production involved applying 6-7% of the weight of the unsalted wheel in salt over the course of 4 days (Morris, 1981). On the first day, salt in the amount of 3.5% of the weight of the wheel was applied to the outside. On the second day, no salt was added. On the third and fourth days, 2.5% and 1.25% of the weight of the wheel was applied (Morris, 1981). The amount of salt and methodology for application varies between producers. This surface application generally creates an area towards the exterior of the finished wheel where very little mold growth is observed.

To prevent surface mold growth, salted cheese wheels can be dipped in antimycotic solution, microcrystalline wax, or wrapped in flexible plastic or aluminum films. The wheels are then punched (through protective films) approximately 150 times on either side with a 1/16 -1/8th inch needle. This punching allows air to reach the interior of the cheese wheel, facilitating the growth of the *P. roqueforti* spores that were trapped in the curd during cheesemaking. The cheese wheels are then moved to the sporulation room for approximately 2 weeks to allow the characteristic *P. roqueforti* to grow within the cheese. Ideal conditions for this sporulation or ripening vary slightly with mold strain (Farahat et al., 1990; Rabie et al., 1988), but in general, are near 50°F and a relative humidity of 90-95%. This allows for proper mold growth without the cheeses drying out. Historically, sporulation has been achieved in limestone or sandstone caves such as Roquefort France or Faribault Minnesota. Once sufficient mold growth has occurred, the wheels of blue cheese may be scraped clean, wrapped in aluminum foil, wax, or other oxygen limiting packaging, and stored at 40-45°F for 5 to 6 months for complete ripening

and flavor development. For a summary of the manufacture of blue cheese, see Figure 2.1.

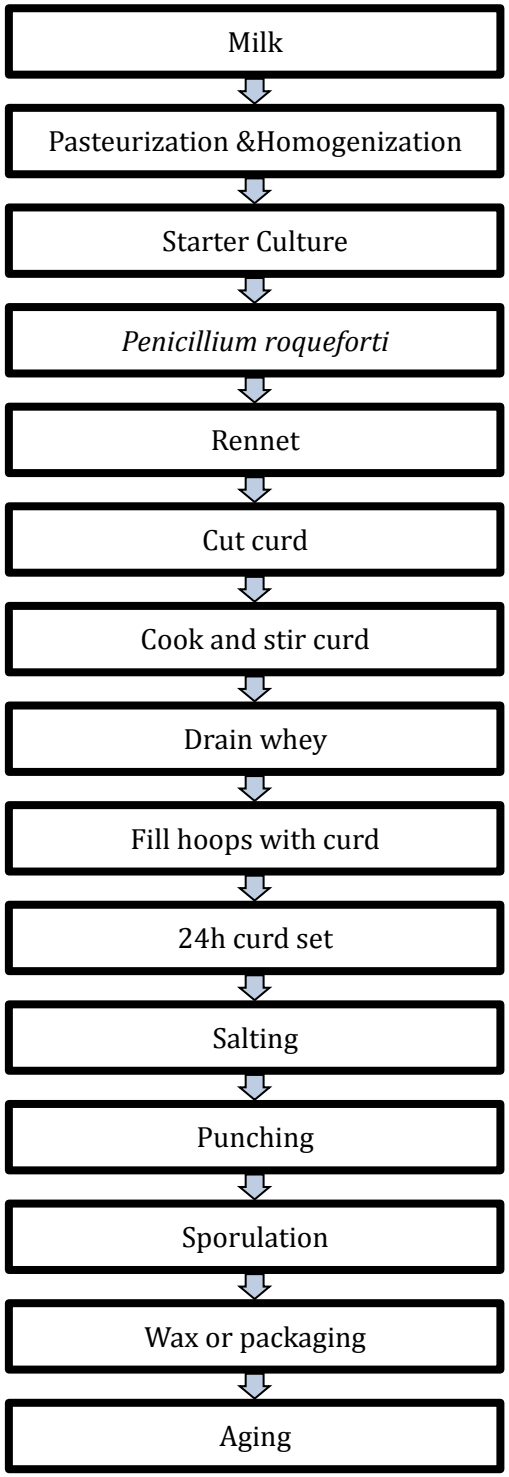

Figure 2.1: An overview of blue cheese manufacture, modified from Morris (1981)

The role of salt in cheese

An excess consumption (or an extreme deficiency) of sodium can have severe negative impacts on human health (USDA, 2013). It has been shown that a reduction in dietary sodium can have both short and long-term positive effects on cardiovascular health (Cook et al. 2007). With the goal of improving the health of consumers, pressure has been placed on the food industry to lower sodium across many food products, including cheese. However, salt has essential functional properties in cheese, which must be considered before changing the sodium levels. These functions include but are not limited to; preservation, flavor development, and quality control (Guinee & Fox, 2004). Further, in blue cheese, salt concentration is important for controlling the growth of *Penicillium roqueforti* (Sampamundo et al. 2013). The functionality of salt with respect to these aspects of blue cheese production is discussed in the following sections.

The role of salt in blue cheese

In blue cheese, the overwhelming majority of the salt in the finished cheese is added after the curd has set into wheels. Surface salted blue cheese may be brined, but is typically dry-rubbed (Morris, 1981). When salt is applied to the cheese wheel surface, it dissolves into the liquid phase of the curd. Water is then drawn to the surface of the cheese, via osmosis, where it will accumulate (Kindstedt, 2005). As the salt continues to diffuse into the cheese, the curd contracts and further expels moisture as whey in a process known as syneresis (Dejmek & Walstra, 2004). A portion of salt, lactose, and soluble protein will be lost as it is expelled with the whey. This affects the final salt content and pH of the cheese (Fox et al., 2004).

Syneresis is driven by temperature, pH, pressure, and concentration of salt

(Dejmek & Walstra, 2004). Morris (1981) estimated that in order to achieve a final salt content of 4.0-5.0%, the salt rubbed onto the surface of a blue cheese wheel should amount to 6.5%-8% of the total weight of the unsalted cheese wheel. However, due to osmosis, with a reduced addition of salt comes a reduced expulsion of whey as less moisture is driven to the surface of the cheese (and expelled) to equilibrate salt concentration. This varying degree of salt loss has been observed by others (Fox et al. 2004, McMahon et al. 2009). Varying degrees of salt and moisture content can have inhibitory or enhancing effects on culture activity.

One characteristic of blue cheese salting is the migration of salt ions from the exterior to the interior of the cheese wheel over aging. The diffusion of Na^+ and Cl^- ions is affected by fat globules, protein aggregates, cheese moisture viscosity, temperature, and cheese geometry (Guinee, 2004). Morris et al. (1985) found that Cheddar curd (2x2x6cm chips) reached an equilibrium level of NaCl within 48 hours while a 20kg unsalted block of milled pressed curd in brine did not reach equilibrium within 24 weeks. These results support the theory that an open, random structure lends to faster adsorption and equilibrium of salt ions. In addition, moisture content was consistently correlated with a higher diffusion constant (D) (Morris et al., 1985; Guinee, 2004). Blue cheese has both a more open texture and higher moisture content than Cheddar cheese, so the diffusion of salt ions is expected to be larger in comparison.

Cantor et al., (2004) reported the surface to core gradient of pH, salt, and a_w in 5 week-old Danablu blue cheese to be 5.0 to 6.4, 4.5 to 2.0, and 0.92 to 0.94 (respectively). These gradients were lower than those reported for week 1 in the same cheese, suggesting the internal composition of the cheese wheel may eventually reach an equilibrium

depending on the extent of aging. It is possible that the time to reach this equilibrium would be different with a reduction in salt added to the surface.

Bona et al., (2007) modeled the diffusion of NaCl and KCl in prato cheese salted in brine using a fixed effects method, modified to fit Fick's second law for simultaneous diffusion. They confirmed with two mathematical models that the diffusion rate of K^+(0.24 to 0.254 cm^2day^{-1})was higher than Na^+(0.225 to 0.242 cm^2day^{-1}),.

It is understood that there is a gradual diffusion of sodium and/or potassium ions into the body of a wheel of blue cheese. These ions may have an affect on the ripening reactions (such as proteolysis and lipolysis) within the cheese, having a more exaggerated effect at the most internal portions of the cheese wheel. Because of the diffusion gradient, it was expected that the most affected region of cheese would be the internal portion of the reduced sodium treatment. For this reason, the current study included a sampling scheme that would test inner and outer samples of each salting treatment for all compositional, proteolytic, lipolytic, and sensory evaluation over the course of 153 days.

Effect of salt on water activity and safety

As previously mentioned, salt works to preserve cheese by lowering the water activity (a_w) of the cheese. Reducing the S/M, the ratio of salt-to-moisture (% salt/ %Moisture, or "S/M"), has been shown to affect the water activity of cheese (Guinee & Fox, 2004, Grummer & Schoenfuss, 2011, Rulikowska et al., 2013). An increase in a_w can create a more hospitable environment for unwanted pathogenic and non-pathogenic microorganisms to survive in the cheese. Most pathogenic microorganisms cannot grow or produce toxins below a limiting water activity of 0.95. Exceptions to this limitation are Listeria monocytogenes, which can grow at an a_w of 0.92 (in addition to being salt

tolerant up to 10% NaCl depending on media) and *Staphylococcus aureus,* which may grow at an a_w of 0.85 (Curtis, 2007, Tapia et al. 2007, McMeekin et al. 1987). The average water activity of blue cheese is around 0.95 (Guinee & Fox, 2004). In addition to unwanted pathogen growth, spoilage bacteria, yeasts, and molds may grow at a_w of 0.6-0.85 (Beuchat, 1981; Tapia et al., 2007). Because reducing the S/M may increase the a_w, reducing the S/M in blue cheese may have an affect on the growth rate of these unwanted organisms.

Recently there have been several recalls in the United States pertaining to the presence of *Listeria monocytogenes* in blue cheese (FDA, 2011, 2012). Some studies have shown a correlation between reducing sodium, increasing a_w, and increasing growth rate of *L. monocytogenes* in tryptone soy broth (Samapundo et al., 2013, Boziaris et al. 2006.) These studies also confirm the possibly similar inactivation mechanisms of NaCl and KCl blends when KCl is used as a replacement salt.

Because cheese is a complex system, there are other factors that affect the a_w of cheese. Numerous studies have found that the a_w of cheese decreases with increasing soluble nitrogen content, indicating an inverse relationship between proteolysis and water activity in Cheddar and blue cheese (Hickey et al. 2013, Esteban et al. 1991, Fernandez-Salguero et al., 1986). In a reduced sodium cheese, a greater extent of proteolysis may drive an initially higher a_w down via the production of soluble low molecular weight solids such as peptides and free amino acids (Rulikowska et al. 2013). However, these effects and their contribution to cheese safety are typically not significant until later in aging, when proteolysis has occurred to a greater extent.

Effect of salt on starter culture and Penicillium roqueforti

The S/M is correlated with lactic acid bacteria (LAB) in that, at high S/M, the initial population of starter culture LAB is reduced and fermentation is inhibited. Rulikowska et al. (2013) found that reducing the S/M in Cheddar cheese resulted in higher initial populations of starter culture LAB, and a greater concentration of lactic acid (mg/100g cheese) at the same time point. Reddy and Marth (1995) measured the predominant LAB species of reduced sodium Cheddar with the use of KCl replacement (wt/wt) and found that salting treatment had no effect on predominance of a given *Lactococcus* species, and that total enumeration was equivalent between the control and reduced treatments with KCl replacement. Ayyash et al. (2010) also found no difference in LAB enumeration between samples of reduced sodium Halloumi cheese using a KCl replacement as compared to a control. This suggests that at least one hurdle of salt reduction in blue cheese may be overcome by the use of replacement salts, namely, KCl.

The S/M also affects fermentation reactions, which affect the resultant pH of the cheese. With an increasing S/M ratio, the starter culture *Lactococci* will ferment less of the lactose present in the cheese.

Blue cheese is characterized by the blue green mold*, P. roqueforti.* The nomenclature of *P. roqueforti* has changed recently. A secondary variety which was called *P. roqueforti* var. *carneum*, produces one mycotoxin (patulin) that *P. roqueforti* var. *roqueforti* does not (Boysen et al., 1996). However, it has been determined that the two "varieties" (*roqueforti* and *carneum*) are distinctly different species based on morphology (Karlshoj & Larsen, 2005). It should be noted that both species are capable of producing mycotoxins such as PR toxin and roquefortine C but ideal conditions for

specific mycotoxin production are not well understood (Chang et al. 1991, Fernandez-Bodega et al., 2009).

Gervais et al. (1988) and Valik et al. (1999) found that growth rates of *P. roqueforti* were stable at $a_w > 0.92$, but below this value growth was hindered considerably. This suggests growth rates of *P.roqueforti* in reduced S/M blue cheese may not differ from controls since the initial a_w is above 0.92 even at normal salting levels. However, below a_w=0.96 sporulation is inhibited (Cantor et al., 2004). The peptidases and lipases secreted by *P.roqueforti* come from both the mycelium and spores (Girolami and Knight, 1958; Kinsella & Hwang, 1976). In addition, it has been shown that a_w may decrease significantly over aging in blue cheese varieties (Prieto et al. 1999). LopezDiaz, et al. (1996) investigated the growth rate and sporulation of 9 strains of P.roqueforti on agar with salt concentrations from 0%-6% and found that, in general, lower salt concentrations correlated with higher growth rates and a more rapid onset of sporulation.

A reduction in S/M and resultant change in a_w could have a large impact on the growth, sporulation, and resultant enzymatic and proteolytic activities of *P. roqueforti.*

Effect of salt on proteolysis

Proteolysis contributes to cheese texture, pH, a_w and the development of flavor compounds. In blue cheese, proteolysis is the hydrolysis of proteins in cheese by enzymes from the rennet coagulant, LAB, NSLAB, *P. roqueforti*, native milk proteinases, and (to a small degree) environmental proteinases (Upadhyay et al. 2004). Mold ripened cheeses exhibit greater proteolysis during ripening than cheese varieties without molds due to the fact that the spores and mycelium of *P. roqueforti* excrete

proteolytic (and lipolytic) enzymes (Kinsella & Hwang, 1976; Gripon 1993). Several studies have found a greater extent of proteolysis in reduced sodium cheeses, but none have measured the extent of proteolysis in reduced sodium blue cheese (Thakur et al., 1975; Schroeder et al.,1988; Gomes et al., 2011; Rulikowska et al. 2013).

The proteolytic systems of *P. roqueforti* show optimum casein hydrolysis in the acidic range and become increasingly inactive below pH 3.0 and above pH 6.5 (Modler, et al. 1974, Gripon 1993, Gente et al. 2001). With a reduction in S/M, the lactic acid production of starter cultures may increase, reducing the initial pH of the cheese and increasing or inhibiting the activity of these proteinases. Rabie et al., 1988 found the pH of ripening blue cheese ranging from approximately 5.3 to 5.9 over aging, well within the reported optimum range of the peptidases of *P. roqueforti.* However, the range of initial pH varied between *P. roqueforti* strains utilized, thus, sodium reduction may affect some cheeses more than others where different strains are used (Rabie et al., 1988).

Together with lipolysis, proteolysis is one of the predominant mechanisms of volatile flavor production in cheese (McSweeney & Sousa, 2000). The hydrolysis of casein is largely driven by the addition of rennet coagulant and results in the production of medium to large sized peptides. These peptides are then broken down further into small peptides, alcohols, aldehydes, and free amino acids (FAA) by the coagulant and the enzymes of the starter cultures and *P. roqueforti* (Tunick, 2007; McSweeny & Sousa 2000). Blue-veined cheeses have been shown to contain proteinases and peptidases that break down α_{s1}, α_{s2} and β-caseins to a greater degree than other cheeses and to varying degrees depending on *P. roqueforti* strain (Marcos et al. 1979, Larsen et al. 1998, Upadhyay et al. 2004). The proteolytic excretions of *P. roqueforti* include aspartyl-

proteinases, metallo-proteinases, aminopeptidases, and carboxypeptidases (Fernandez-Salguero, 2004).

As proteolysis progresses, free amino acids (among other compounds) are produced. The FAA profile and concentration of blue cheese can contribute to sweet, salty, sour, bitter, and umami tastes (Lawlor et al. 2003). The aldehydes produced can contribute to flavor and aroma characteristics such as “pungent” , “green”, “bread”, “malt”, and several others (Lawlor et al. 2003). A modified summary of the break down of protein into flavor components is shown in Figure 2.2.

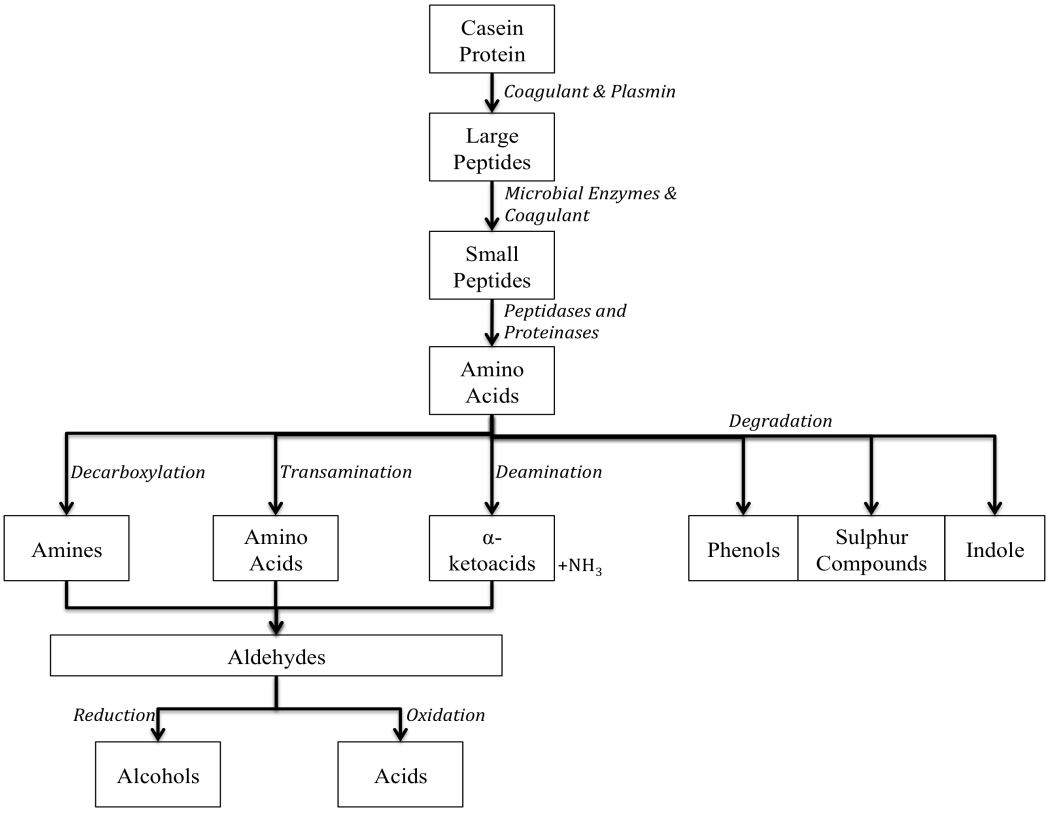

Figure 2.2: Proteolysis of casein into flavor components. Modified from McSweeney & Sousa (2000) and from Tunik (2007).

While the effects of proteolysis in reduced sodium blue cheese have not been investigated, the use of KCl as a replacement salt in reduced sodium Cheddar, Mozzarella, Akawi, and Feta resulted in similar levels of proteolysis as compared to full sodium controls (Reddy & Marth, 1993; Katsiari et al., 2000; Ayyash et al. 2011; Ayyash et al. 2012).

Methods for determining proteolysis involve the identification of nitrogen containing compounds, based on the structure of proteins and their metabolites. The Kjeldahl procedure can be used for total nitrogen or total protein determination and involves the complete digestion of a sample (15.12 Bradley et al., 1992). The peptides and free amino acids formed during proteolysis can indicate the extent of proteolysis and are commonly measured by such methods as the Lowry method, Bradford assay, trinitrobenzene sulphonic acid (TNBS), pH-4.6 soluble nitrogen, and fluorimetric OPA method (Wallace & Fox, 1998; Chang, 2010).

The OPA (o-phthaldialdehyde) method involves the reaction of OPA with primary amino groups and a SH-compound (either DTT or β-mercaptoethanol) to form a compound which absorbs light at 340nm (Nielsen et al., 2001;). The method had been used for quantification of total free amino acids using either a Serine or Lysine standard curve in milk and cheese products, compared against the ninhyrdin and TNBS methods for accuracy and convenience (Church et al., 1983; Rohm et al., 1996). The present study will utilize both the OPA and biuret methods for detection of total amino acids and soluble small molecular weight compounds, respectively.

Effect of salt on Lipolysis

Lipids present in milk can undergo enzymatic hydrolysis (lipolysis) and lipid oxidation. Because of its negative oxidation-reduction potential, oxidation is typically limited in cheese (McSweeny and Sousa, 2000; Woo and Lindsay 1984). Enzymatic lipolysis leads to the formation of free fatty acids (FFA), glycerol, and mono- or di-glycerides, which are essential to both the flavor profile and flavor development of cheese (Fox et. al 2004). FFAs are the precursors to many more volatile compounds responsible for cheese flavor such as secondary alcohols, lactones, methyl ketones, and acids (Molimard et al., 1996, Fox et al., 2004).

It is understood that lipolysis in mold ripened cheese (including blue cheese) is more extensive than in other types of cheese (Molimard et al,. 1996). This difference in lipolysis is largely due to the activity of the lipases produced by *P. roqueforti* (Farahat 1990). In the literature, the extent of lipolysis in blue cheese varies based on degree of ripening and methodology of measurement (Farahat et al. 1990). Typically, the extent of lipolysis is determined by quantification of FFAs and has been reported within the range of 19,000ppm -38,000 total FFA (Woo & Lindsay, 1982;Wolf et al., 2011). While these FFA contribute flavors (such as “rancid”, “soapy”, and “waxy” and many others) their degradation products also contribute to the flavor profile of blue cheese (Figure 2.3).

Methyl ketones are of principle importance in blue cheese flavor, especially 2-heptanone, 2-pentanone, and 2-nonanone, produced via the β-decarboxylation of FFA (Tomasini et al. 1995, Kilcawley et al. 1998, McGorrin 2007). The flavor production pathways are complex and occur simultaneously within the cheese. The lipase activity of *P. roqueforti* has been studied in slurry systems used for enzyme modified cheeses to produce characteristic methyl ketones as quickly and as in high a quantity as possible for

commercial use (King & Clegg, 1979; Kilcawley et al. 1998). King & Clegg simulated blue cheese lipolysis in slurry systems (68% moisture) and found that methyl ketone production was higher when additional octanoic and hexanoic acids were introduced to the system, indicating enhanced methyl ketone formation with higher levels of FFA. Tomasini et al. (1993) found that the addition of esterases or the use of lipolyzed cream increases the production of FFA in these model slurries, providing more reagent for the formation of methyl ketones. However, while the addition of specific FFA to a slurry system was found to increase corresponding methyl ketone production, lipolysis of other FFA ceased, indicating the potentially toxic effect of high FFA concentraitons (King and Clegg 1979).

A blue cheese produced with a reduced S/M may experience a resultant increase in lipolytic activity, generating a larger amount of free fatty acids. These FFA may limit further activity of *P.roqueforti* while continuing to be metabolized, contributing to methyl ketone concentration.

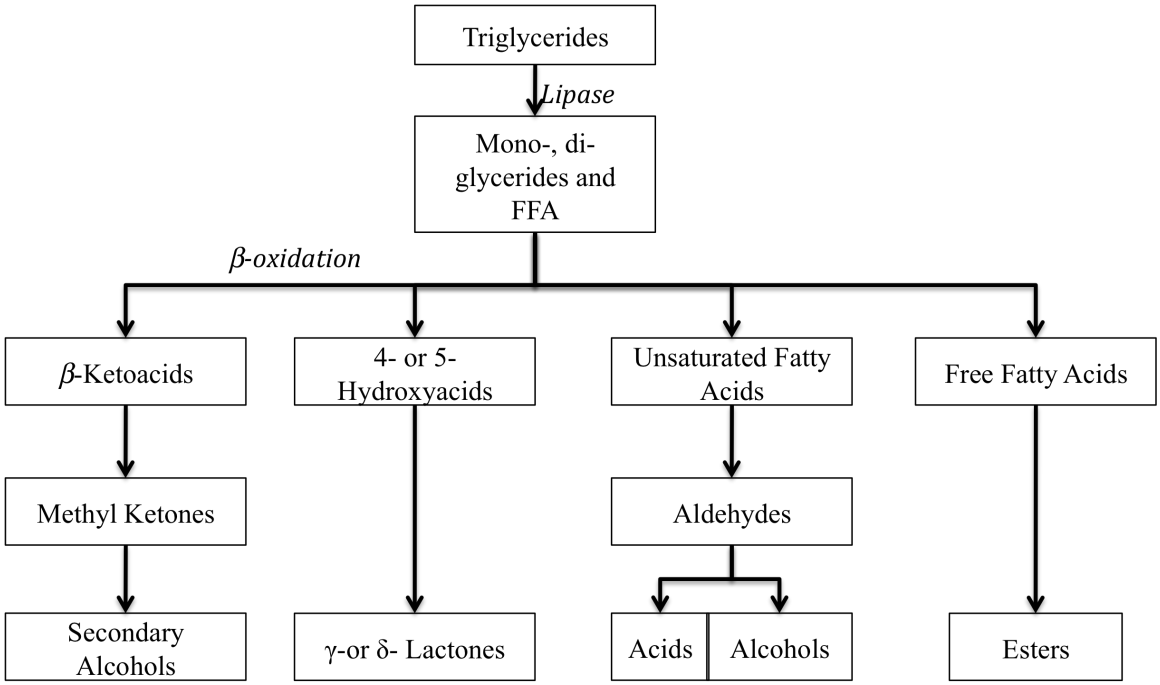

Figure 2.3: Lipolysis of cheese triglycerides leading to flavor compounds. Modified from Tunick (2007).

Effect of sodium reduction and KCl on sensory attributes

Blue cheese has a characteristic fruity, musty,"blue cheese" aroma and flavor, which is believed to be predominantly caused by 2-heptanone and other ketones such as 2-pentanone, 2-octanone, and 2-nonanone (Cadwallader et al., 2007; Larroche et al., 1994). While the overall profile of blue cheese can be descriptively evaluated for it's intricacies, these ketones are so specific to blue cheese that they are commonly described as having simply a blue cheese flavor and aroma (Cadwallader et al., 2007; Larroche et al., 1994).

The aroma, flavor, taste, and aftertaste profile (and corresponding descriptive lexicon) of blue cheese has been evaluated in previous research and includes descriptive terms such as pungent, fruity, moldy, sweaty, floral, alcohol, burn, cabbage, and peppery

(to name a few) (Lawlor et al., 2003; Retiveau et al., 2005; Talavera-bianchi & Chambers, 2007). The moldy attribute was positively correlated with pH 4.6-soluble nitrogen, histidine and serine levels, indicating the importance of proteolysis to the development of moldy flavor (Lawlor et al., 2003). Salty flavor was correlated with S/M, pH, and pH 4.6-SN content and strength of flavor was correlated with S/M, pH 4.6-SN, FAA, and FFA concentration (Lawlor et al., 2003). The blue cheese variety with the highest intensity of strength of flavor was notably more pierced more than the others, providing more *P. roqueforti* growth and proteolytic activity. Similar correlations are expected for the present study, as changing S/M is expected to affect pH, mold growth, proteolysis, and lipolysis.

Lawlor et al. (2003) noted that highly experienced assessors of 6 varieties of blue cheese varied in their ability to discriminate between certain attributes and sample replicates. They attributed some of this variation to the heterogeneity of blue cheese, as the open texture can lead to differences in mold growth throughout the cheese and would lead to sensory differences. To account for variation, their testing involved a high number of replicates from many locations in the cheese wheel. In the present study, location within the cheese wheel is a desired predictor. In the present study, to account for sample variation, samples from a given location (inner, outer) will be homogenized. Evaluation of texture differences will be sacrificed in an attempt to gain a more accurate description of aroma, flavor, taste, and aftertaste profile between treatments and locations.

Another sensory concern in regards to sodium reduction is the use of replacement salts and bitterness. Kamleh et al., (2012) found that bitterness was significantly higher in reduced sodium Cheddar cheese containing 280mg Na/100g cheese with 183mg K/100g

cheese compared to the full sodium control (523mg Na/100g cheese). Alternatively, Grummer et al., (2013) found that bitterness did not vary between reduced sodium Cheddar cheese containing 360mg Na/100g cheese and 700mg K/100 g cheese and a full sodium control (664mg Na/100g cheese). Lindsay et al., (1982) also reported similar bitterness ratings for Cheddar cheese produced with a 1.5% reduction in sodium using molar replacement by KCl. These studies suggest that total sodium level may be a large factor in perceived bitterness as discrepancy between studies includes a 100mg Na/100g cheese difference in the amount of NaCl used in the control cheese. In Cheddar, this difference could also influence the creation of bitter peptides (Cadwallader et al., 2007). In blue cheese, however, the salt content is nearly twice that of Cheddar. In addition, bitterness is typically included in blue cheese lexicon and flavor profile (Retiveau, et al., 2005). In addition, the present study will reduce the sodium and implement KCl replacement at a lower level than those studies which determined an increase in bitterness between control and reduced sodium samples.

With the preconceived acceptance of strong pungent flavors, and the practice of reducing the sodium by only 25% as compared to 50%, the flavor profile of the reduced sodium blue cheese with KCl replacement is thought to be comparable to the control in acceptance by the consumer.

Chapter 3: Determining the effect of sodium reduction on blue cheese

3.1 Introduction

The food industry is under pressure to lower sodium levels in foods as overconsumption of sodium has been linked to cardiovascular problems and adverse health effects (Geleijnse et al., 2003; CDC, 2012; Institute of Medicine, 2013). The USDA dietary guidelines for sodium consumption advise the average adult consume no more than 2300 mg Na/day. According to the USDA nutrient database, blue cheese contains approximately 1395mg sodium (Na) per 100g cheese, which equates to 17% of our daily recommended intake of sodium in a single 28g serving. This is more than twice the amount of Cheddar (612mg Na/ 100g cheese). The reduction of sodium in blue cheese may have a positive impact on average dietary sodium intake. While there have been several studies on sodium reduction strategies in Cheddar and other cheeses, none so far have investigated blue cheese (Lindsay et al., 1982; Schroeder et al., 1988; Katsiari et al., 1997; Grummer et al., 2013; Rulikowska et al., 2013).

Salt is essential to cheese manufacture and influences microbial growth, water activity, syneresis, enzyme activity, protein stability, and flavor (Guinee & Fox, 2004). Previous studies on sodium reduction in cheese have utilized potassium chloride (KCl) as a replacement for NaCl to reach a similar a_w and effective S/M ratio as the full salt cheese, maintaining microbial control and enzymatic activity (Linsday et al., 1982; Katsiari et al., 1997; Grummer et al., 2013).

Blue cheese is characterized by the blue green mold, *Penicillium roqueforti* which excretes endo and exo peptidases, resulting in a greater extent of proteolysis than many

cheese varieties (Marcos et al., 1979; Larsen et al. 1998; Upadhyay et al., 2004). Blue cheese has a high salt concentration in order to control the growth of *P. roqueforti.* Though there is variation between strains, *P. roqueforti* has been reported as surviving in salt concentrations up to 13%, with growth stimulated around 3.5% NaCl and a_w = 0.98 – 0.94 (Morris 1981; Godinho & Fox, 1981; Cantor et al., 2004). In addition, blue cheese is often surface salted, creating a gradient in salt concentration from the surface of the wheel to the center. The diffusion of NaCl in a cheese matrix has been reported between 0.1-0.4 cm^2day^{-1} and is influenced by factors such as temperature, small molecular weight compounds, and fat (Thuran et al., 1992; Guinee, 2004; Floury et al., 2010). With a reduction in sodium, it is expected that the S/M, moisture content, rate of proteolysis, and production of small molecular weight compounds will differ and could impede the overall diffusion of NaCl into the center of the cheese wheel. The diffusion coefficient of KCl was found to be higher than that of NaCl in a study on brined Prato cheese, which may impact the ability of KCl to have the same functionality as NaCl in reduced sodium blue cheese (Bona et al., 2007).

We hypothesized that 25% reduced sodium blue cheese could be manufactured using a molar replacement of KCl to maintain a similar a_w and ripening reactions. Further, it was hypothesized that by not utilizing KCl, the effects of sodium reduction would be more pronounced, especially in regards to a_w, proteolysis, moisture, and flavor profile. Finally, it was hypothesized that sodium reduction would have an impact on the salt gradient from the surface of the wheel to the interior, which may exaggerate the effects of sodium reduction especially within the interior of the cheese wheel.

To test these hypotheses, 6-lb surface-salted blue cheese wheels were produced from the

curd of the same cheese make and given one of three salting treatments (Control, 25% Reduced NaCl, and 25% Reduced NaCl with KCl added at a molar replacement to the NaCl reduced). Cheese samples for all salting treatments were tested at inner and outer wheel locations for fat, moisture, water activity, pH, sodium concentration, potassium concentration, proteolysis, production of free fatty acids, and production of flavor volatiles at monthly intervals for 5 months. A trained descriptive sensory panel analyzed the cheeses at months 3 and 5. A consumer panel (n=95) evaluated the cheeses at the completion of aging. Cheese production and testing was completed in duplicate, one month apart, in order to account for variation in initial milk composition

3.2 Materials and Methods

Design of experiment and sampling

This study evaluated the effects of sodium reduction in blue cheese by comparing three salting treatments over 5 months of aging for both inner and outer wheel sample locations. The salting treatments are termed Control, Reduced, and Reduced with KCl (Table 3.1). The curd from one vat of milk was formed into wheels, and treatments were assigned at the salting step of the cheese wheels. A detailed description of cheese manufacture can be found in the following sections.

Table 3.1: Description of salting treatment names

Treatment	Description
Control	Full sodium control
Reduced	25% Reduced sodium cheese
Reduced with KCl	25% Reduced sodium cheese with molar replacement by KCl

The targeted final reduction of sodium between the control and either reduced or reduced with KCl treatments was 25%. A 30% reduction in sodium was targeted during

cheese manufacture in an effort to achieve a final reduction of 25% as compared to the control. The reduced with KCl treatment targeted the same reduction in sodium, with a molar replacement by potassium chloride (Table 3.2).

Table 3.2: Salt (%wt. of un-salted wheel) applied at manufacture for treatment groups.

	Treatment		
	Full Sodium[1]	Reduced Sodium[2]	
Salt, %	Control	Reduced	Reduced with KCl
NaCl	3.5	2.45	2.45
KCl	0	0	1.34

[1] 1200mg Na/100g sample targeted
[2] 900mg Na/100g sample targeted

At each sampling time point, two wheels of cheese were analyzed from each treatment group (Figure 3.1). This was done to account for variation between wheels. From each of these wheels, two samples of cheese were extracted from both the inner and outer portions of the wheel. Thus, for a given salting treatment, location, and time point, each measured attribute is an average of 4 separate cheese samples, per cheese-making replicate (8 total samples). The timing of the analytical and sensory tests are described in Table 3.3.

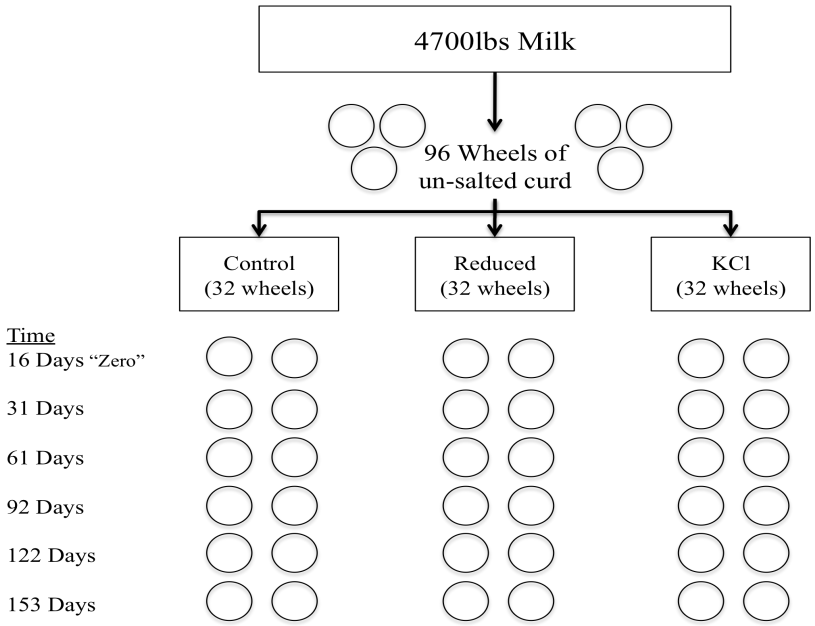

Figure 3.1: Cheese salting treatment schematic and sampling schedule. Because sporulation takes 2 weeks, "time zero" is 16 days after the manufacture of curd. All other time points correspond to monthly intervals of sampling.

Table 3.3: Schedule of compositional and sensory testing for blue cheese treatments

	Age of cheese when analyzed (days from curd manufacture)						
Analysis Performed	16	31	61	92	122	153	183[2] 203[1]
Water Activity	x	x	x	x	x	x	
pH	x	x	x	x	x	x	
Moisture	x	x	x	x	x	x	
Fat	x	x	x	x	x	x	
Sodium	x	x	x	x	x	x	
Potassium	x	x	x	x	x	x	
Total Protein	x						
OPA/BCA	x	x		x	x	x	
Volatile Flavor Analysis				x		x	
Free Fatty Acids				x		x	
Descriptive Sensory Analysis				x		x	
Consumer Analysis							x

1,2: Consumer analysis was done on a single day with make 1=203 days aged, make 2=183 days aged

When sampled, a wheel of cheese was cut in half along its 7inch diameter. The exterior 0.25" (0.63cm) "rind" was removed from the side, top and bottom of the cheese

wheel (portion “A” in Figure 3.2). The next most exterior 0.25”-1.75” (0.63-4.4cm)was saved as the “outer” location sample (portion “B” in Figure 3.2). This portion of cheese was lightly homogenized in a plastic bag, by hand, for 30 seconds before being divided in half, to provide sample replication for “outer” location. Another 0.25inches (0.63cm) was then removed and discarded (portion “C” in Figure 3.2) in order to provide a substantial distance between “outer” and “inner” samples. The remaining center-most portion, approximately 2”-3.5”from wheel surface (5.1cm-8.9cm), was saved as the “inner” location sample and was homogenized and divided in the same manner as the outer portion (“D” in Figure 3.2).

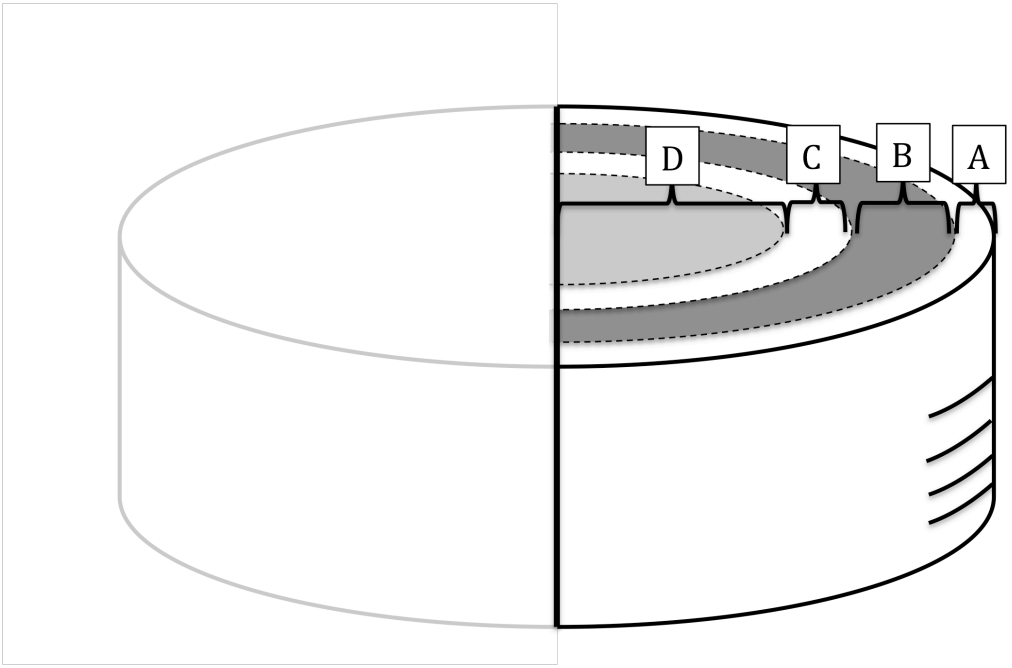

Figure 3.2: Sample preparation diagram for 6lb wheel of blue cheese. Diameter=18cm Height=15cm. A = Surface-0.63cm, B = Outer sample portion 0.63cm-4.4cm from surface, C = portion 4.5-5.1 from surface, D = Inner sample portion 5.1-8.9cm from surface.

Blue cheese manufacture

Surface-salted blue cheese wheels were manufactured from a single vat of milk, in duplicate cheese makes, in the J. Warthesen Food Processing pilot plant at the University of Minnesota (Table 3.4).

Table 3.4: A summary of blue cheese manufacture including major steps, target time points, temperatures, pH, and TA where appropriate

Step	Time (h)	Temp (C)	pH	TA
Starter Added	0.0	90	N/A	0.21
Mold powder added	0.0	90	N/A	0.21
Calcium added	0.9	90	N/A	0.15
Rennet added	1.0	90	drop of 0.1 from start	0.14
Cut coagulum	1.5	90	N/A	0.13
Stir	1.8	90	N/A	N/A
Whey drain #1	2.0	90	N/A	N/A
Add salt to curd	2.3	N/A	N/A	N/A
Whey drain #2	2.8	N/A	6.0	N/A
Hooping	3.3	N/A	N/A	N/A
Drain on table	4.0	N/A	N/A	N/A
	5.0	N/A	N/A	N/A
	6.0	N/A	5.3	N/A
Salting Day 1	24.0	N/A	N/A	N/A
Salting Day 2	48.0	N/A	N/A	N/A
Salting Day 3	96.0	N/A	N/A	N/A
Antimycotic and Punching	120.0	N/A	N/A	N/A
Sporulation	120.0	N/A	N/A	N/A
Sealed and moved to storage	456 (2 wks of sporulation)	N/A	N/A	N/A

For each cheese make, an average of 4,800 lbs of milk sourced from Land ‘O Lakes was received and separated into skim and cream portions. The skim milk portion was pasteurized at 165.5°F (74°C) with a hold time of 20 seconds on a plate and frame milk pasteurizer. The cream portion of the milk was heated to 163°F (73°C) and held for 30 seconds (100 gallon univat tank, MFG Co, Chicago, IL) then cooled to 130°F (54°C) before being homogenized (Gaulin 2-stage homogenizer, Manton-Gaulin Co., Massachusetts) at 2000 psi and 500psi for the first and second stage, respectively. Once heat-treated and homogenized, cream and milk were blended into a Damrow rectangular open cheese vat (Tetra Damrow, Vernon Hills, IL) and warmed to 90°F (32°C).

Starter culture was added as a bulk culture. The commercial starter culture used was Flora Danica, a freeze-dried culture composed of a mixture of strains, the most predominant of which are lactic acid producers *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*, and flavor bacteria *Lactococcus lactis* ssp. *lactis* biovar *diactetylactis*, and *Leuconostoc mesenteroides* ssp. *cremoris* (Chr. Hansen, Inc., Milwaukee, WI). Flora Danica bulk set starter was prepared the day before cheese making in a 16.2g/860lb milk concentration and allowed to ferment for 16-18 hours at 72°F (22°C).

The *P. roqueforti* used for this study was commercial strain Choozit *P. roqueforti* BCM1 LIQ 250D (Danisco, Copenhagen, Denmark). The same supply of *P. roqueforti* culture was used for both cheese replicates. Just before addition to the prepared milk, 25g of BCM1 was added directly to the prepared bulk set starter and mixed. The starter was

then added to the warmed milk and stirred gently by the automatic agitators on the vat for approximately 1 hour at 90°F (32°C).

After 45minutes to an hour (as determined by a slight reduction in pH, indicating the activity of starter culture) calcium chloride (CalSol 71257, CHR Hansen) was added to the vat at a level of 90mL/1000L of starting milk. Calcium chloride was stirred into heated milk for 10 minutes.

At approximately 1 hour after the addition of starter culture, or when the pH of the heated milk had dropped by a tenth (0.1) from the pH at the time of starter addition, rennet was added. The coagulating enzyme was fermentation chymosin (ChyMax 2X, CHR Hansen) and was stirred into the milk at a level of 1.5oz/1000lbs (40mL/454kg) milk.

A gel coagulum was allowed to form over the next 30 minutes and was then cut into curds using a 3/8" (0.95cm) wire cheese knives. After healing for 5 minutes, the cut curd was gently stirred for 10 minutes at 90°F (32°C). The whey was then drained to the level of the curd, approximately half the vat, in order to relieve osmotic pressure and to induce further expelling of moisture from the curd. The partially drained curds were stirred out for 40 minutes, during which time NaCl (0.6% of the starting weight of the milk) was added to the curd in order to firm the curd, further encourage syneresis, and improve the buoyancy of the curd in whey. At a target whey pH of 6.0, the remaining whey was drained until only enough remained in the vat to keep the curd from drying out during hooping.

Curds were "hooped" into stainless steel hoop forms on a stainless steel draining table and allowed to set and drain for 1 hour before being inverted. Set curds in hoops

were inverted every hour for the next 4 hours, and again at 12 hours. The set wheels (still in hoop forms) were left on the draining table over night.

The four-day salting process started approximately 24 hours from the time of starter culture addition, when the pH of the whey draining from the set wheels was approximately 5.3. For the control treatment, salt (Morton Purex Food Grade NaCl, CAS 7647-14-5) was added at a level of 2% of the weight of the wheel on day 1, 1% on day 2, and 0.5% on day 4. No salt was added on day 3. Salt was precisely measured according to the weight of each individual wheel and surface rubbed onto cheese wheels one at a time. On each day, once salted, cheese wheels were loaded onto plastic draining trays and stored at 45°F (7°C) and 80% RH.

The reduced and reduced with KCl treatments were salted in a similar manner to the control, with the %wt of NaCl (or NaCl/KCl blend) applied reflecting the amount required to reach the targeted sodium reduction from the control. KCl treatments were always salted last so as not to contaminate the control or reduced treatment cheese wheels with potassium chloride.

When salting was complete, the wheels were dipped in Delvocid antimycotic solution. To create the antimycotic solution, 15g of Delvocid (50% natamycin on xanthan gum, DSM Food Specialties, Netherlands) was diluted in a 10% NaCl and water solution. Wheels were dipped in antimycotic and punched with 50 1/16th inch needles, three times on either face of the wheel, using a cheese wheel punch (BIMBA Manufacturing, University Park, Il). The wheels were placed in large plastic bags (3 per bag) which were loosely sealed, and moved to the sporulation room. Sporulation was allowed to occur for

16 days. At the end of sporulation, the containment bags were heat-sealed and moved to 40°F (4°C) storage for aging.

Chemical analysis of blue cheese

Titratable Acidity

Titratable acidity (TA) of the milk or whey, where appropriate, was measured during cheese manufacture to monitor the activity of starter culture and development of acid within the cheese. Titratable acidity testing was performed in accordance standard method 15.3 (Bradley et al., 1992). To determine TA, 2 drops of phenolphthalein indicator was added to 9grams of milk or whey and titrated, drop wise, with 0.1N sodium hydroxide (NaOH) using a standardized burette. When the phenolphthalein indicator turned a faint pink color, the reaction was complete and the volume of NaOH used to titrate the sample was recorded. Acidity is expressed as a percentage of lactic acid where 1mL 0.1N NaOH = 0.009g lactic acid.

$$\%\text{acidity} = \frac{(mL\ \text{NaOH}) \times (\text{Normality of NaOH}) \times (9)}{\text{Sample weight in grams}}$$

pH

The pH of the blue cheese samples was taken in duplicate at each month of aging using an Accumet Basic AB15 pH meter fitted with an Accumet glass body, gel-filled, spear tip electrode. The electrode was standardized using standard solutions (pH=4, pH=7 and pH=10) before being submerged in homogenized cheese samples. The electrode was carefully rinsed between cheese samples and periodically used to read the pH=7 standard to ensure the calibration had not drifted.

Moisture

Cheese samples were analyzed in duplicate for moisture using the vacuum oven moisture and solids method 15.10 (Bradley et al, 1992). Moisture was determined at 2 weeks ("time zero") as well as 1, 2, 3, 4, and 5 months of aging. To obtain a more accurate measurement, blue cheese samples were homogenized by gently mixing the cheese sample into a blue-green paste just before moisture determination. Disposable aluminum moisture dishes were pre-dried for 3 hours at 100°C in a force draft oven. The aluminum pans were cooled in a dessicator and pre-weighed before 3 ± 0.5g cheese sample was spread into the pans in a uniform thickness. Each cheese sample was tested in duplicate and two blank pans were included for standardization. The aluminum pans with sample were then placed in the vacuum oven (Napco Vacuum oven model 5851) with filter paper covers for 4.75hours ± 15min. The oven was maintained at 100°C with a vacuum of 26.6 inHg (-90kPa) and 117 mL/min air flow. The dried sample pans were removed and placed in a dessicator to cool before being weighed. Percent moisture was calculated based on weight lost during drying.

$$\% \text{ Moisture} = \frac{(\text{Loss in cheese sample weight (g) - loss in blank weight (g)})}{\text{Original sample weight (g)}} * 100$$

Water Activity (a_w)

Water activity (a_w) was measured using a CX-2 Aqua Lab water activity meter (Aqua Lab Technologies, Riverside CA) for day 16- day 153. Day 153 measurements were also taken using an Aqua Lab VSA. Results from the two a_w meters were comparable. Homogenized cheese samples were packed into the bottom 1/3 of a Decagon disposable sample dish, completely covering the bottom of the dish. A lid was placed on the dish and the cheeses were allowed to sit for 1 hour before measurement. Four

standard solutions of NaCl were prepared with a_w = 0.92, 0.94, 0.96, and 0.98 according to Raoult's law, using a $\gamma = 1.004$ the true activity coefficient of sodium chloride (Labuza, 2004). These solutions were run in triplicate at each sampling time and used as a calibration curve for the CX-2 device.

Ash

Cheese samples were analyzed for ash content in duplicate according to gravimetric ash method 15.4 (Bradley et al.,1992) with additional steps for the determination of sodium and potassium concentration (see "Sodium and Potassium" below). Porcelain crucibles were marked with heat-safe marking ink and cleaned with aqua regia solution (3 parts HCl and 1 part HNO_3, diluted 1:1 with deionized water) by submersion for 3 hours. The crucibles were then rinsed and dried in a forced air oven (Lab-line Imperial II Thermo Fischer) for 1 hour at 100°C.

Approximately 2.5g cheese sample was accurately measured into clean, dry, pre-dried crucibles. Two empty crucibles were included for blanking calculations. Crucibles were pre-heated for 1 hour in a forced air oven at 100°C before being carbonized over a Bunsen burner (housed within a safety hood). The carbonized samples in crucibles were then placed in a muffle furnace to be incinerated at 550°C for 12 hours. At the end of 12 hours, the muffle furnace was shut off and the crucibles were allowed to cool for 8 hours before opening the furnace and transferring the crucibles to a dessicator.

The crucibles containing incinerated sample were then weighed a final time. Percent ash was determined according to the following equation:

$$\%\text{Ash} = \frac{\text{Ash residue (g)}}{\text{Weight of Cheese sample (g)}} \times 100$$

Sodium and potassium

Sodium and potassium concentrations were determined using atomic absorption. The applied method was adapted from the International Organization for Standardization ISO 8070:2007(E). The ash obtained from the total ash procedure (see previous section) was suspended in 1mL of 0.1N nitric acid in order to completely remove all mineral substances from the wall of the crucible. The suspended ash in acid was quantitatively transferred to a 250mL volumetric flask and brought to full volume with deionized water. Each sample was diluted a second time 20-fold and stored in conical centrifuge tubes for analysis.

Atomic absorption standards were created for both sodium and potassium by diluting 1000 mg/L (1000ppm) standard solutions obtained from Fischer Scientific. Dilutions used for standardization included 0.05, 0.5, 1.0, 2.5, 3.0, 5.0, and 7.5 mg/L (ppm). Standard solutions were created using 1mL 0.1N nitric acid brought to volume in 250mL deionized water to mimic sample dilutent. Atmoic absorption measurements were obtained for each sample using a Perkin Elmer AAnalyst spectrophotometer 100 (Perkin Elmer, Inc.) equipped with a Lumina Lamp K-Na cathode lamp. Compressed air was used as the flame oxidant and acetylene gas was used for fuel. The conditions for absorption measurements are listed below:

Wavelength: 589m

Slit width: 0.2 (Na), 0.7 (K)

Read time: 5 seconds

Read delay: 2 seconds

Oxidant flow: 10

Fuel flow: 3

Re-slope: 2.5mg/L

Fat

Total fat analysis was performed in duplicate for each cheese sample according to the Babcock method 15.8 (Bradley et al., 1992). Precisely 9g of cheese was weighed into a Paley bottle (0-50% graduations) before adding 9mL of hot (60°C) deionized water to melt and suspend the cheese sample. The bottle was capped and placed on a mechanical shaker (Lab Line, Lab Rotator) for approximately 10min to allow the cheese slurry to come to room temperature. With the bottle tilted away from the researcher, 17mL of concentrated sulfuric acid (APHA for fat Babcock method, specific gravity 1.825) was added in three additions of approximately 7mL, 5 mL, 5 mL while swirling to dissolve the sample. The addition of acid was complete within 20 seconds. The Paley bottles were then placed on the shaker for 15min to allow the acid to fully dissolve the cheese sample.

The Paley bottles were then placed into a Jalco Motor Co. Babcock centrifuge and centrifuged for 5 minutes. After 5 minutes, warm deionized water was added to each bottle to that the level of liquid inside the bottle reached the base of the neck. The bottles were centrifuged for an additional 2 minutes. Warm deionized water was added until the visible liquid fat later was completely suspended in the neck of the bottle. The bottles

were centrifuged for a final 1 minute before being placed in a 57°C water bath (VWR Scientific 1245 PC, VWR International, PA) for 15 minutes. The transparent fat columns were then immediately read to the nearest 0.5% using a standardized caliper. Fat columns were determined between the bottom of the lower meniscus to the top of the upper meniscus, with cloudy or brown fat columns being rejected for re-analysis.

Free Fatty Acids

Free fatty acids were extracted and analyzed for samples at month 3 and month 5 to be compared with sensory analysis and volatile flavor analysis. Extraction and detection methods were modified from previous work by De Jong and Badings (1990), Kaluzny et al. (1985), and Innocente et al. (2000). One gram of blue cheese sample was combined with 3g anhydrous sodium sulfate and ground using a glass motar and pestle in order to absorb moisture. Samples were transferred to a 15mL conical centrifuge tubes and 0.3mL 2.5M sulfuric acid and 1mL of internal standard were added to each tube. Internal standard consisted of 500mg/L C_7 (heptanoic acid) in ether. Three mL of a 1:1 mixture ethyl ether/heptane was added to each tube. The sample tubes were well mixed and centrifuged to clarify the solution. The clear aqueous solvent layer was removed and saved in a glass screw top test tube. The extraction with 1:1 ethyl ether/heptane was repeated two more times, each time saving the solvent layer. The extracts were stored frozen until solid phase extraction.

Solid phase extraction was performed in order to remove neutral lipids from the samples. Solid phase extraction was done using Supelco Discovery DSC-LC NH_2 3mL solid phase extraction tubes (Supelco, PA). The SPE tubes were conditioned twice with 5mL heptane. The vacuum manifold was held at constant vacuum of 5mmHg with a

condenser trap containing dry ice to prevent ether fumes from entering the vacuum pump. Once conditioned, the sample was applied to the column and filtered drop-wise. To elute neutral lipids, 2mL of 2:1 (chloroform: 2-propanol v/v) was applied to the column, twice. The final step was to elute fatty acids using 2 mL of 2% formic acid in ether solution, twice.

The collected extract (4mL) was concentrated down to 2mL using a nitrogen flush and brought back to 3mL with pure ethyl ether in order to ensure all fatty acids were in solution and none had attached to the side of the collection vial.

Fatty acid extracts were analyzed by gas chromatography on a Finnigan Focus gas chromatograph with flam ionization detector (Thermo Finnigan, Rodando, Milan). The column used was a fused silica capillary Nukol column, 30m x 0.25mm x 0.25mm (Supelco, PA). A Restek triple filter (Single position baseplate, #22020, SGT, Singapore) cleaned incoming He carrier gas. Two mL of sample extract was injected by auto injection using an AI 3000 auto injector (Therm Elecron Corp., Waltham, Massachusetts). The sample was injected in split mode (split flow = 20mL/min) at 230°C inlet temperature. Thermo GC Link software (Ver 2.0, 2002) was used for heating profile programming. The heating profile of each run was as follows: Initial hold 125°C for min, Ramp 1: 12°C/min to 190°C and hold for 28 min, Ramp 2: 12°C/min to 195°C and hold for 7 min, Ramp 3: 15°C/min to 125°C and hold for 1min. Fatty acids were quantified by use of standard solution relative peak intensities to the internal standard using ChromStar (Version 5.03, SPCA, 2001). The free fatty acids quantified were C2, C4, C6, C8, C10, and C12. There was a 5min read delay in data collection to allow for the ether solvent peak to pass without interfering with fatty acid data. Detector temperature was 220°C.

Total Protein

Total protein was determined by Kjeldahl method using modified protocol from the Kjeldahl method 15.12 (Bradley et al., 1992). Total protein was measured in duplicate for each cheese treatment and location at 2 weeks of aging. Nitrogen free weigh papers were used as blank calibration and to hold samples to make transfer into the digestion tubes easier. Approximately 0.5g of cheese sample was accurately weighed into a nitrogen free weigh paper. Samples in weigh papers were placed into individual digestion flasks with a single 5g kjeldhal catalyst tablet (Acros Mecrucry and Selenium free, K_2SO_4/Na_2SO_4 $CuSO_4$ and TiO_2) and 15mL concentrated sulfuric acid. The Kjeldahl digestion system used was a BUCHI block digestion system K-437 with Scrubber 414 and Distillation unit K-350 (BUCHI, Switzerland). Once the heating block had reached 420°C, the digestion flasks were lowered onto the heating element and digested at 420°C for 90minutes. The digestion flasks were allowed to cool completely, covered, overnight.

50mL deionized water was added to each digested flask prior to being distilled in the BUCHI K-350 with approximately 70mL of 32% NaOH. The Distillation time was approximately 4 minutes and the receiving flask contained 50mL 4% Boric Acid and 2 drops methyl red indicator. The receiving flask with distilled sample (yellow in color) was then titrated with 0.1 N HCl on a Metler Toledo DL22 auto titrator to the reaction end point pH of 5.2 (confirmed by a faint pink solution color.)

Total % nitrogen was determined by mL 0.1N HCl required to titrate the ammonium borate in the receiving flask according the following equation:

$$\%N=\frac{(\text{mL 0.1N HCl for sample - mL 0.1N HCl for blank}) \times 0.1\text{N HCl} \times 14\text{g/mol (Nitrogen)}}{\text{Sample weight in grams} \times 1000} \times 100\%$$

The % protein was determined from the % nitrogen using the standard conversion for dairy products, found in SMEDP (Bradley et al., 1992).

$$\%P = \%N \times 6.38$$

Proteolysis

Proteolysis was determined using two methods; the bicinchoninic acid (BCA) assay for total water soluble protein, and the ortho-phthaldialdehyde (OPA) assay for determination of water soluble free amino acid concentration. For both assays, sample was prepared according to methods described by Nielsen et al. (2001). Approximately 0.8g of homogenized cheese sample was combined with 6mL DD water into a 20mL DT-20 mixing vessel with dispersion tube (#3703100) fit atop an IKA ultra-turrax tube drive homogenizer, 3645000 (IKA Works, Inc., Wilmington, NC). The ultra-turrax was set to level 5 for 60 seconds. This slurry was transferred to a 15mL centrifuge tube with 4mL of DD water used to rinse the remainder of the slurry from the sample cup. This slurry was then centrifuged at 1800rpm for 10 minutes and the aqueous top layer was removed. The aqueous layer was passed through a 0.45um RC syringe filter and stored frozen for later use in both the BCA and OPA assays. When assays were implemented, the sample was diluted 10 fold to match the calibration curve provided in both assay protocols.

A Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) was used for the determination of total water-soluble peptides. Instructions were followed for the micro-plate procedure using a Synergy HT micro-plate reader equipped with KC 4 software (Version 3.3 Rev. 10, BIO-TEK Instruments, Inc. Winooski, VT). Samples were compared to a bovine serum albumin standard curve. BSA standards were provided in the BCA kit.

The o-phthaldialdehyde method is a dye binding method in which, under alkaline conditions and in the presence of a reducing agent (diethiothreitol), OPA binds to the a-amino group of free amino acids and small peptides (MW <6000 Da) to form a fluorescent compound which can be detected using spectrophotometry. The procedure used in this study was based on the method described by Nielsen et al., (2001) and Gencor International Inc. OPA reagent was made by dissolving 7.620g disodium tetraborate decahydrate and 200mg sodium dodecyl sulfate in a 200mL volumetric flask with 150mL warm (20°C) deionized water. 160mg o-phthaldialdehyde was dissolved in 4mL ethanol and quantitatively transferred to the tetraborate/SDS solution. Finally, 176mg diethiothreitol was added to this mixture and the solution was brought to 200mL with deionized water. OPA reagent is light sensitive and was used within 2.5 hours of preparation. A standard solution ladder L-Serine was made by dissolving L-Serine in deionized water at concentrations of 100, 140, 200, and 400 mg/L. Sample was prepared as previously discussed and was thawed for 30 minutes at room temperature before immediate use in the assay.

Spectrophotometer readings were performed at 340nm with a read time of 0.75 seconds using a Varian Cary 50 Scan UV-Vis spectrophotometer (Varian Australian Pty

Ltd.) and Cary WinUV software (3.1.3.1). Deionized water was used as the control. A total of 4 blanks were measured before each set of samples. To run a sample, or blank, 3.0mL OPA reagent was added to a clean dry test tube. 400uL sample (or blank, or standard) was added to this test tube and agitated for 5 seconds before being transferred to a plastic cuvette and held at room temperature for exactly 2minutes. At the end of 2 minutes, exactly, the absorbance was measured at 340nm. The average of 4 sample measurements was used for a given sample. Blanks and a 100mg/L Serine standard were used to standardize all samples. Absorbance values were correlated to equivalent mg free amino acid based on the serine standard curve. Final values were reported as mg FAA/g cheese.

Flavor Volatiles

Analysis of flavor volatiles was performed at the Department of Food Science, University of Wisconsin-Madison by Mingkai Cao. Contamination was prevented by autoclaving of all glassware and tubing materials at 121°C. The method of volatile profile analysis was modified from Verzera, 2004, Gkatzionis, 2009, and Wolf, 2010. A 1.0g sample of blue cheese was placed in an 8mL glass GC vial containing 1.0g 4-methyl-2-pentanone water solution (50ppm) for internal standard. A 85-um Carboxen/Polydimethylsiloxane SPME fiber (Supelco, Bellefonte, PA)fiber was exposed to the vial head space for 30 min at 45°C.

Analysis of flavor volatiles was performed using a gas chromatograph (Agilent 6890N; Agilent Technologies Inc., Palo Alto, CA) with a fused silica capillary column (RTx-5MS, 30-m long x 0.25-mm i.d. x 0.5-µm film thickness; Restek Corp., Bellefonte, PA) which was coupled to a mass selective detector (Agilent 5973 MS; Agilent

Technologies Inc.). The carrier gas was high purity (99.999%) helium, at a constant flow rate of 2.0mL/min. The injection temperature was 250°C. The initial column temperature was 70°C for 2 min, increased to 140°C at a rate of 6°C /min, held at 140°C for 2 min, increased to 220°C at a rate of 6°C /min and held at 220°C for 5 min. Identification of volatiles reported was made by matching the sample mass spectra with those from a published database (NIST Version 1.7 Mass Spectral Database; Agilent Technologies Inc.) and comparing retention times with those of authentic standards.

Statistical analysis of compositional data

Means of compositional data were compared using a linear mixed model with cheese make (1 or 2) as a blocking group (R statistical software v. 2.15.3, R foundation for statistical computing). Cheese treatment (control, reduced, reduced with KCl), time of testing (Day 16, 31, 61, 92, 122, 153) and Location (inner, outer) were main predictors. Cheese wheels were nested within cheese make and contributed to random error. Comparisons were made by Anova Type II technique using the "nlme" and "car" packages (Pinheiro, et al. 2013, Fox & Weisberg 2011, R core team, Vienna, Austria) to analyze significance of predictors and strength of models when non-contributing interactions were removed. Tukey's HSD was utilized for comparison of levels within a predictor and combinations of predictors such as Cheese treatment K at Day 16 v. Day 153.

Sensory analysis

Descriptive sensory analysis

Sensory evaluation and data analysis was completed by the University of Minnesota Sensory Center within the Department of Food Science and Nutrition. Descriptive analysis was completed at day 92 and 153 (month 3 and 5, respectively) for both cheese makes.

Subjects

Panelists (n=10) for descriptive analysis were members of the panel hired and trained by the Sensory Center at the University of Minnesota. All members were PROP tasters or supertasters and received monetary compensation for their participation. Recruiting and experimental procedures were approved by the University of Minnesota's Institutional Review Board.

Products

Three treatments of blue cheese were tested from both cheese makes at 92 and 153 days of aging. Samples were tested from both the inner and outer location of the cheese wheel. Before testing, cheese samples were processed for 30 seconds in a 3-cup capacity food processor (KitchenAid®, St. Joseph, MI). Panelists received about 1 tablespoon of blended blue cheese at room temperature (70°F) in a random 3-digit coded plastic 2 oz. (Propak™, Mooresville, NC) cup with lid.

Training

Prior to make 1 testing, panelists participated in four 1-hour training sessions in which they individually described pairs of blue cheese samples using descriptive terms provided. Any terms not included were discussed and added to create a final lexicon, which was used for all testing (Table 3.5). Definitions of each descriptive term were well

established within the first two training sessions. In the second two training sessions, panelists practiced rating attributes and discussed which were most prominent in a given sample. Before make 2 testing, panelists participated in two 1-hour training sessions to recalibrate their understanding of descriptive terms. The same testing procedure was repeated at day 153. No additional attributes were added to the lexicon for day 153 in comparison to day 92.

Testing

For each cheese make and time point (ex: Make 1, day 92) panelists participated in 4 testing sessions. In the first two sessions, panelists evaluated a set of three samples. These were replicated in the third and fourth sessions. Within a given testing session, sample order was balanced for position and carry over effects using Williams Latin Square design. During a testing session, panelists evaluated a sample by rating the intensity of attributes on a 20 point scale with 'none' on the left (0) side and 'intense' on the right (20) side. Flavor and taste intensity scales were calibrated to the standard citric acid scale while aroma intensities were calibrated to the standard butanol scale.

Data Analysis

Descriptive sensory analysis data was analyzed using SAS PROC GLM (version 9.1) to determine differences between product attributes. ANOVA models compared average attribute intensity scores for fixed effects of treatment (C, R, RK), location (inner, outer), cheese make (1 and 2) and all possible combinations of interactions. Significance was determined for $P<0.05$.

Table 3.5: Descriptive lexicon established for the evaluation of blue cheese aroma, flavor, taste, and aftertaste.

Descriptive Term	Definition	Reference
Aroma and Flavor		
Overall Intensity	The overall intensity of the aroma/flavor	
Rancid	Aromatics associated with short chain fatty acids	Butyric acid (20 ppm)
Waxy/Crayon	Aromatics associated with medium chain fatty acids	Crayons (Crayola™, Easton, PA)
Soapy	Aromatics associated with medium/long chain fatty acids	Soap (Dove®, Unilever, Trumball, CT)
Oxidized	Aroma associated with oxidized fat	Oxidized vegetable oil
Cardboard	Aroma reminiscent of cardboard	Moistened cardboard
Methyl Ketone	Aroma associated with blue-veined cheeses	2-octanone (40 ppm)
Moldy	Aroma associated with molds	2-ethyl-1-hexanol (10,000 ppm in glycol)
Musty	Aroma associated with freshly turned soil	Geosmin (25-30 ppb)
Milky	Aromatic/flavor associated with milk or milk derived products	Land O'Lakes Whole Milk
Creamy	A sweet, dairy note associated with cream or other high fat dairy products	Land O'Lakes Heavy whipping cream
Barny	Aromatic characteristic of a barn. Combination of manure, urine, moldy hay, feed, livestock odors.	10% whey protein concentrate in distilled water
Grassy	Aromatic associated with sweet, dry grasses	Hay
Burn	Chemical feeling factor associated with high concentrations of irritants	50% vinegar in distilled water
Basic Tastes		
Sweetness	The taste stimulated by sucrose and other sugars.	5.0% sucrose in distilled water (25g/500ml)
Sourness	The taste stimulated by acids, such as citric, malic, phosphoric, etc.	0.075% citric acid in distilled water (0.375g/500ml)
Saltiness	The taste stimulated by sodium salts, such as sodium chloride and sodium glutamate, and in part by other salts, such as potassium chloride	0.44% NaCl in distilled water (2.2g/500ml)
Bitterness	The taste stimulated by substances such as quinine, caffeine, and hop bitters	0.057% caffeine in distilled water (.285g/500ml)
Umami	Oral sensation stimulated by monosodium glutamate.	1.0% MSG in distilled water (5g/500ml)

Table 3.5 continued

Aftertaste		
Bitter	The taste stimulated by substances such as quinine, caffeine, and hop bitters	0.057% caffeine in distilled water
Salty	The taste stimulated by sodium salts, such as sodium chloride and sodium glutamate, and in part by other salts, such as potassium chloride	0.44% NaCl in distilled water
Sweet	The taste stimulated by sucrose and other sugars.	5.0% sucrose in distilled water
Sour	The taste stimulated by acids, such as citric, malic, phosphoric, etc.	0.075% citric acid in distilled water
Rancid	Aromatics associated with short chain fatty acids	Butyric acid (20 ppm)
Waxy/Crayon	Aromatics associated with medium chain fatty acids	Crayons (Crayola™, Easton, PA)
Soapy	Aromatics associated with long chain fatty acids, saponification	Soap (Dove®, Unilever, Trumball, CT)
Oxidized	Aroma associated with oxidized fat	Oxidized vegetable oil
Methyl Ketone	Aroma associated with blue-veined cheeses	2-octanone (40 ppm)
Moldy	Aroma associated with molds	2-ethyl-1-hexanol (10,000 ppm in glycol)
Musty	Aroma associated with freshly turned soil	Geosmin (25-30 ppb)
Barny	Aromatic characteristic of a barn. Combination of manure, urine, moldy hay, feed, livestock odors.	10% whey protein concentrate in distilled water
Grassy	Aromatic associated with sweet, dry grasses	Hay
Burn	Chemical feeling factor associated with high concentrations of irritants	50% vinegar in distilled water

Consumer Study

A consumer study to evaluate flavor, appearance, and texture liking was performed through the Sensory Center of the University of Minnesota Food Science and Nutrition Department. A total of 95 panelists were recruited from the students and staff of the University of Minnesota. Panelists were 18 years or older, had no food allergies, and had consumed blue cheese within the past 6months. Panelists were given monetary compensation for their participation in the study. The University of Minnesota's Institutional Review Board approved recruiting and testing procedures.

Products

Seven blue cheese samples were tested together in the consumer study. Cheese samples of Control, Reduced, and KCl salting treatments from both Make 1 and Make 2 as well as an industry standard were tested. The industry standard was St. Pete's Select Blue Cheese, Amablu, which was labeled as aged for 100 days (Faribault Dairy Company Inc., Faribault, MN). Two wheels of industry standard were used to account for variation in wheel composition. The ingredients of the industry standard blue cheese were: Whole milk, salt, cheese culture, rennet, and select *P. roqueforti* species.

Cheese samples were prepared by removing the outer most 0.5inch portion of the cheese wheel, and sampling the remainder of the wheel in a crumble form. Two wheels were prepared and mixed to account for wheel variation. Approximately 1 tablespoon of cheese crumbles was placed in a 3-digit coded plastic 2oz. cup (Propak^{TM}, Mooresville, NC) with lid and brought to room temperature before being served to the panelist.

Testing

Panelists received a single set of all 7 samples. The serving order was balanced for position and carryover effects. Panelists were instructed to taste the sample and evaluate it for overall liking, liking of appearance, liking of flavor, and liking of texture on a 120 point affective magnitude scale. The leftmost side of the scale (0) read "greatest imaginable disliking" and the rightmost end (120) read "greatest imaginable liking". Panelists could re-taste samples at any point during testing. Panelists were asked to rank the intensity of flavor, pungency/sharpness, stickiness, and firmness on a 20 point scale with the leftmost end reading "none" and the rightmost end reading "Extremely Flavorful, Extremely Pungent/Sharp, Extremely Sticky, or Extremely Firm".

Sensory Data Analysis

SAS PROC GLM (version 9.2) was used to determine differences of mean product attributes. Attribute intensities were dependent variables. Judge, sample, and taste position were predictors.

3.3 Results

Analysis of chemical properties

Water activity (a_w)

The water activity of salting treatments was determined over 153 days of aging and is summarized in Table 3.6 and Figure 3.3.

Table 3.6: The effect of sodium reduction with and without KCl on water activity (a_w). Values represent the mean of 8 cheese samples ±2SE except for Day 16, which represents the mean of 4 cheese samples.

Salt Treatment	*Age of Cheese (days)*					
	16	*31*	*61*	*92*	*122*	*153*
Control Inner[1]	0.955 ±0.003^{Aa}	0.944 ±0.008^{Ba}	0.944 ±0.002^{Ba}	0.943 ±0.005^{Ba}	0.941 ±0.003^{Ba}	0.932 ±0.001^{Ca}
Control Outer[2]	0.953 ±0.002^{Aa}	0.946 ±0.006^{Ba}	0.944 ±0.002^{Ba}	0.945 ±0.004^{Ba}	0.943 ±0.004^{Ba}	0.938 ±0.001^{Cb}
Reduced Inner[3]	0.963 ±0.004^{Ac}	0.949 ±0.008^{Ba}	0.952 ±0.002^{Bb}	0.952 ±0.006^{Bb}	0.949 ±0.003^{Bb}	0.943 ±0.001^{Dc}
Reduced Outer[4]	0.961 ±0.002^{Ab}	0.954 ±0.007^{Bb}	0.954 ±0.001^{Bc}	0.954 ±0.005^{Bb}	0.948 ±0.003^{Bb}	0.945 ±0.001^{Cd}
KCl Inner[5]	0.957 ±0.004^{Aa}	0.952 ±0.002^{Ba}	0.947 ±0.003^{Cd}	0.943 ±0.004^{Da}	0.941 ±0.002^{Da}	0.937 ±0.002^{Ea}
KCl Outer[6]	0.956 ±0.005^{Aa}	0.950 ±0.004^{Ba}	0.949 ±0.002^{Bd}	0.945 ±0.004^{Ca}	0.942 ±0.002^{Da}	0.938 ±0.002^{Eb}

A-E: Mean values within the same row with the same letter superscript are not significantly different, indicating changes for a specific treatment over time.
a-e: Mean values within the same column with the same letter superscript are not significantly different, indicating changes for all treatments at a single time
[1]Control Inner = Full sodium cheese, sampled from center of wheel
[2]Control Outer = Full sodium cheese, sampled 0.6cm below wheel surface
[3]Reduced Inner = 25% sodium reduction target, sampled from center of wheel
[4]Reduced Outer = 25% sodium reduction target, sampled 0.6cm below wheel surface
[5]KCl Inner = 25% sodium reduction with KCl, full sodium cheese target, sampled from center of wheel
[6]KCl Outer = 25% sodium reduction with KCl, sampled 0.6cm below wheel surface

Time, salting treatment, and location were significant factors affecting the water activity of a cheese sample. Water activity decreased over time for all samples (on average, from 0.958 to 0.939 day 16 to 153, respectively). The reduced treatment had a higher water activity than both the control and KCl treatment for all time points measured except day 31. The control and KCl treatments did not differ at any time during aging. The inner and outer samples co-varied with time, within a given salting treatment. At day 16, reduced inner samples had higher water activities than reduced outer sample. At day 153, the inverse relationship was true.

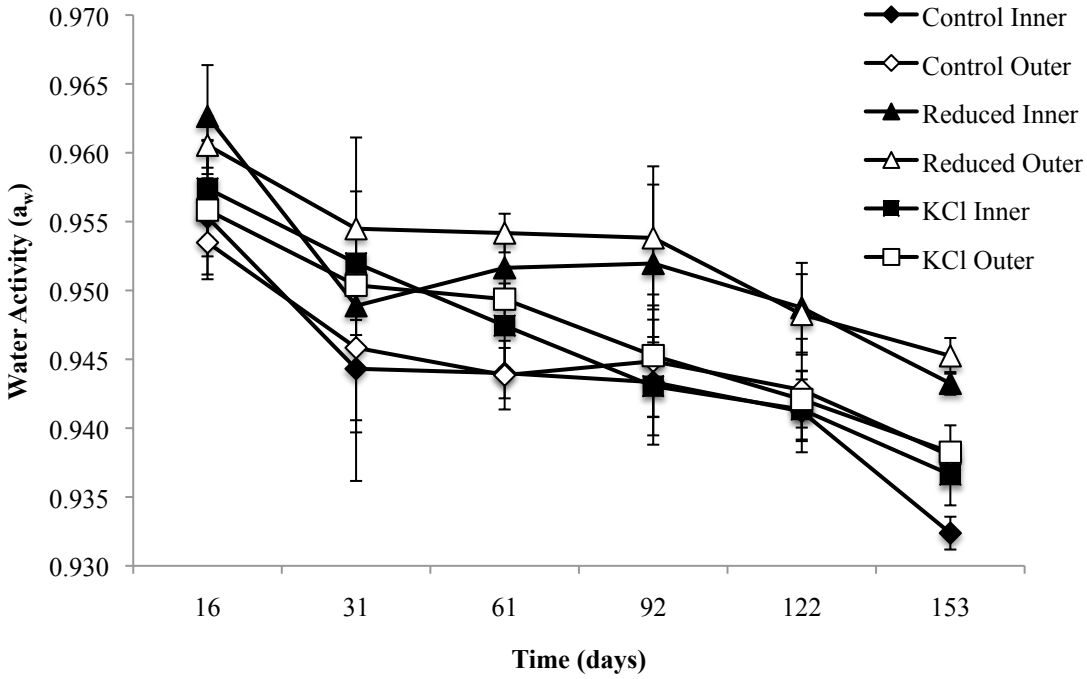

Figure 3.3: The effect of sodium reduction, with and without KCl replacement, on water activity (aw). Error bars represent 2SE. Data points represent an average of 8 measurements except for day 16, which is the average of 4 samples.

Moisture

The percent moisture of cheese treatments was monitored over 153 days of aging. The moisture content of all salting treatments and locations decreased over time (Figure 3.4). Reduced salt treatments were higher in moisture than control and KCl treatments at time 31, 61, and 153 days at corresponding locations. There were no differences in moisture between KCl and Control treatments or locations for any time points. Location of sampling did not impact moisture for any salting treatment.

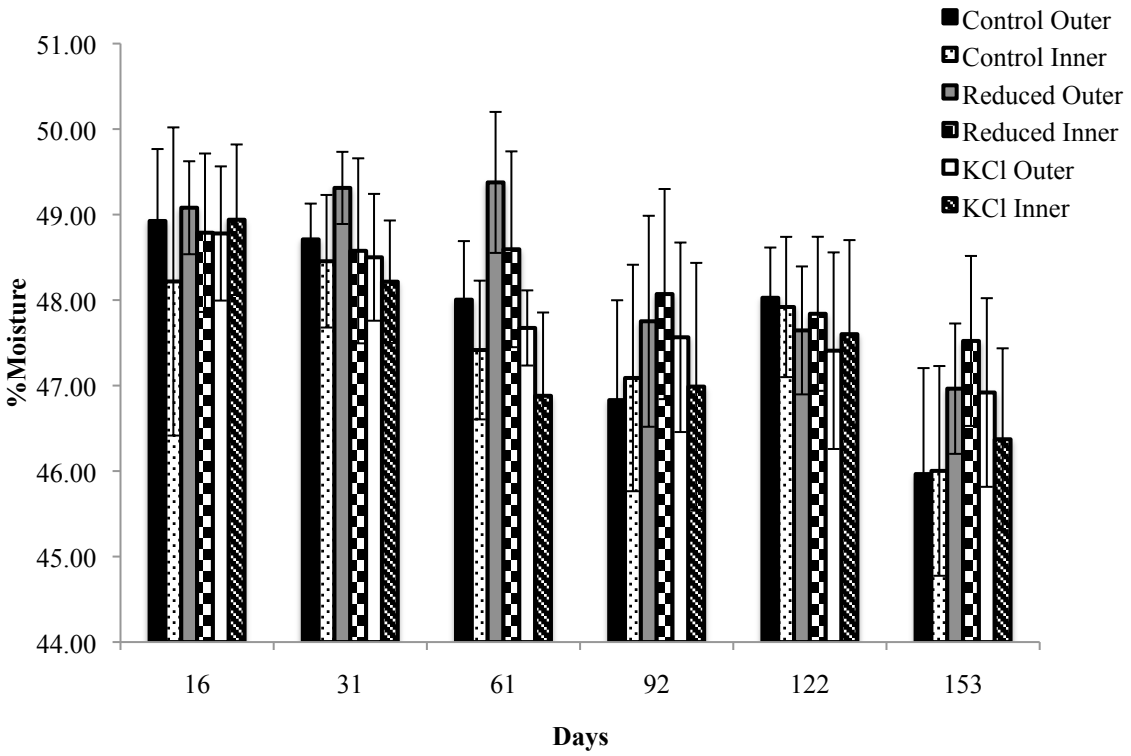

Figure 3.4: The effect of sodium reduction, with and without KCl replacement, on Moisture. Data represent an average of 8 measurements. Error bars represent 2SE.

Salt to Moisture

The salt to moisture (% Salt / %Moisture, **S/M**) ratios were determined for all cheese treatments over aging (Table 3.7). In the case of the reduced sodium with KCl treatment, S/M_{eff} represents the effective S/M with %KCl converted to effective %NaCl using the molar ratio of potassium and sodium.

Table 3.7: S/M during aging. Data represent an average of 8 measurements, ±2SE.

	Age of Cheese (days)					
Salt Treatment	***16***	***31***	***61***	***92***	***122***	***153***
Control Inner	5.26 ±0.08	6.18 ±0.37	6.59 ±0.19	6.72 ±0.16	6.52 ±0.30	6.36 ±0.16
Control Outer	5.98 ±0.18	6.19 ±0.34	6.66 ±0.28	6.54 ±0.18	6.26 ±0.48	6.49 ±0.27
Reduced Inner	4.70 ±0.34	4.91 ±0.23	5.28 ±0.32	5.54 ±0.14	5.35 ±0.15	5.13 ±0.39
Reduced Outer	5.08 ±0.22	5.20 ±0.33	5.30 ±0.39	5.60 ±0.13	5.31 ±0.14	5.31 ±0.23
KCl Inner[1]	5.81 ±0.51	6.08 ±0.26	6.59 ±0.42	6.98 ±0.14	6.60 ±0.29	6.75 ±0.24
KCl Outer[1]	6.55 ±0.63	6.93 ±0.62	6.40 ±0.39	6.81 ±0.16	6.46 ±0.32	6.54 ±0.27

[1] Values for treatments containing KCl blends are Effective S/M (S/M_{eff}) where %KCl has been converted to equivalent %NaCl based on molecular weight.

S/M_{eff} were higher for both control and KCl treatments over time as compared to reduced. KCl treatments had higher S/M_{eff} than control treatments until day 31. At day 61 onward, KCl and control treatments had the same S/M_{eff}.

Inner locations had lower S/M_{eff} than outer locations at the early stages of aging. For the control treatment, the inner samples had the same S/M_{eff} as outer samples by day 31. Reduced and KCl treatments had the same S/M_{eff} in inner and outer treatments by day 61 (Figure 3.5).

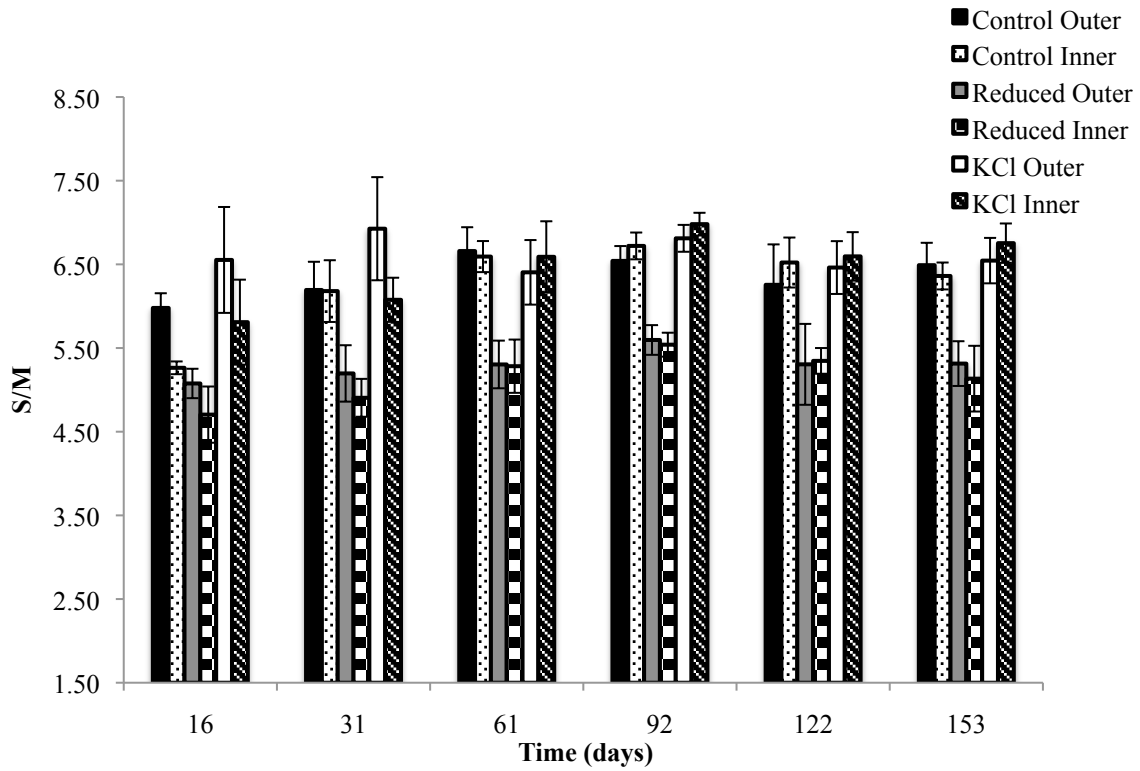

Figure 3.5: S/M_{eff} during aging. Data represent an average of 8 measurements. Error bars represent 2SE.

Sodium

The concentration of sodium (mg/100g cheese) was higher in control treatments than reduced or KCl (Figure 3.6).

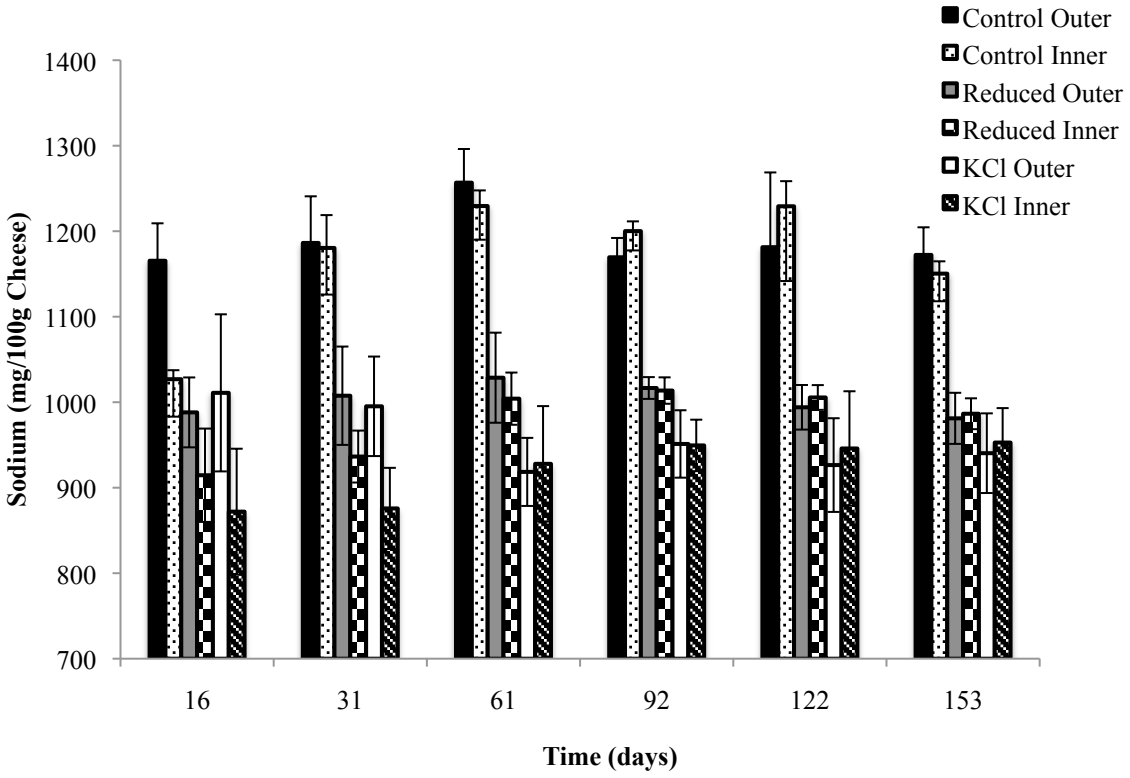

Figure 3.6: The effect of sodium reduction, with and without KCl replacement, on Sodium concentration. Data represent an average of 8 measurements. Error bars represent 2SE.

Control treatments were manufactured targeting 1200mg Na/100g cheese.

Reduced and KCl treatments were manufactured to target 900mg Na/100g cheese (Table 3.8).

Table 3.8: Sodium concentration (mg/100g Cheese) during aging. Values represent the average of 8 samples, ±2SE.

Salt Treatment	*Age of Cheese (days)*					
	16	***31***	***61***	***92***	***122***	***153***
Control Inner	1027 ±21	1180 ±77	1229 ±37	1200 ±23	1229 ±59	1150 ±29
Control Outer	1165 ±44	1186 ±54	1256 ±39	1169 ±23	1181 ±87	1172 ±32
Reduced Inner	914 ±54	936 ±30	1004 ±31	1013 ±15	1005 ±15	986 ±18
Reduced Outer	988 ±41	1007 ±57	1028 ±53	1016 ±13	994 ±26	981 ±30
KCl Inner	871 ±73	875 ±47	927 ±67	949 ±30	945 ±67	952 ±40
KCl Outer	1010 ±92	995 ±58	918 ±30	951 ±40	926 ±55	940 ±47

Cheese samples from the inner wheel location had a lower concentration of sodium than the outer wheel samples at 16 and 31 days for both the reduced and KCl treatments. Control treatment inner wheel samples had lower concentrations of sodium than the outer wheel samples only at day 16. Sodium concentration did not change for any treatment or location after day 31.

To evaluate the sodium reduction achieved for the reduced and KCl treatments, a check-point was established at day 61 and utilized an entire wheel of each cheese type. Full wheels were homogenized and compared . KCl treatments achieved a 23% reduction in sodium as compared to the control. Reduced treatments achieved a 21% reduction in sodium as compared to the control.

Potassium

Potassium levels were higher in KCl treatments as compared to the reduced and control treatments, as expected (Figure 3.7). Reduced and control treatments had similar potassium levels at both inner and outer locations, for all time points.

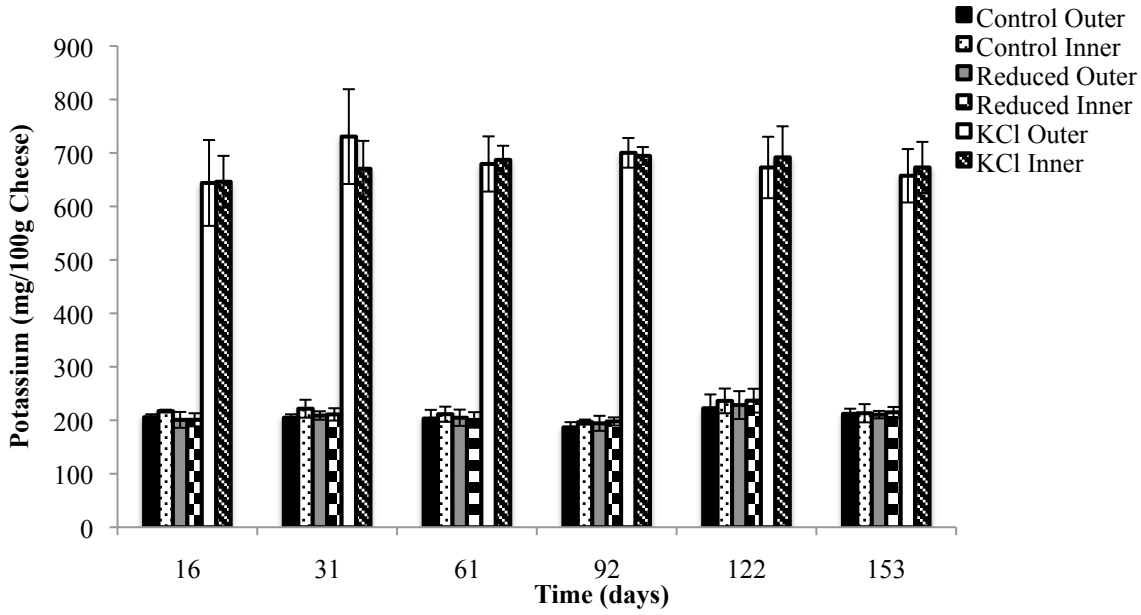

Figure 3.7: The effect of sodium reduction, with and without KCl replacement, on potassium concentration. Data represent an average of 8 measurements. Error bars represent 2SE.

Ash

The % Ash for all samples over 153 days of aging is shown in Figure 3.8. The % Ash was higher in control and reduced with KCl samples in comparison to reduced samples. The % Ash was similar between control and reduced with KCl samples, with reduced with KCl samples sometimes having larger %Ash due to the larger molecular weight of potassium in comparison to sodium.

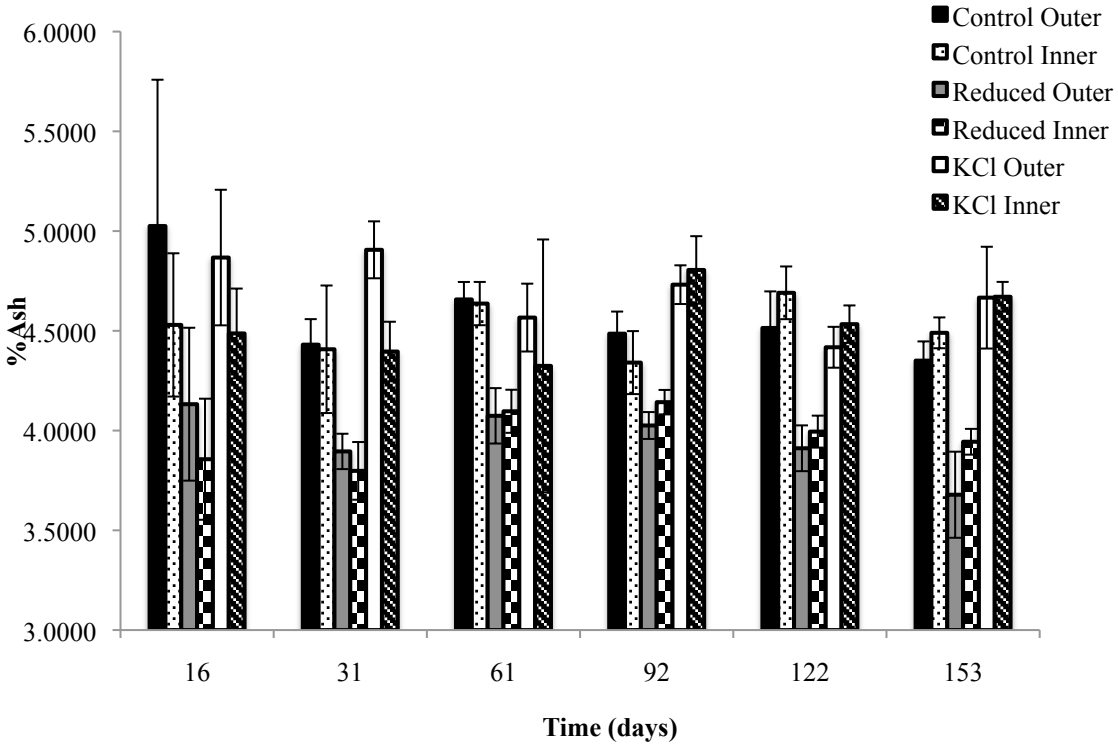

Figure 3.8: The effect of sodium reduction, with and without KCl replacement, on % Ash. Data represent an average of 8 measurements. Error bars represent 2SE.

pH

The pH of cheese treatments was monitored over 153 days (Table 3.9, Figure 3.9). There was no overall trend in pH over time, and there was no difference in pH between the salting treatments. The pH of inner cheese samples was higher than the pH of outer samples for aging time points 16 days – 61 days, and at 122 days ($p < 0.05$).

Table 3.9: The effect of sodium reduction with and without KCl on pH. Values represent the mean of 8 samples ±2SE.

	Age of Cheese (days)					
Salt Treatment	***16***	***31***	***61***	***92***	***122***	***153***
Control Inner[1]	5.58 ±0.13[a]	5.22 ±0.11[a]	5.25 ±0.18[a]	5.09 ±0.08[a]	5.51 ±0.49[a]	5.11 ±0.05[a]
Control Outer[2]	5.15 ±0.25[b]	4.97 ±0.04[b]	4.99 ±0.13[b]	4.89 ±0.08[a]	5.23 ±0.34[b]	4.97 ±0.05[a]
Reduced Inner [3]	5.30 ±0.30[a]	5.24 ±0.11[a]	5.50 ±0.41[a]	5.07 ±0.08[a]	5.06 ±0.12[a]	5.16 ±0.07[a]
Reduced Outer[4]	5.02 ±0.23[b]	5.01 ±0.08[b]	5.13 ±0.14[b]	4.99 ±0.09[a]	4.94 ±0.12[b]	4.94 ±0.05[a]
KCl Inner [5]	5.45 ±0.14[a]	5.25 ±0.08[a]	5.52 ±0.35[a]	5.18 ±0.10[a]	5.37 ±0.28[a]	5.06 ±0.10[a]
KCl Outer[6]	4.98 ±0.15[b]	4.97 ±0.05[b]	5.17 ±0.21[b]	5.05 ±0.09[a]	5.09 ±0.14[b]	5.04 ±0.06[a]

a-b: Mean values within the same column with the same letter superscript are not significantly different, indicating changes for all treatments at a single time

[1]Control Inner = Full sodium cheese, sampled from center of wheel

[2]Control Outer = Full sodium cheese, sampled one inch below wheel surface

[3]Reduced Inner = 25% sodium reduction target, sampled from center of wheel

[4]Reduced Outer = 25% sodium reduction target, sampled one inch below wheel surface

[5]KCl Inner = 25% sodium reduction with KCl, full sodium cheese target, sampled from center of wheel

[6]KCl Outer = 25% sodium reduction with KCl, sampled one inch below wheel surface

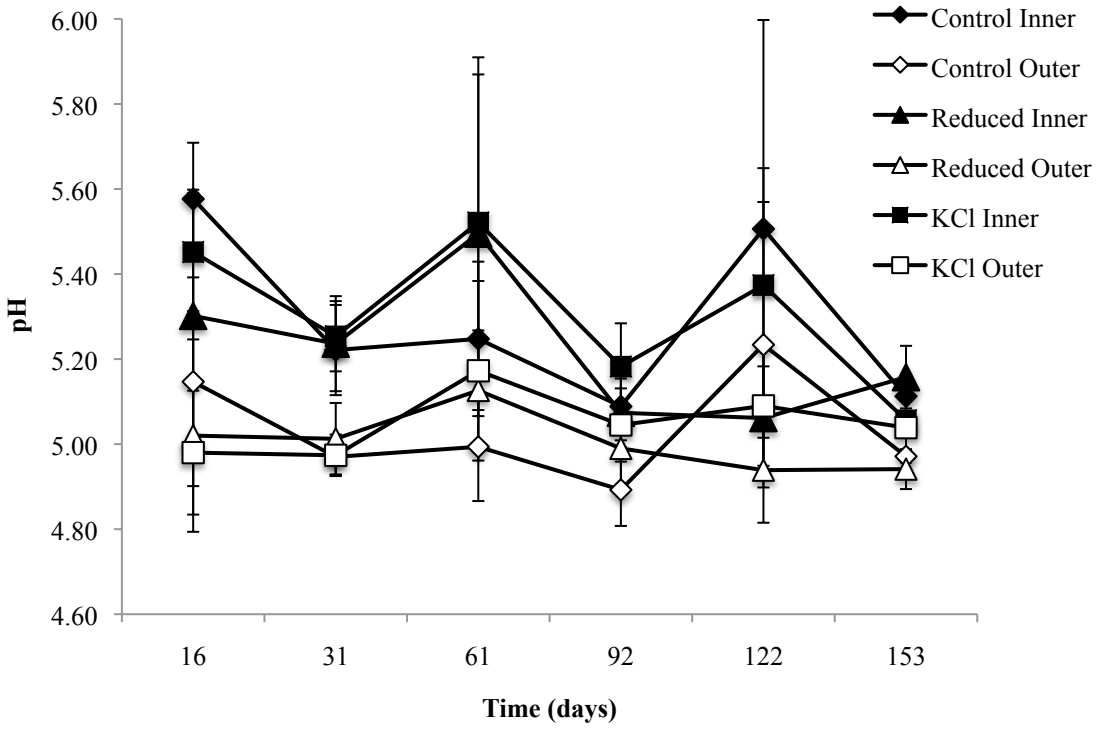

Figure 3.9: The effect of sodium reduction, with and without KCl replacement, on pH. Data represent an average of 8 measurements. Error bars represent 2SE.

%Fat in dry matter

Fat in dry matter increased over time for all treatments (Figure 3.10). Reduced samples from the inner wheel were higher than all other samples at day 122 and 153. For all treatments, inner wheel samples were higher than outer wheel samples after day 61.

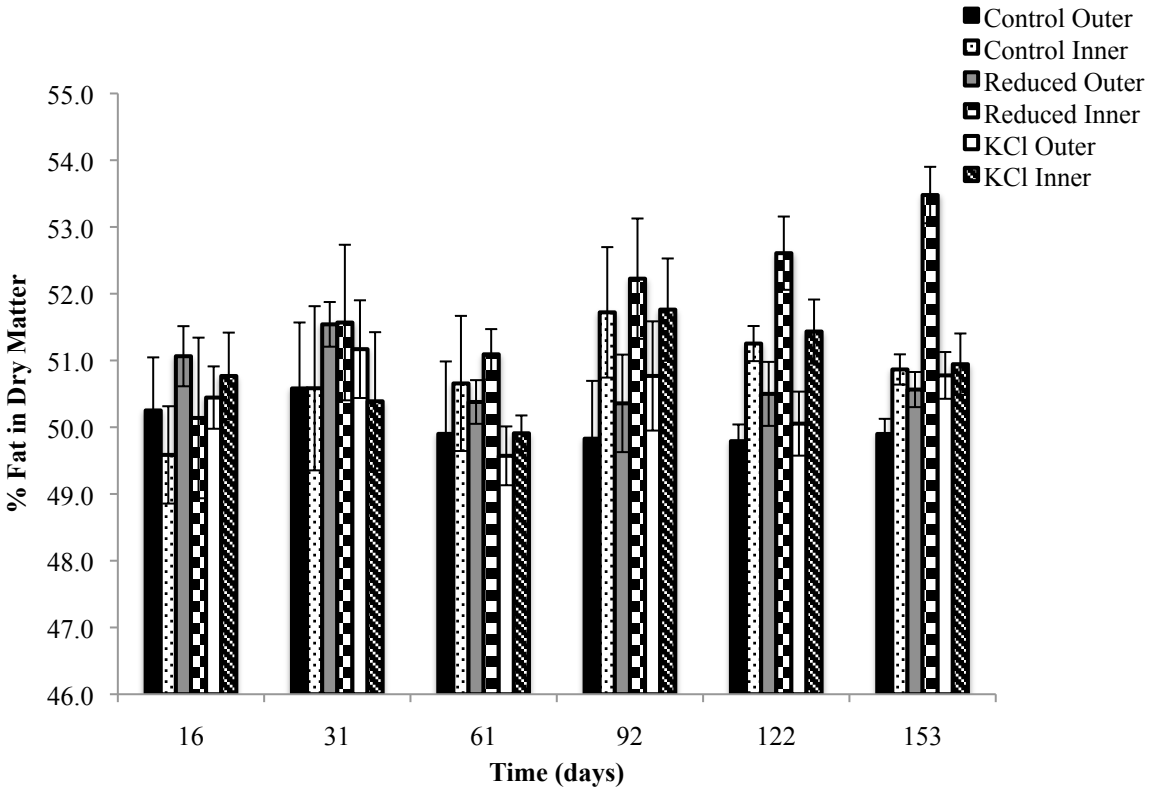

Figure 3.10: The effect of sodium reduction, with and without KCl, on %fat in dry matter. Data represent an average of 8 measurements. Error bars represent 2SE.

Free Fatty Acids

Five short to medium chain fatty acids and 2 iso-compound derivatives were detected in all samples at both 92 and 153 days of aging (Table 3.10).

Table 3.10: Free fatty acids and isomers detected in all treatments of blue cheese, at day 92 and 153 and previously described associated aroma note (Cadwallader et. al., 2007)

Compound	Associated Aroma
Free Fatty Acid	
Butyric	fecal, vomit, cheesy
Caproic	sweaty, goat-like
Capric	body odor, rancid
Lauric	waxy, soapy
Myristic	soapy
Branched Chain	
isobutryic	rancid butter, sweaty, sweet, apple-like
isovaleric	sweaty,sweet, rotten fruit

Quantified values for FFA can be found in Table 3.11. The quantified values had extremely high variation. Significance of quantified FFA was determined by ANOVA (Table 3.12). Butryic acid concentration decreased over time for all treatments and was not different between treatments. Isobutyric acid concentration increased with aging in inner samples and decreased with aging in outer samples. Isobutryic acid concentrations increased during aging for control, but not KCl or reduced treatment cheese. Concentrations of isovaleric acid were higher in KCl treatments compared to control or reduced. Isovaleric acid concentration decreased over time for KCl and reduced treatments, but was unchanging in the control treatment. Caproic acid levels did not vary for any treatments over aging. Concentrations of capric, lauric, and myristic acid were higher in inner wheel samples compared to outer. Myristic and lauric acid concentraionts were higher in the control treatment compared to reduced or KCl.

Table 3.11: Mean values and standard deviations of quantified free fatty acids at 92 and 153 days of aging

	Butryic Acid				*Isobutryic Acid*				*Isovaleric Acid*				*Caproic Acid*			
	Day 92		*Day 153*		*Day 92*		*Day 153*		*Day 92*		*Day 153*		*Day 92*		*Day 153*	
Treatment	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD
Control Inner	**7323.82**	1037.81	**4235.02**	833.16	**1.43**	0.19	**4.18**	1.98	**3.44**	1.13	**3.97**	1.16	**41.87**	47.49	**5.41**	2.48
Control Outer	**7057.71**	2390.58	**3944.30**	538.58	**3.19**	4.66	**2.33**	0.90	**2.38**	1.40	**3.48**	2.21	**11.22**	24.44	**6.26**	4.23
Reduced Inner	**7097.08**	593.99	**3785.33**	404.35	**2.32**	0.99	**2.12**	0.80	**3.73**	1.59	**2.04**	1.44	**23.54**	52.25	**7.21**	4.63
Reduced Outer	**7063.12**	1153.59	**3965.59**	708.96	**1.62**	0.77	**1.38**	0.86	**3.52**	7.55	**1.07**	0.64	**4.56**	5.17	**6.30**	3.85
KCl Inner	**6642.60**	990.96	**4256.80**	599.96	**3.39**	2.73	**2.65**	1.03	**6.43**	5.53	**3.16**	0.97	**6.58**	5.72	**7.62**	2.58
KCl Outer	**6486.24**	590.38	**4743.80**	370.71	**2.80**	1.56	**1.88**	0.75	**5.56**	2.86	**0.83**	0.63	**7.34**	8.50	**11.57**	9.70

Table 3.11 Continued

Treatment	*Capric Acid*				*Lauric Acid*				*Myristic Acid*			
	Day 92		*Day 153*		*Day 92*		*Day 153*		*Day 92*		*Day 153*	
	mg/Kg Cheese	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD	**mg/Kg Cheese**	SD
Control Inner	**184.03**	323.02	**102.44**	42.11	**480.86**	301.84	**857.44**	556.67	**362.35**	537.93	**189.75**	31.39
Control Outer	**73.82**	91.46	**45.67**	18.06	**637.68**	373.47	**339.48**	307.44	**154.52**	88.95	**107.58**	52.34
Reduced Inner	**117.71**	91.86	**121.64**	101.72	**289.09**	336.66	**330.06**	346.38	**181.69**	82.05	**165.60**	91.52
Reduced Outer	**49.25**	19.70	**57.50**	46.05	**370.64**	235.63	**367.59**	215.07	**89.00**	25.76	**96.16**	48.97
KCl Inner	**72.74**	32.24	**84.59**	44.59	**446.47**	424.90	**494.34**	437.67	**175.58**	52.59	**139.41**	52.33
KCl Outer	**57.35**	22.27	**27.92**	12.50	**475.60**	356.23	**300.77**	168.26	**119.02**	39.19	**75.26**	22.39

Table 3.12: Values within each cell represent probabilities (p) from a full-factorial ANOVA of free fatty acid relative quantities in blue cheese. Cells with (-) indicate p-value >0.05.

		Probability (p) values for free fatty acids in blue cheese						
Model Factor	**df[4]**	*butyric*	*isobutyric*	*isovaleric*	*caproic*	*capric*	*lauric*	*myristic*
Salting Treatment (S)[1]	2	-	-	<0.05	-	-	<0.05	<0.05
Location (L)[2]	1	-	-	-	-	<0.001	-	<0.001
Time (T)[3]	1	<0.001	-	<0.01	-	-	-	-
S * L	2	-	-	-	-	-	-	-
S * T	2	-	<0.05	<0.001	-	-	-	-
L * T	1	-	<0.05	-	-	-	<0.01	-
S * L * T	2	-	<0.05	-	-	-	<0.05	-

[1] Salt Treatment = Control, Reduced, or KCl salting treatment
[2] Location = Inner or Outer sampling region
[3] Time = 92 or 153 days of aging
[4] df = degrees of freedom of model factor

Water Soluble Peptides

Percent water soluble peptides increased from day 16 to day 31 (Figure 3.11). From day 61 to 152, the percent WSP were not significantly different for any treatment. Inner wheel samples had higher %WSP than outer wheel samples at day 16 for all treatments and for control treatments until day 61. While not statistically significant, reduced treatment samples tended to have larger WSP than control or KCl.

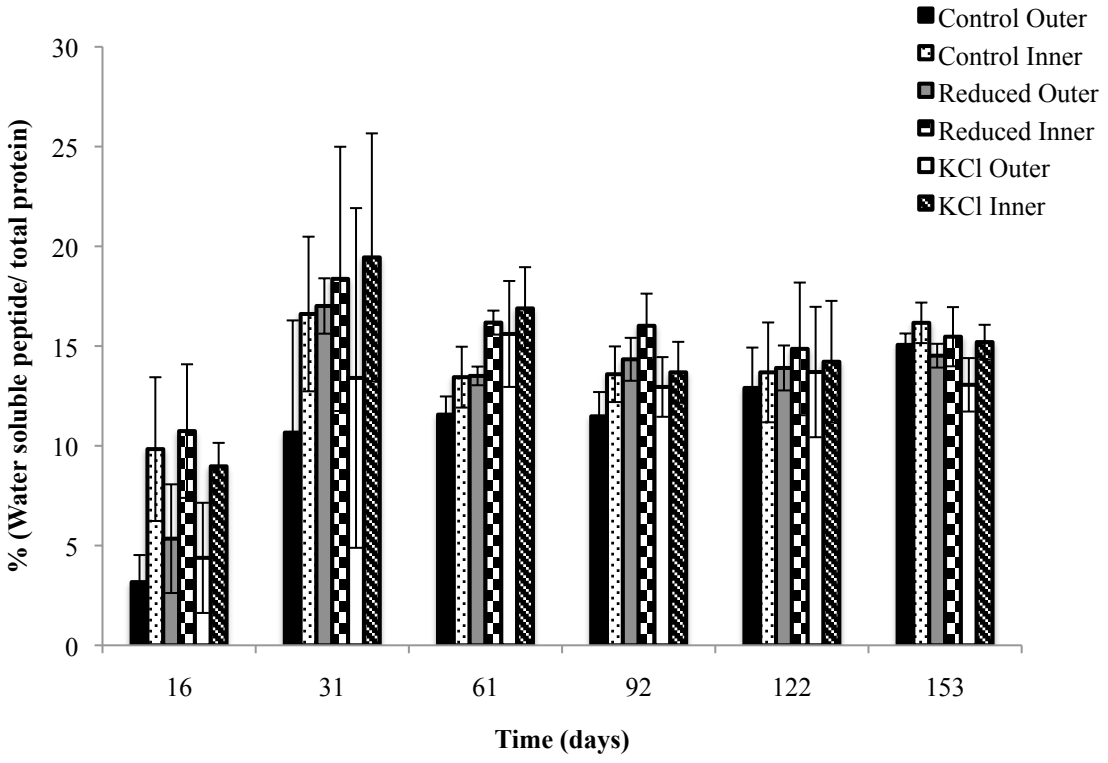

Figure 3.11: The effect of sodium reduction, with and without KCl, on %water soluble peptides. Data represent an average of 8 measurements. Error bars represent 2SE.

Free Amino Acid

Free amino acid (FAA, mg/g Cheese) concentration was higher in inner wheel samples than outer wheel samples for all time points and treatments (Figure 3.12). FAA increased from day 16 to day 31 and remained constant for the remainder of aging. There was no difference in FAA concentration between treatments, though reduced samples trended higher.

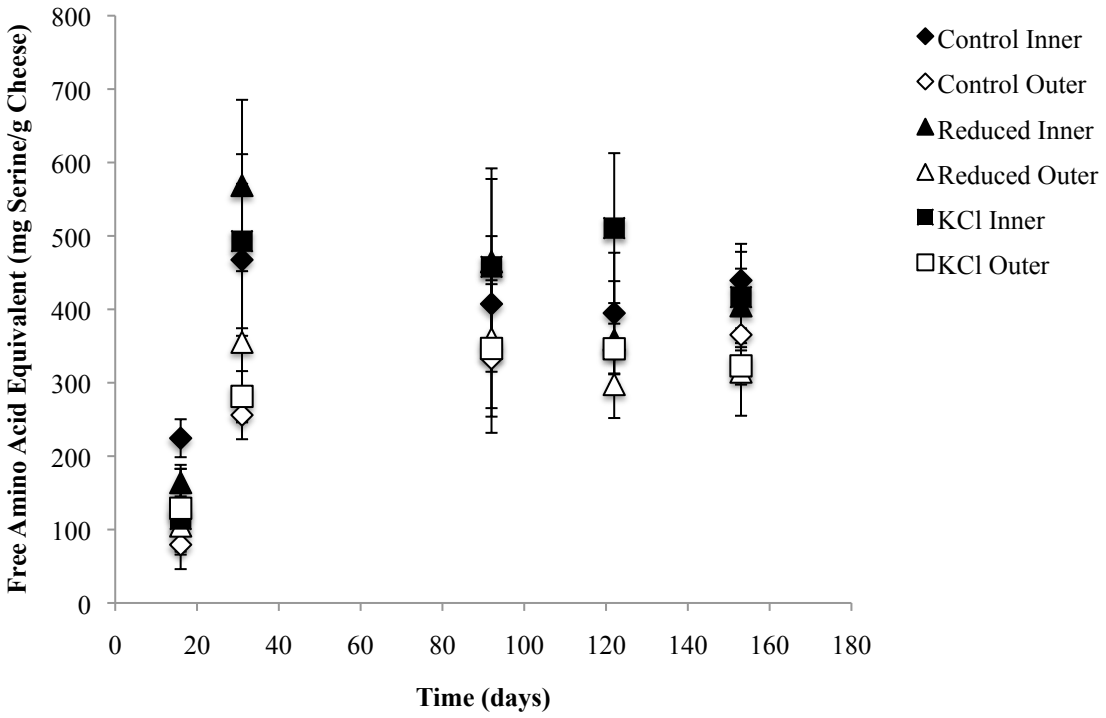

Figure 3.12: The effect of sodium reduction, with and without KCl, on Free Amino Acid concentration (mg/g cheese). Data represent an average of 8 measurements. Error bars represent 2SE.

Flavor Volatiles

15 volatile flavor compounds were detected in blue cheese sampled at day 92 and 153 (Table 3.13). Relative intensities compared by ANOVA found 7 volatile compounds impacted by location, age, or an interaction of treatment and time (Table 3.14), one of which was an unidentified hexanone. There were no differences attributed solely to salting treatment for any volatile detected. Ethyl hexanoic and methyl propanoate were measured at higher intensity in inner wheel samples than outer wheel samples and increased from day 92 through 153 within inner wheel samples. Dimethyl sulfide intensity decreased from day 92 to 153 for C and RK treatment, but increased over time for R treatment. 2-Heptanone, a methyl ketone typically associated with blue cheese flavor, had higher intensity in inner samples within a treatment at day 153, but not day 92. Inner samples had higher relative intensities of ethyl butyrate, ethyl hexanoate, and methyl propanoate.

Table 3.13: Flavor volatiles detected in all treatments of blue cheese, at day 92 and 153 and previously described associated aroma note (Molimard and Spinnler, 1995; Cadwallader et. al., 2007)

Compound	Associated Aroma
Acid	
Butanoic Acid	rancid, cheese
Alcohol	
2-Methylpropanol	alcohol, fuel
1-Butanol	floral, fruity, sweet
3-Methyl-1-Butanol	fruity, alcohol, solvent-like, grainy
Ester	
Ethyl butyrate	sweaty,sweet, rotten fruit
Ethyl hexanoate	fruity, pineapple, banana
Methyl propanoate	sweet, fruity, apple-like
Ketone	
2-Butanone	etheric, acetone
2-Pentanone	blue cheese
2-Hexanone	blue cheese
2-Heptanone	blue cheese
2-Octanone	floral, fruity, musty, soapy
8-Nonen-2-one	fruity, mushroom, musty
2-Nonanone	green, fatty, varnish, blue cheese
Sulfur Compounds	
Dimethyl sulfide	cabbage, cauliflower, goat, onion, potato

Table 3.14: Values within each cell represent probabilities (p) from a full-factorial ANOVA of flavor volatile relative intensities in blue cheese. Cells with (-) indicate p-value >0.05.

		Propability (p) values for flavor volatiles in blue cheese					
Model Factor	***df*** [4]	*2-Pentanone*	*Dimethyl Sulfide*	*Ethyl butyrate*	*2-Heptanone*	*Ethyl hexanoate*	*Methyl Propanoate*
Salt Treatment (S)[1]	2	-	-	-	-	-	-
Location (L)[2]	1	-	-	<0.05	<0.05	<0.005	<0.0001
Time (T)[3]	1	-	-	-	-	-	-
S * L	2	-	-	-	-	-	-
S * T	2	-	<0.005	-	-	-	-
L * T	1	<0.005	-	<0.05	-	-	<0.05
S * L * T	2	<0.01	-	-	-	-	-

[1] Salt Treatment = Control, Reduced, or KCl salting treatment
[2] Location = Inner or Outer sampling region
[3] Time = 92 or 153 days of aging
[4] df = degrees of freedom of model factor

Sensory Evaluation

Descriptive Sensory Analysis: Day 92 (Month 3)

Descriptive sensory analysis of both cheese makes at 92 days of aging provided an overall comparison of salting treatment, location, and cheese make. The pertinent results ($P < 0.05$) are displayed in Table 3.15. In general, at month three, the large differences existed between reduced treatments and control or KCl.

Reduced treatments were scored higher for intensities of creamy aroma, barny aroma, and sweetness compared to the control. KCl had more intense waxy crayon, overall aroma, barny aroma, sweetness and sourness as compared to the control. Between reduced and KCl treatments, the KCl treatments had more waxy/crayon flavor.

In general, inner wheel samples had much higher intensities than outer wheel samples (Table 3.16)

Table 3.15: Descriptive sensory analysis between treatments, Day 92

Sensory Attribute	Control	Reduced	KCl	p value
Overall Intensity of Aroma	7.7^{b}	7.7^{b}	7.9^{a}	0.036
Creamy Aroma	1.1^{b}	1.5^{a}	1.3^{ab}	0.008
Barny Aroma	0.7^{b}	1.6^{a}	1.4^{a}	0.01
Sweetness	0.5^{b}	0.9^{a}	0.9^{a}	0.017
Sourness	1.9^{b}	2.2^{ab}	2.3^{a}	0.037
Waxy/Crayon Flavor	4.9^{b}	4.6^{b}	5.0^{a}	0.006

Table 3.16: Descriptive sensory analysis between location, Day 92

Sensory Attribute	Inner	Outer	p value
Overall Intensity of Aroma	7.9[a]	7.6[b]	0.005
Rancid Aroma	5.1[a]	4.5[b]	0.001
Methyl Ketone Aroma	5.1[a]	4.7[b]	0.005
Milky Aroma	0.8[a]	1.1[a]	0.013
Overall Intesity of Flavor	6.5[a]	6.1[b]	0.004
Rancid Flavor	3.6[a]	3.4[b]	0.022
Waxy/Crayon Flavor	5.0[a]	4.6[b]	0.002
Soapy Flavor	3.5[a]	3.2[b]	0.006
Musty Flavor	4.9[a]	4.6[b]	0.046
Sweet Aftertaste	1.7[a]	1.4[b]	0.046
Sour Aftertaste	4.1[a]	3.8[b]	0.018
Rancid Aftertaste	3.4[a]	3.0[b]	0.001
Methyl Ketone Aftertaste	3.7[a]	3.5[b]	0.042

Descriptive Sensory Analysis: Day 153 (Month 5)

Descriptive analysis at day 153 had far fewer differences between attribute intensities for salting treatment (Table 3.17). KCl cheese were more bitter than control cheeses and were more oxidized than control or reduced samples.

As was seen on day 92 analysis, inner wheel samples were rated higher in intensity than outer wheel samples. The quantity of attributes that differed between inner and outer wheel samples was higher than at day 92 (Table 3.18).

Table 3.17: Significant ($p<0.05$) descriptive sensory attributes between treatments, Day 153

Sensory Attribute	Control	Reduced	KCl	p value
Bitterness	3.4[b]	3.7[ab]	3.9[a]	0.03
Oxidized flavor	2.0[b]	2.1[b]	2.5[a]	0.01
Bitter Aftertaste	2.6[b]	2.9[ab]	3.0[a]	0.03

Table 3.18: Significant (p<0.05) descriptive sensory attributes between locations, Day 153

Sensory Attribute	Inner	Outer	p value
Waxy aroma	1.6[a]	1.3[b]	0.013
Soapy aroma	1.9[a]	1.4[b]	0.002
Methyl Ketone aroma	4.8[a]	4.4[b]	0.006
Moldy aroma	3.9[a]	3.2[b]	0.000
Creamy aroma	1.4[b]	1.7[a]	0.048
Bitterness	3.9[a]	3.5[b]	0.024
Umami	2.0[a]	1.5[b]	0.000
Overall flavor	8.7[a]	8.2[b]	0.000
Rancid flavor	4.9[a]	4.6[b]	0.030
Soapy flavor	2.9[a]	2.5[b]	0.023
Methyl Ketone flavor	5.7[a]	5.3[b]	0.001
Moldy flavor	5.1[a]	4.4[b]	0.000
Grassy flavor	0.6[a]	0.4[b]	0.017
Burn	2.5[a]	2.2[b]	0.038
Overall aftertaste	6.1[a]	5.7[b]	0.000
Sour aftertaste	2.5[a]	2.2[b]	0.010
Methyl ketone aftertaste	3.8[a]	3.5[b]	0.001
Moldy aftertaste	3.5[a]	3.0[b]	0.002

Consumer Study

A consumer panel (n=95) evaluated all cheese treatments from both makes and an industry standard for attribute acceptance (Table 3.19). Control and reduced treatments from make 1 and KCl treatments from both makes had the same overall liking, appearance liking, texture liking, and pungent/sharp intensity. The industry standard had similar liking scores for appearance and firmness as compared to all samples with the exception of reduced treatment from make 1.

Table 3.19: Consumer scores for attributes of all cheese treatments and an industry standard blue cheese in an n=92 consumer study

Attribute	Control 1	Control 2	KCl 1	KCl 2	Reduced 1	Reduced 2	Industry Standard	p-value
Overall liking*	68^{c}	81^{a}	78^{ab}	79^{a}	72^{bc}	77^{ab}	73^{bc}	<0.001
Appearance liking*	76^{ab}	80^{a}	82^{a}	81^{a}	74^{b}	80^{a}	80^{a}	0.005
Flavor liking *	68^{c}	81^{a}	77^{ab}	80^{a}	70^{bc}	76^{ab}	71^{cb}	<0.001
Texture liking*	71^{cd}	82^{a}	80^{ab}	80^{ab}	69^{d}	78^{ab}	75^{bc}	<0.001
Flavor Intensity**	13^{ab}	13^{ab}	14^{a}	14^{a}	13^{ab}	12^{b}	13^{ab}	0.005
Pungent/ Sharp**	12^{a}	12^{a}	13^{a}	13^{a}	13^{a}	12^{a}	13^{a}	0.045
Stickiness**	11^{ab}	10^{bc}	10^{abc}	10^{bc}	11^{a}	9^{c}	10^{abc}	<0.001
Firmness**	7^{b}	9^{a}	8^{a}	9^{a}	7^{b}	9^{a}	8^{a}	<0.001

1 = Cheese treatment from Make 1 (aged 203 days)
2 = Cheese treatment from Make 2 (aged 183 days)
* = Liking score on a 120 point scale
** = Intensity score on a 20 point scale

3.4 Discussion

The goal of this research was to evaluate the effects of 25% sodium reduction in blue cheese. Treatments of reduced sodium blue cheese and reduced sodium blue cheese with molar KCl replacement were created with an overall final sodium reduction of 21% and 23%, respectively.

The total sodium content of the control cheese treatment was approximately 1100mg Na/100g cheese, which is lower than the USDA database average (1395 mg Na/100g cheese). The reduced treatment and reduced treatment with KCl averaged total sodium content of 850mg Na/100g cheese and 830mg Na/100g cheese, respectively. The diffusion of NaCl through the cheese was different between salting treatments. In sampling from inner and outer locations, the sodium content of the inner cheese samples for all treatment groups was approximately 100mg Na/100g cheese *less* than outer samples until day 31 for control treatment cheese and until day 61 for reduced and reduced with KCl treatments. Thus, equilibration of sodium concentration was more rapid for the control treatment as compared to reduced or reduced with KCl samples (Figure 4.4).

The diffusion of Na^+ and Cl^- ions is driven by the difference in osmotic pressure between the surface and body of the cheese and is thought to be inhibited by fat globules, protein aggregates, cheese moisture viscosity, temperature, and cheese geometry (Guinee, 2004; Cantor, 2004). The diffusion of salt ions, then, would differ between varieties of cheese with different compositions. Gobbetti et al., (1997) observed a salt gradient from

exterior to interior portions of Gorgonzola cheese (S/M = 6.7%) that equilibrated upon 63 days of aging, longer than the time required for salt to equilibrate in the current study. At the same time point (63 days of aging) the measured water soluble N was >50% total N at the core, which is far higher than the current study and might indicate a correlation between extent of proteolysis and the time required for salt to diffuse through a cheese body (Gobbetti et al., 1997). In the current study, proteolysis was measured to be similar between treatments, though reduced samples trended higher. Inner samples had a higher degree of proteolysis than outer samples at many initial time points. Reduced samples took approximately 30 days longer than the control samples to reach sodium equilibrium between inner and outer wheel samples which may validate the theory that proteolysis impedes diffusion. However, the initial sodium gradient between surface and core was smaller for the reduced cheese than for the control, which may have had a larger impact on the rate of diffusion.

In previous studies, moisture content has been positively correlated with larger sodium diffusion constants (D) (Morris et al., 1985; Guinee, 2004). At the same moisture level, D also increases with increasing fat content and subsequent decreasing protein (Guinee, 2004). The present study found a higher moisture content in the reduced treatment cheese, where diffusion of salt was slower in comparison to the full sodium control, suggesting the inhibitory effects of protein structure on salt diffusion is greater than the effect of moisture to increase the rate of diffusion.

Cantor et al., (2004) reported the surface to core gradients in 5 week old Danablu blue cheese to be pH 5.0 to 6.4, % salt 4.5 to 2.0, and a_w 0.92 to 0.94. The reduced with

KCl treatment had slower sodium ion diffusion than control cheese despite similar moisture, a_w, pH, fat, and protein levels. The inhibited diffusion of NaCl in the reduced with KCl treatment was thought to be predominantly caused by the reduction in osmotic pressure gradient between the surface and the cheese wheel center.

Potassium concentration, however, was not varied from outer wheel to inner wheel samples, indicating a complete equilibration of KCl by day 16 for all treatments (Figure 4.5). In comparing control and KCl samples, this relationship is important to the function of replacement salts. Bona et al., (2007) modeled the diffusion of NaCl and KCl in prato cheese salted in brine using a fixed effects method, modified to fit Fick's second law for simultaneous diffusion. They found that the diffusion coefficient (D) of KCl, in two mathematical model comparisons of diffusion in Cheddar cheese was higher than that of NaCl. Floury et al. (2010) concluded that while the fixed effects model of diffusion may be sufficient in compositionally similar cheese, more research is needed to account for the migration of small molecular weight compounds which might interfere with solute migration (such as FAA and small peptides). This is particularly relevant to blue cheese, where proteolysis and lipolysis are often more pronounced.

Water activity can directly affect microbial activity, safety, enzymatic activity, and the overall quality cheese (Fox et al., 2004). In the current study, molar replacement of NaCl by KCl was essential for maintaining similar water activity in the cheese. This replacement was based on Raoult's Law. Without replacement of this solute content, the water activity was expected to be higher. The correlation between direct reduction of

sodium in cheese and increasing a_w is well established (Schroeder et al., 1988; Thakur et. al. 1975; Hickey et al., 2013; Rulikowska et al., 2013).

Our study demonstrated this relationship very well, as reduced treatment cheese samples had higher measured a_w than control or KCl treatments for all time points (except day 31 in which R treatment group was numerically higher, though not significantly) (Figure 3.1).

In addition, the a_w of all samples decreased over time (avg. 0.958 to 0.939 16 to 153 days), correlating with a decreasing % moisture over time (avg. 48.8%±0.3 to 46.6%±0.6, 16 to 153 days) and increasing proteolysis over time. Hickey et al., (2013) measured the compositional attributes of Cheddar cheese over time and found the predominant factor in model prediction of a_w (based on their data) was proteolysis. That is, as the cheese aged the water activity decreased as proteolysis increased.

The present study also confirms that the use of replacement salt (KCl) can maintain similar a_w to a full sodium control over the full course of aging. Previous research of reduced sodium Cheddar using replacement salts corroborates with these findings (Katsiari et al. 1997; Grummer et al., 2012).

Total moisture (%) decreased over aging for all samples (avg. 48.8%±0.3 to 46.6%±0.6, 16 to 153 days), as was expected and is commonly observed in aging cheese. There was no difference in moisture content of inner and outer cheese wheel samples. It was expected that with higher NaCl concentrations, the surface or outer portion of the cheese wheel would have lower moisture than the interior at the early stages of ripening, equilibrating over time. Malacarne et al. (2009) observed higher % moisture at inner

wheel locations compared outer wheel locations, which equilibrated at 24wks of aging in Parmigiano-Reggiano cheese. It has been observed that moisture content in cheese will migrate towards area of high solute, to equilibrate differences in osmotic pressure (Guinee, 2004). It follows, then, that at the early stages of ripening when salt was most concentrated towards the surface of the wheel, moisture was drawn towards this area of high solute and thus was not significantly lower than inner wheel locations.

Reduced treatments were higher in total % moisture than control or KCl treatments at several points in aging. At days 31 and 61, reduced treatment cheese averaged 49% moisture compared to control and reduced with KCl, which averaged 48% moisture. With less NaCl added to the cheese, less moisture is driven off during production and aging, resulting in a cheese with higher moisture content (Morris et al., 1984). Rulikowska et al., (2013) observed a difference in moisture between two Cheddar cheeses of 42% and 38% in cheeses containing 0.5% and 3.0% NaCl, respectively. The difference in moisture between reduced treatments and control treatments for the current study is thought to be a driving force behind other differences such as proteolysis and lipolysis in the cheese.

For the present study, there were no differences in pH between salting treatments and no trend (increasing or decreasing) over aging for any treatment. Reddy & Marth (1995) found no difference in the pH of Cheddar cheese made with 1:1, 1:2, or 3:4 mixtures (wt/wt) of NaCl and KCl. Fitzgerald & Buckley (1985) also found no difference in pH between Cheddar cheese made with 1:1 wt/wt replacement KCl. However, it was expected that the reduced sodium blue cheese would have a higher pH than the control cheese or reduced with KCl treatment as a result of enhanced proteolysis driven by

reduced S/M, higher a_w, and higher moisture. Proteolysis creates free amino groups and peptides, which could increase the pH. Reduced samples had higher trending proteolysis than control or KCl, but were not significantly higher. In addition, proteolysis was observed to increase over time for all treatments.

The inner wheel samples (where sodium concentration and S/M was initially lower) had higher pH than did the outer samples for the same treatment over time. In contrast to these findings, Rulikowska et al. (2013) observed Cheddar cheese with 0.5% NaCl had a lower pH than did Cheddar cheese with 3.0% NaCl. However, proteolysis is known to be more extensive in blue cheese and could influence the pH to a greater degree than in Cheddar (Cantor et al., 2004). Rabie et al. (1987) observed that blue cheese with lower % salt had higher pH values, corroborating what we observed and confirming the relationship between extensive proteolysis and increased pH within the center of the wheel. Thus, the higher overall pH in the inner wheel samples is thought to be caused by proteolysis.

It is unclear why there was not a trend of increasing pH for all treatments as proteolysis increased over time for all treatments. It is possible that for both inner and outer treatments, as amino acids and small peptides were produced, they were rapidly metabolized to methyl ketones and thus did not continue to influence the pH over time as they were not accumulating.

The higher % moisture and reduced S/M of the reduced sodium treatment was thought to have an effect on the activity of the starter culture. While starter cultures were not enumerated, their activity could be represented by the pH of the cheese. With more

active Lactococcus species would come a greater extent of lactose fermentation, creating more lactic acid, resulting a lower pH. Reddy & Marth, (1995) found that sodium reduction and reduction with replacement by KCl had no effect on the predominant LAB species in ripening Cheddar cheese. Previous research on reduced sodium Feta cheese (1-4% NaCl) concluded that lower NaCl concentrations resulted in a significant increase in total bacteria, yeast and mold, and coliform counts (Aly, 1994). Their study involved cheeses of much higher moisture content than of the present study. In future work, if a greater degree of sodium reduction is considered for blue cheese, enumeration of total bacteria and yeast and mold may be necessary to maintain consumer safety. In the present study, it is theorized that an increase in lactic acid bacteria (and thus, lactic acid) would lower the pH, while stimulated sporulation and growth of *P. roqueforti* would to a greater extend of proteolysis (raising the pH), thus, the overall net change in pH from a full sodium control is minimal, as measured.

The main cultures in the Flora Danica bulk starter used for this study were lactic acid producers *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*, and flavor bacteria *Lactococcus lactis* ssp. *lactis* biovar *diactetylactis*, and *Leuconostoc mesenteroides* ssp. *cremoris* (Chr. Hansen, Inc., Milwaukee, WI). The *Penicillium roqueforti* used for this study was a single strain, BCM1 (Danisco, Copenhagen, Denmark). The activity of some starter cultures may be pertinent to the development of blue cheese flavor as they can enhance or inhibit the growth and sporulation of *P. roqueforti* in addition to providing some lipolytic activity themselves (Fox et al., 2004).

Hansen and Jakobsen (1997) investigated the interactions between 15 strains of

LAB and 20 strains of *P. roqueforti.* It was determined that sporulation and growth rates of *P. roqueforti* were stimulated by interactions with *Leuconostoc mesenteroides* ssp. *cremoris* as compared to independent growth or growth with *Streptococcus thermophilus*. The ratio of stimulated sporulation and growth rate of interacting cultures as compared to independent cultures was intensified as NaCl concentration increased, but total sporulation decreased. These data suggest that for a reduced sodium blue cheese, where NaCl concentration does not inhibit *L. mesenteroides cremoris* as much as a full sodium control, the growth of *P. roqueforti* maybe enhanced. A result of starter culture enhancement of *P. roqueforti* sporulation is flavor production. It is theorized that *P. roqueforti* spores contribute to ketone formation as much if not more than the mycelium (Kinsella & Hwang, 1976;Girolani and Knight, 1958). It follows, then, that if the reduced treatment cheese had more spore formation, there would be a larger amount of ketone formation in these treatments. The formation of ketones and other flavor volatiles is discussed in the following sections.

Proteolysis during cheese ripening is the hydrolysis of peptide bonds within the cheese protein matrix. This hydrolysis leads to the release of peptides, free amino groups, and free carboxylic acid groups which can raise the water activity, lower the pH, and affect the texture of the cheese over time (Upadhyay et al., 2004).

In this study, proteolytic activity was measured by determining water-soluble peptide fraction (%WSP) and equivalent concentration of free amino acids (FAA). Both %WSP and FAA increased from day 16 to day 31, and were unchanging for the remainder of aging, though reduced treatments values trended higher for both %WSP and

FAA (Figures 4.9 and 4.10, respectively). The lower a_w and lower S/M of the reduced treatment cheeses were expected to result in a significant increase in proteolysis as compared to the control. Previous studies have reported the inhibitory effects of NaCl on the extracellular lipases of *Penicillium roqueforti* (Kinsella & Hwang, 1976). While the reduced treatments (especially at inner wheel samples) showed occasionally higher signs proteolytic activity, there was no statistical significance determined.

In general, as cheese ripens, casein proteins are hydrolyzed into peptides and free amino acids by proteolytic enzymes from starter bacteria, NSLAB, residual coagulant, plasmin, and in the case of blue cheese, proteolytic enzymes secreted by *Penicillium roqueforti* (Cantor & Fox, 2004; Fernandez-Salguero, 2004). Proteolysis has been observed in previous studies as correlating with decreasing water activity (McSweeney, 2004;Hickey et al., 2013). Because the a_w of all treatments in the present study continued to decreased over time, it was expected that the concentration of FAA and %WSP would increase during aging, corroborating with previous studies. However, while a_w decreased over time, the %WSP and FAA equivalents increased initially and were unchanging after 31 days.

The degradation products of proteolysis include amino acids, aldehydes, alcohols, acids, and sulfur compounds. These products are often quite volatile and make up the flavor profile of aged cheese. It is possible the measured quantities of %WSP and FAA equivalent did not change after day 31 because the constituents were continuing to degrade, resulting in no overall change in %WSP and FAA. Continuous measurement of specific peptides with capillary electrophoresis or SDS-PAGE could help in confirming

this theory.

There were no significant differences in the relative intensities of alcohols, acids, and aldehydes between treatments as measured at 92 and 153 days. However, these compounds were present in the cheese (Table 3.13 & 3.14) and may have been present in higher/lower amounts between samples at earlier stages of aging, or between 92 and 153 days. Dimethyl sulfides were increasing in reduced sodium treatment over time, but decreased or stayed the same for control and KCl treatment. The presence of dimethyl sulfide is typically associated with aged cheeses because the sulfur compounds involved are produced, in part, by the breakdown of sulfur containing amino acids, cysteine and methionine (Cadwallader et al., 2007). One of the contributing compounds to dimethyl sulfide in cheese is methanethiol, produced from methionine. Methianethiol is produced by either coryneform bacteria, or proteolytic enzymes (Adda, et al., 1982). However, coryneform bacteria are typically associated with surface ripened cheeses or smear-cheeses and were likely not present in this study (Fox et al., 2004). Therefore, in the present study, the formation and presence of higher levels of dimethyl sulfide in reduced sodium cheese is likely due to proteolysis and further supports the theory that these treatments underwent a more extensive degree of proteolysis than the other treatments, despite %WSP and FAA equivalent measurements being similar. Dimethyl sulfide can impart a sulfurous, pungent, ripe-cheese aroma and flavor and has a very low detection threshold in water of 60ppb (Cadwallader et al., 2007; Molimard & Spinnler, 1996). Because of its pungency and low threshold, the slightly higher levels of dimethyl sulfide in the reduced treatments as compared to the control or reduced with KCl may have

contributed to the higher intensity rating for overall aroma and flavor associated with reduced samples rated by the descriptive sensory panel.

In the present study, the decreasing a_w over time as well as the higher a_w associated with reduced sodium blue cheese provides strong evidence of proteolytic activity, given the conclusions of previous research. While total %WSP and FAA equivalent did not continue to increase, the increase in their derivatives indicates the continued extent of proteolysis. Further, the larger the concentrations of some secondary metabolites of proteolysis (dimethyl sulfides) in reduced samples support the theory that proteolysis may have been more extreme in this treatment. In continued research, FAA identification in conjunction with flavor volatile detection would be helpful in determining the composition of proteolytic end products and whether sodium reduction and KCl treatments varied in the pathway of proteolysis. This is especially important in differentiation between the flavor compounds produced as the result of proteolysis and lipolysis, as both mechanisms can yield compounds with similar flavors and aromas.

Short to medium chain fatty acids were detected for this study. These are: butyric, isobutyric, isovaleric, caproic, capric, lauric, and myristic acids. Free fatty acids are produced via hydrolysis of the triglycerides of the milk fat. This lipolysis has been observed to occur to a lesser degree in conditions of high NaCl (Cantor et. al, 2004). However, A study on environmental conditions affecting *P. roqueforti* lipase activity in agar showed an increase in FFA production on 2%NaCl concentration media as compared to 0%NaCl (Larsen & Jensen, 1999). Therefore, it is thought that a given strain of *P.roqueforti* has an optimum salt concentration for FFA production, which may or

may not be the same as that used for cheese production.

In the present study, salting treatments had minimal effects on FFA production. Medium-chain FFA were observed in larger quantities in inner wheel samples as compared to outer wheel samples, suggesting a greater inhibition of lipase activity at the exterior of the cheese wheel where the concentration of NaCl was initially greater. Lauric and myrisitic acids were observed in larger quantities in control treatments as compared to reduced or reduced with KCl. Lindsay et al., (1982) observed higher quantities of lauric and myristic acid in Cheddar cheese with 1:2 NaCl:KCl salting treatments. It is possible that the lipases of the *P. roqueforti* used in this study were somewhat more selective for the hydrolysis of C_{12} and C_{14} fatty acids in comparison to the lipolytic enzymes of the Cheddar in the aforementioned study.

Penicillium roqueforti produces both an acidic and alkaline lipase. The pH optimum of these lipases are between 3.7-6.0 and 8.0-9.0, respectively (Lamberet & Menassa, 1983). The pH of the cheeses in this study were entirely within the range of 4.89-5.68, well within the optimal range for the acidic lipases of *P. roqueforti.* Thus, it is assumed that the pH was non-inhibitory and differences in lipolysis were more influenced by salt content than pH.

The lipases excreted by *Penicillium roqueforti* also act to oxidize free fatty acids to methyl ketones, which contribute to the aroma and flavor of blue cheese (Girolami & Knight, 1955). Methyl ketones most commonly associated with blue cheese aroma and flavor are (largely) 2-heptanone and to some degree, 2-pentanone, 2-hexanone, and 2-nonanone (Cadwalladar et al., 2007; Karahadian et al., 1985). In the present study, 2-

pentanone and 2-heptanone were found to have relative intensities significantly affected by cheese wheel location, time, and salting treatment (to some degree). These ketones are specifically produced from the oxidation of a FFA with one carbonyl carbon more than the ketone itself. For example, 2-heptanone is produced by the oxidation of caprylic (octanoic) acid and 2-pentanone is produced by the oxidation of caproic (hexanoic) acid (Gehrig & Knight, 1958). Thus, with an increase in 2-heptanone and 2-pentanone in the inner wheel samples compared to the outer wheel samples, we would expect to see a correlating larger concentration of caprylic and caproic acids in the same location, as these are the precursors to methyl ketone formation. The results of this study showed no variation in caproic acid for location or salting treatment, and we were unable to quantify caprylic acid. The lack of information on caprylic acid may be due to extraction procedure or column type, and could be a point of focus for further research. Caproic acid, on the other hand, may have been in higher concentration at earlier time points and had already been oxidized to 2-pentanone as some studies have shown the rapid oxidation of FFA in model systems.

Konstantinos et al., 2009 observed the blue mold portions of Stilton cheese to have more methyl ketone production in comparison to the white cheese portion, which contained more alcohols and aldehydes. While not measured quantitatively, the reduced sodium treatments in this study were observed by the researchers to have a larger amount of blue green mold throughout the cheese wheel in comparison to the control and reduced with KCl treatments. In addition, the interior samples of all treatments appeared to have more mold growth and softer texture as compared to the outer wheel. These observations

fit well with correlating data on methyl ketones in inner wheel samples and sensory results of more intense flavors in inner wheel samples and reduced sodium samples.

The concentration of isobutyric acid increased over time in the inner wheel portions of the control treatment cheese. The reduced and reduced with KCl treatment cheeses were not observed to have an increasing concentration of isobutyric acid. It is understood that bacteria synthesize branched short chain fatty acids (iso compounds) and straight chain fatty acid compounds based on enzymatic specificity of acyl-CoA (Kaneda, 1991). These compounds affect cell membrane fluidity and their synthesis, while important to cheese flavor, is strain specific (Dherbecourt et al., 2008). Because a difference in synthesis was only observed in the full sodium control, it is possible there exists a relationship between NaCl concentration and the activity or specificity of the cellular enzymes within *P. roqueforti.* Isovaleric acid was not observed to have an increasing concentration in any treatment. The isomeric compounds detected in this study were observed at very low concentrations and more precise detection is required for further analysis.

Descriptive panels evaluating the cheeses in this study described much higher intensities of attributes such as methyl ketone aroma, overall aroma, rancid aroma, musty flavor, and rancid flavor for inner wheel samples compared to outer wheel samples at both day 92 and day 153, for all treatments. In addition, reduced sodium treatments and KCl treatments had higher intensity scores for creamy aroma, and barny aroma (Table 4.9-4.12). The relative intensity of saltiness was never significantly different between any treatment or location within a treatment. Lindsay et al., (1982) reported significantly

reduced saltiness scores for Cheddar cheese made with 1:1 (NaCl:KCl). In contrast, Grummer et al., (2011) observed similar saltiness intensities for 50% reduced sodium Cheddar cheese using KCl replacement which targeted the same a_w. The difference between the two studies described is the total level of sodium. Grummer et al., (2011) had higher levels of sodium in their control and reduced Cheddars as compared to Lindsay et al., (1982). The sodium levels in this study (even for reduced and reduced with KCl) are higher, still, than those reported by Grummer et al., 2011. This leads to the conclusion that saltiness is only affected below a certain threshold of sodium reduction, which was not reached in the present study.

Another taste associated with KCl replacement is bitterness (Lindsay et al., 1982; Fox et al., 2004). The bitterness and bitter aftertaste intensities of reduced and reduced with KCl samples were higher for day 153 as compared to the control. Bitterness was not different between samples at day 92. Grummer et al., (2011) observed no difference in bitterness between reduced with KCl samples and control Cheddar cheese. Lawlor et al., (2003) did not observe differences in bitterness between 6 varieties of blue cheese ranging in sodium content from 1.8-4.2%, however, these cheeses did not contain any additional sodium. Because the bitterness observed in the present study was higher for both reduced and reduced with KCl treatment cheese, it is not certain that the bitterness is caused by potassium chloride alone. Sensory observations associated with bitterness were more intense at the later stages of aging, and may be more correlated to age than to salting treatment. The peak age of sodium reduced blue cheese may be earlier than that of full sodium blue cheese, if the goal is to avoid bitterness entirely. However, a large

(n=92) consumer study did not show differences in overall liking between any treatments (discussed below).

Compounds which contribute to blue cheese aroma and flavor include FFA, FAA, methyl ketones, acids, alcohols, esters, sulfur compounds, and some peptides (Rabie et al., 1988; Molimard & Spinnler, 1996). In the present study, between salting treatments, significant differences in the quantity or relative intensity of dimethyl sulfide (having a musty, mushroom flavor), 2-heptanone and 2-pentanone (characteristic blue cheese or fermented flavor), and a series of esters (fruity flavors) were measured and expected to alter the sensory evaluation of the cheeses (Cadwallader et al., 2007; Wolf et al., 2009). T

At 92 days of aging, attributes that could have been associated with these compounds were overall intensity of aroma (higher in reduced with KCl treatment) and sweetness (higher in reduced and reduced with KCl treatment).

The more impressive correlation between measured volatile components and descriptive sensory analysis is the long list of attributes which were observed as more intense in the inner wheel samples as compared to outer wheel samples for both day 92 and day 153 (Table 4.10 and 4.12, respectively). Konstantinos et al., (2009) observed higher amounts of ketones in the moldy, interior portions of Stilton cheese and higher amounts of alcohols and aldehydes in the white portions of Stilton cheese. As previously mentioned, the inner wheel samples of the present study were quantitatively observed by the researcher to have a greater amount of mold growth. This agrees with previous research that the overwhelmingly important factor to blue cheese flavor and intensity of

flavor perception is the production and accumulation of methyl ketones (Kinsella & Hwang 1976; Cantor et al., 2004; Cadwallader et al., 2007).

A consumer panel of 95 people sampled all cheese treatments from both cheese makes alongside a high quality industry standard (St. Pete's Select Amablu Blue Cheese, aged 100 days, Faribault Dairy Inc.) and scored liking of overall product, appearance, flavor, and texture. In addition they ranked the flavor intensity, pungency, stickiness, and firmness.

The same overall liking was determined for control, reduced, and reduced with KCl from cheese making replicate 2 as well as reduced with KCl from replicate make 1. Gomes et al., (2011) observed similar overall liking for Minas fresh cheese using a 25% sodium reduction and replacement by KCl, but reducing liking for 50% and 75% reduction and replacement. Consumer scores were also similar for all treatments except reduced from make 1 for liking of appearance. Grummer et al., (2013) also observed similar consumer liking scores for Cheddar cheese made with molar replacement by KCl.

Despite descriptive sensory differences at day 92 and 153, all cheese samples had the same rating for pungent/sharp, indicating an equilibration of ripening effects with extended aging.

3.5 Conclusions

In the study presented, full sodium blue cheese containing 1100mg Na/ 100g cheese was compared to reduced sodium blue cheese containing 850mg Na/100g cheese and reduced sodium blue cheese with molar KCl replacement containing 830mg Na/100g cheese. Over the course of 5 months of aging, the compositional analysis was observed at both the inner and outer wheel locations. Volatile flavor components and free fatty acids were measured at 92 and 153 days in conjunction with descriptive sensory analysis.

Salt reduction of 21 and 23% was achieved for the reduced and reduced with KCl treatments, respectively. A gradient of high to low sodium concentration was observed from the outer to inner wheel samples for up to 61 days in the reduced and reduce with KCl treatment but only 31 days for the control. These mineral gradients between the surface and center of the cheese wheel correlated with gradients in a_w, pH, and at some points, moisture and proteolysis. This resulted in significant differences in free fatty acids, free amino acids, volatile flavor compounds, and descriptive analysis of the inner wheel samples as compared to the outer wheel samples, confirming the hypothesis that location within a blue cheese wheel will have a large effect on these attributes.

The overall effects of salting treatment were most pronounced in the water activity of the reduced sodium treatment, as well as % moisture and extent of proteolysis which were higher for the reduced sodium treatment at some time points. In general, the reduced with KCl treatment cheese was not compositionally different from the control except in some free fatty acid and methyl ketone formation. This is thought to be caused by the maintaining of water activity between the reduced with KCl and control cheese

treatments, a method of sodium reduction which has been further justified as a means to mimic the mechanisms of NaCl by the results of this study.

While a descriptive sensory panel reported more intense attributes in the reduced and reduced with KCl treatments at day 92, there were fewer differences at day 153. At day 183 and 203 of aging, a consumer panel rating similar overall, appearance, and texture liking of all treatments from make 2, and both reduced with KCl treatments (make 1 and 2).

Overall, reducing NaCl in blue cheese by 21% resulted in some compositional and sensory differences, which would be expected to be more pronounced if sodium reduction (without replacement salts) was increased. Further, a 23% reduction in sodium with molar replacement of KCl resulted in a similar cheese composition with some sensory differences that the consumer rated as liking equally for all replicate cheese makes.

In further research, the more interesting topics of study (as indicated by this research) include the diffusion coefficient of KCl as compared to NaCl in a matrix of cheese similar to blue, where proteolysis and lipolysis could lead to inhibiting agents of diffusion. This could be a limiting factor to the level of sodium reduction and replacement by KCl in blue cheese.

Far more dramatic effects of sodium reduction were expected, as the literature supports the idea that salt levels are so high in blue cheese because of the high salt tolerance of *Penicillium roqueforti* (Candor et al., 2004; Kinsella & Hwang, 1976). However, at the levels of sodium used in this study, a 21-23% reduction did not have

dramatically deteriorating effects and it is hypothesized that further reduction with replacement could produce a similarly acceptable product. To test the extremes of this hypothesis, a blue cheese made with up to 100% KCl and no NaCl could serve as an outlying comparison.

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Appendix

The following tables are expanded versions of those found in Chapter 4

Table A.1: Compositional analysis summary data

		Treatment					
		Control		Reduced		Reduced with KCl	
Measurement	**Age (Days)**	Inner	Outer	Inner	Outer	Inner	Outer
a_w	*16*	0.955	0.953	0.963	0.961	0.957	0.956
	31	0.944	0.946	0.949	0.954	0.952	0.950
	61	0.944	0.944	0.952	0.954	0.947	0.949
	92	0.943	0.945	0.952	0.954	0.943	0.945
	122	0.941	0.943	0.949	0.948	0.941	0.942
	153	0.932	0.938	0.943	0.945	0.937	0.938
S/M	*16*	5.26	5.98	4.70	5.08	5.81	6.55
	31	6.18	6.19	4.91	5.20	6.08	6.93
	61	6.59	6.66	5.28	5.30	6.59	6.40
	92	6.72	6.54	5.54	5.60	6.98	6.81
	122	6.52	6.26	5.35	5.31	6.60	6.46
	153	6.36	6.49	5.13	5.31	6.75	6.54
Moisture, %	*16*	48.2	48.9	48.8	49.1	48.9	48.8
	31	48.5	48.7	48.6	49.3	48.2	48.5
	61	47.4	48.0	48.6	49.4	46.9	47.7
	92	47.1	46.8	48.1	47.8	47.0	47.6
	122	47.9	48.0	47.8	47.6	47.6	47.4
	153	46.0	46.0	47.5	47.0	46.4	46.9
pH	*16*	5.58	5.15	5.30	5.02	5.45	4.98
	31	5.22	4.97	5.24	5.01	5.25	4.97
	61	5.25	4.99	5.50	5.13	5.52	5.17
	92	5.09	4.89	5.07	4.99	5.18	5.05
	122	5.51	5.23	5.06	4.94	5.37	5.09
	153	5.11	4.97	5.16	4.94	5.06	5.04
FDM,%	*16*	49.6	50.3	50.1	51.1	50.8	50.4
	31	50.6	50.6	51.6	51.5	50.4	51.2
	61	50.7	49.9	51.1	50.4	49.9	49.6
	92	51.7	49.8	52.2	50.4	51.8	50.8
	122	51.3	49.8	52.6	50.5	51.4	50.1
	153	50.9	49.9	53.5	50.6	50.9	50.8

Sodium, mg/100g	*16*	1027	1165	914	988	871	1010
	31	1180	1186	936	1007	875	995
	61	1229	1256	1004	1028	927	918
	92	1200	1169	1013	1016	949	951
	122	1229	1181	1005	994	945	926
	153	1150	1172	986	981	952	940
Potassium, mg/100g	*16*	217	206	201	201	646	644
	31	222	205	211	209	670	731
	61	211	203	201	205	687	679
	92	197	187	198	194	695	700
	122	236	223	237	228	692	673
	153	213	212	215	211	673	657
WSP, %	*16*	9.8	3.2	10.7	5.3	9.0	4.4
	31	16.6	10.7	18.4	17.0	19.4	13.4
	61	13.4	11.6	16.2	13.5	16.9	15.6
	92	13.6	11.5	16.0	14.3	13.7	13.0
	122	13.7	12.9	14.9	13.9	14.2	13.7
	153	16.2	15.1	15.5	14.5	15.2	13.1
AA equivalent, mg/g Cheese	*16*	224	79	205	105	175	129
	31	467	256	569	355	493	281
	92	443	346	500	374	554	398
	122	380	368	317	332	440	402
	153	456	442	440	345	427	274

Table A.2: Day 92 mean values and p-values of all sensory attributes for Control, Reduced, and Reduced with KCl treatments. Means followed by the same letter are not significantly different.

Sensory Attribute	Control	KCl	Reduced	p value
Overall Intensity of Aroma	7.7a	7.9a	7.7a	0.036
Rancid Aroma	4.8a	4.8a	4.8a	0.661
Waxy/Crayon Aroma	0.9a	1.1a	1.0a	0.454
Soapy Aroma	1.1a	1.2a	1.1a	0.491
Oxidized Aroma	1.9a	2.0a	2.2a	0.365
Cardboard Aroma	0.3a	0.4a	0.4a	0.656
Methyl Ketone Aroma	5.1a	4.8a	4.9a	0.354
Moldy Aroma	3.4a	3.7a	3.5a	0.221
Musty Aroma	3.1a	3.1a	3.0a	0.978
Creamy Aroma	1.1b	1.3ab	1.5a	0.008
Milky Aroma	0.8a	0.9a	1.1a	0.112
Grassy Aroma*	0.4a	0.8a	0.8a	0.201
Barny Aroma*	0.7b	1.4a	1.6a	0.01
Sweetness	0.5b	0.9a	0.9a	0.017
Sourness	1.9b	2.3a	2.2ab	0.037
Saltiness	2.9a	3.0a	2.6a	0.124
Bitterness	4.2a	4.5a	4.3a	0.338
Umami	3.3a	3.5a	3.4a	0.561
Overall Intensity of Flavor	6.3a	6.4a	6.3a	0.709
Rancid Flavor	3.5a	3.6a	3.4a	0.514
Waxy/Crayon Flavor	4.9a	5.0a	4.6b	0.006
Soapy Flavor	3.3a	3.4a	3.4a	0.683
Oxidized Flavor	2.2a	2.0a	2.0a	0.611
Cardboard Flavor	1.0a	1.0a	0.9a	0.445
Methyl Ketone Flavor	4.2a	4.0a	4.0a	0.426
Moldy Flavor	2.5a	2.6a	2.7a	0.469
Musty Flavor	4.9a	4.9a	4.5a	0.086
Creamy Flavor	2.6a	2.9a	2.7a	0.097
Milky Flavor	2.0a	2.3a	2.0a	0.122
Grassy Flavor*	0.5a	0.8a	0.7a	0.518
Barny Flavor*	0.8a	1.2a	1.1a	0.245
Burn	1.8a	1.8a	1.8a	0.812
Overall Intensity	3.7a	3.6a	3.6a	0.822

Table A.1 Continued

Bitter Aftertaste	2.2a	2.3a	2.5a	0.249
Salty Aftertaste	2.1a	2.2a	2.0a	0.54
Sweet Aftertaste	1.4a	1.7a	1.4a	0.154
Sour Aftertaste	3.8a	4.0a	4.0a	0.238
Rancid Aftertaste	3.2a	3.3a	3.1a	0.561
Waxy Crayon Aftertaste	2.2a	2.1a	2.0a	0.359
Soapy Aftertaste	0.9a	0.8a	0.8a	0.584
Oxidized Aftertaste	2.3a	2.3a	2.3a	0.957
Methyl Ketone Aftertaste	3.7a	3.5a	3.6a	0.745
Moldy Aftertaste	1.8a	2.1a	2.0a	0.407
Musty Aftertaste	1.6a	1.7a	1.6a	0.919
Grassy Aftertaste*	0.2a	0.4a	0.3a	0.534
Barny Aftertaste*	0.6a	1.0a	0.7a	0.079
Burn Aftertaste	1.1a	1.2a	1.2a	0.771

Table A.2: Day 92 mean values and p-values of all sensory attributes for Inner and Outer Locations. Means followed by the same letter are not significantly different.

	In	Out	p
Overall Intensity of Aroma	7.9a	7.6b	0.005
Rancid Aroma	5.1a	4.5b	0.001
Waxy/Crayon Aroma	1.1a	1.0a	0.454
Soapy Aroma	1.2a	1.1a	0.291
Oxidized Aroma	2.2a	1.9a	0.102
Cardboard Aroma	0.4a	0.3a	0.391
Methyl Ketone Aroma	5.1a	4.7b	0.005
Moldy Aroma	3.6a	3.5a	0.42
Musty Aroma	3.1a	3.1a	0.959
Creamy Aroma	1.2a	1.4a	0.226
Milky Aroma	0.8b	1.1a	0.013
Grassy Aroma*	0.6a	0.7a	0.467
Barny Aroma*	1.2a	1.3a	0.96
Sweetness	0.7a	0.8a	0.73
Sourness	2.1a	2.2a	0.389
Saltiness	2.9a	2.7a	0.237
Bitterness	4.3a	3.9a	0.885
Umami	1.9a	1.6a	0.091
Overall Intensity of Flavor	6.5a	6.1b	0.004
Rancid Flavor	3.6a	3.4b	0.022
Waxy/Crayon Flavor	5.0a	4.6b	0.002
Soapy Flavor	3.5a	3.2b	0.006
Oxidized Flavor	2.1a	2.0a	0.599
Cardboard Flavor	1.0a	1.0a	0.975
Methyl Ketone Flavor	4.2a	4.0a	0.191
Moldy Flavor	2.6a	2.6a	0.916
Musty Flavor	4.9a	4.6b	0.046
Creamy Flavor	2.7a	2.7a	0.739
Milky Flavor	2.0a	2.1a	0.272
Grassy Flavor*	0.5a	0.8a	0.11
Barny Flavor*	1.0a	1.0a	0.646
Burn	1.8a	1.8a	0.873
Overall Intensity	3.6a	3.6a	0.968

Table A.2 Continued

Bitter Aftertaste	2.5a	2.3a	0.155
Salty Aftertaste	2.1a	2.1a	0.56
Sweet Aftertaste	1.7a	1.4a	0.046
Sour Aftertaste	4.1a	3.8b	0.018
Rancid Aftertaste	3.4a	3.0b	0.001
Waxy/Crayon Aftertaste	2.1a	2.0a	0.468
Soapy Aftertaste	0.8a	0.8a	0.664
Oxidized Aftertaste	2.4a	2.2a	0.309
Methyl Ketone Aftertaste	3.7a	3.5b	0.042
Moldy Aftertaste	2.0a	1.9a	0.638
Musty Aftertaste	1.6a	1.6a	0.936
Grassy Aftertaste*	0.4a	0.3a	0.436
Barny Aftertaste*	0.8a	0.8a	0.97
Burn Aftertaste	1.2a	1.1a	0.319

Table A.3: Day 92 mean values and p-values of all sensory attributes for Control, Reduced, and Reduced with KCl treatments. Means followed by the same letter are not significantly different.

Attribute	Control	KCl	Reduced	p
Overall aroma	7.5	7.5	7.5	1
Rancid aroma	4	4	4.1	0.93
Waxy aroma	1.4	1.4	1.5	0.92
Soapy aroma	1.7	1.6	1.6	0.86
Oxidized aroma	1.4	1.7	1.7	0.2
Cardboard aroma	0.5	0.5	0.5	0.78
Methyl ketone aroma	4.6	4.5	4.7	0.59
Moldy aroma	3.5	3.4	3.7	0.47
Musty aroma	2.6	2.8	2.8	0.56
Creamy aroma	1.6	1.5	1.4	0.42
Milky aroma	0.8	0.9	0.8	0.52
Barny aroma	2	2.3	1.9	0.27
Grassy aroma	0.7	0.6	0.5	0.44
Sweetness	0.6	0.7	0.7	0.57
Sourness	3.8	4.2	4	0.12
Saltiness	4.7	4.6	4.5	0.41
Bitterness	3.4^{b}	3.9^{a}	3.7^{ab}	0.03
Umami	1.6	1.8	1.7	0.43
Overall flavor	8.4	8.6	8.4	0.2
Rancid flavor	4.6	4.7	4.9	0.19
Waxy flavor	2	2	2.2	0.51
Soapy flavor	2.6	2.9	2.7	0.5
Oxidized flavor	2.0^{b}	2.5^{a}	2.1^{b}	0.01
Cardboard flavor	0.6	0.4	0.4	0.13
Methyl ketone flavor	5.5	5.4	5.5	0.66
Moldy flavor	4.8	4.6	4.7	0.68

Table A.3 Continued

Musty flavor	3	3.2	3	0.51
Creamy flavor	2.2	2.1	2.3	0.65
Milky flavor	1.1	0.8	1.2	0.06
Barny flavor	2	2.4	2.2	0.15
Grassy flavor	0.5	0.6	0.5	0.66
Burn	2.3	2.5	2.3	0.59
Overall aftertaste	5.9	6	5.9	0.88
Bitter aftertaste	2.6[b]	3.0[a]	2.9[ab]	0.03
Salty aftertaste	2.9	3	2.8	0.25
Sweet aftertaste	0.5	0.5	0.5	0.39
Sour aftertaste	2.3	2.4	2.3	0.77
Rancid aftertaste	3.3	3.4	3.5	0.45
Waxy aftertaste	1.6	1.6	1.5	0.7
Soapy aftertaste	1.9	2	2	0.82
Oxidized aftertaste	1.5	1.7	1.4	0.25
Methyl ketone aftertaste	3.7	3.7	3.6	0.72
Moldy aftertaste	3.3	3.3	3.2	0.78
Musty aftertaste	2.3	2.4	2.2	0.76
Barny aftertaste	1.4	1.6	1.4	0.29
Grassy aftertaste	0.3	0.4	0.4	0.74
Burn aftertaste	1.2	1.4	1.3	0.71

Table A.4: Day 92 mean values and p-values of all sensory attributes for Inner and Outer Locations. Means followed by the same letter are not significantly different.

Attribute	Inner	Outer	p
Overall aroma	7.6	7.4	0.119
Rancid aroma	4.1	4	0.512
Waxy aroma	1.6[a]	1.3[b]	0.013
Soapy aroma	1.9[a]	1.4[b]	0.002
Oxidized aroma	1.7	1.5	0.103
Cardboard aroma	0.5	0.6	0.138
Methyl ketone aroma	4.8[a]	4.4[b]	0.006
Moldy aroma	3.9[a]	3.2[b]	0
Musty aroma	2.7	2.8	0.439
Creamy aroma	1.4[b]	1.7[a]	0.048
Milky aroma	0.7	0.9	0.11
Barny aroma	1.9	2.2	0.091
Grassy aroma	0.6	0.6	0.803
Sweetness	0.6	0.7	0.522
Sourness	4.1	3.9	0.488
Saltiness	4.7	4.5	0.083
Bitterness	3.9[a]	3.5[b]	0.024
Umami	2.0[a]	1.5[b]	0
Overall flavor	8.7[a]	8.2[b]	0
Rancid Flavor	4.9[a]	4.6[b]	0.03
Waxy flavor	2.2	1.9	0.092
Soapy flavor	2.9[a]	2.5[b]	0.023
Oxidized flavor	2.3	2.1	0.347
Cardboard flavor	0.5	0.4	0.744
Methyl ketone flavor	5.7[a]	5.3[b]	0.001
Moldy flavor	5.1[a]	4.4[b]	0
Musty flavor	3.1	3.1	0.922

Table A.4 continued

Creamy flavor	2.2	2.2	0.782
Milky flavor	1	1	0.998
Barny flavor	2.3	2.1	0.201
Grassy flavor	0.6[a]	0.4[b]	0.017
Burn	2.5[a]	2.2[b]	0.038
Overall aftertaste	6.1[a]	5.7[b]	0
Bitter aftertaste	2.9	2.7	0.077
Salty aftertaste	3	2.8	0.188
Sweet aftertaste	0.5	0.5	0.789
Sour aftertaste	2.5[a]	2.2[b]	0.01
Rancid aftertaste	3.5	3.3	0.118
Waxy aftertaste	1.6	1.5	0.353
Soapy aftertaste	2.1	1.9	0.114
Oxidized aftertaste	1.5	1.6	0.624
Methyl ketone aftertaste	3.8[a]	3.5[b]	0.001
Moldy aftertaste	3.5[a]	3.0[b]	0.002
Musty aftertaste	2.2	2.4	0.052
Barny aftertaste	1.5	1.5	0.675
Grassy aftertaste	0.4	0.3	0.311
Burn aftertaste	1.4	1.3	0.558

Product Information

Freeze-Dried Culture

Flora Danica

Description:

Chr. Hansen's Flora Danica is a freeze-dried culture which contains multiple and mixed strains of the acid producing bacteria *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* and flavor producing bacteria *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*.

Applications:

Flora Danica cultures can be used as a direct inoculant (DVS) or to inoculate a bulk starter. This culture will produce a full cultured flavor. Flora Danica is ideal for making the following products:

- Buttermilk/Sour Cream
- Cream Cheese
- Specialty Cheeses (Havarti, Baby Swiss, Goats Milk Cheese, Gouda, Edam, etc.)
- Cultured Butter

Characteristics:

- Flora Danica has an outstanding tradition for making quality cheese and cultured products.
- Flora Danica is freeze-dried which makes for easy shipment and storage.
- Flora Danica is resilient to a variety of plant conditions which allows for more consistent performance.

Packaging:

Flora Danica cultures are aseptically packaged in laminated white foil pouches (the actual contents of each pouch may vary), which are then packaged in a cardboard box. They are available as freeze-dried cultures in the following sizes:

50 unit DVS = 16.2 gm

16.2 g / 86016 milk = 8 / 46

Package	Inoculation (Bulk or Direct)
10 x 1,000 liter -- Redi-Set	300 gallons
10 x 50 unit -- DVS	100 gallons
25 x 200 unit -- DVS	500 gallons
20 x 500 unit -- DVS	700-1,000 gallons
* 130 ml can (KFP) -- Redi-Set	300 gallons

= 860 lbs.

*Kosher for Passover Flora Danica is produced as a frozen liquid and packaged in 130 ml easy-open aluminum cans.

CHR HANSEN

Chr. Hansen, Inc.
9015 West Maple Street
Milwaukee, WI 53214-4298
Telephone: 414-607-5700
Customer Service: 800-543-4422
Technical Service: 800-247-8321
Fax: 414-607-5704

Chr. Hansen Ltd.
1146 Aerowood Drive
Mississauga, Ontario L4W 1Y5
Telephone: 905-625-2560
Fax: 905-625-8157

Cultures

Figure A.1 Product Description of Flora Danica Culture

CONSENT FORM
Blue Cheese Study

You are invited to be in a research study of the quality of blue cheese. You were selected as a possible participant because of your interest, availability and lack of any food allergies to dairy. We ask that you read this form and ask any questions you may have before agreeing to be in the study.

This study is being conducted by: Amanda Peck and Zata Vickers, from the Sensory Center in the Department of Food Science and Nutrition.

Background Information:
The purpose of this study is to characterize the sensory qualities and compare blue cheese samples. All products will be commercially available foods, foods prepared using FDA approved ingredients and good manufacturing procedures, or food or pharmaceutical grade substances.

Procedures:
If you agree to be in this study, we would ask you to do one or more of the following things: Develop a vocabulary for describing the sensory attributes of blue cheese and rate the cheese for the intensity of these attributes.

Risks and Benefits of being in the Study:
The study has no risks beyond those of normally consuming nutritional supplement beverages.
The study has no benefits for you other than the compensation.

Compensation:
You will receive payment at the rate of $13.00 an hour for participating in these sessions. You will receive a minimum payment of $5.00 for any sessions that last less than ½ hour. Payments will be made to you on a monthly basis.

Confidentiality:
The records of this study will be kept private. In any sort of report we might publish, we will not include any information that will make it possible to identify a subject. Research records will be stored securely and only researchers will have access to the records.

Voluntary Nature of the Study:
Participation in this study is voluntary. Your decision whether or not to participate will not affect your current or future relations with the University of Minnesota. If you decide to participate, you are free to not answer any question or withdraw at any time with out affecting those relationships.

Contacts and Questions:
The researchers conducting this study are: Amanda Peck and Zata Vickers. You may ask any questions you have now. If you have questions later, **you are encouraged** to contact them at Room 97 or 140, Food Science and Nutrition, 612 625 3712, peckx137@umn.edu and zvickers@umn.edu.

If you have any questions or concerns regarding this study and would like to talk to someone other than the researcher(s), **you are encouraged** to contact the Research Subjects' Advocate Line, D528 Mayo, 420 Delaware St. Southeast, Minneapolis, Minnesota 55455; (612) 625-1650.

You will be given a copy of this information to keep for your records.

Statement of Consent:

I have read the above information. I have asked questions and have received answers. I consent to participate in the study.

Signature:_____ Date: _____

IRB Code #
Version Date: 1 of 1

Figure A.2: Consumer consent form for descriptive panel sessions

Statistical Code

R software input code for pH data. All measurements were run in a similar fashion, eliminating models with insignificant terms until the most efficient model was determined. Text following (#) symbols is not code, but is instruction or comments.

```
 ## Necessary libraries
library(nlme)
library(car)
library(multcomp)

## Load and check data
dat <- read.csv("pH.csv", nrow=276)
dat <- within(dat, {
  Make <- factor(Make)
  Wheel <- factor(Wheel)
  Time <- factor(Time)
})
head(dat)
tail(dat)
str(dat)

## Fit full model
nlmepH <-lme(pH ~ Cheese*Time*Location, random = ~1|Make/Wheel/Location, data=dat)
summary(nlmepH)
anova(nlmepH)
Anova(nlmepH) # Anova preferred

## Take out three way interaction because it wasn't significant
nlmepH2 <-lme(pH ~ (Cheese + Time + Location)^2, random = ~1|Make/Wheel/Location, data=dat)
#summary(nlmepH2)
anova(nlmepH2)
Anova(nlmepH2)

## remove Cheese:Location interaction (now just additive)
nlmepH3 <-lme(pH~Cheese*Time+Time*Location, random = ~1|Make/Wheel/Location, data=dat)
#summary(nlmepH3)
Anova(nlmepH3)

## remove Cheese:Time
nlmepH4 <-lme(pH~Cheese+Time*Location, random = ~1|Make/Wheel/Location, data=dat)
summary(nlmepH4)
Anova(nlmepH4)

## fit with ML and compare AIC and LRT test
x1 <- update(nlmepH, method="ML")
x2 <- update(nlmepH2, method="ML")
x3 <- update(nlmepH3, method="ML")
x4 <- update(nlmepH4, method="ML")
AIC(x1, x2, x3, x4)
anova(x1, x4)
```

```
## show that the time location interaction was significant, so time and location are each important to the
  model.
## Cheese is not significant. no signif difference in ph by cheese type
##  you can say that we fit the full model and then eliminated the least significant term each time.
## we left Cheese in here just to report in order to show there is no significant difference from one cheese
  type to another.

## do for cheese
g1 <- glht(nlmepH4,linfct=mcp(Cheese="Tukey"))
summary(g1)
confint(g1)

## fit with Time:Location as a single effect to get comparisons for Time/Location
dat$LocationTime <- with(dat, factor(paste(Location, Time)))
nlmepH4b <-lme(pH~Cheese+LocationTime, random = ~1|Make/Wheel/Location, data=dat)
## check that fit is the same
AIC(nlmepH4, nlmepH4b)
anova(nlmepH4, nlmepH4b)
## do TukeyHSD
g2 <- glht(nlmepH4b,linfct=mcp(LocationTime="Tukey"))
#summary(g2)

## that's a lot of comparisons (66), are we sure we want that many?
nrow(g2$linfct)


## perhaps there's a better way but I don't know it...
tmp <- do.call(rbind, strsplit(rownames(g2$linfct), " - "))
tmp <- data.frame(x1=substring(tmp[,1],1, nchar(tmp[,1])-1),
                  n1=substring(tmp[,1],nchar(tmp[,1])),
                  x2=substring(tmp[,2],1, nchar(tmp[,2])-1),
                  n2=substring(tmp[,2],nchar(tmp[,2])) , stringsAsFactors=FALSE)
#do this each time (not specifically for pH)

#this finds the rows that have the same location or the same time
k <- with(tmp, c(which(x1==x2), which(n1==n2)))
#this does only the comparisons for the rows specified in the previous line
g2a <- glht(nlmepH4b, linfct=g2$linfct[k,])
#this is the number of comparisons we're doing
nrow(g2a$linfct)

#here are the results of the comparison of the pH means.
#note that we are comparing the inner PH at differet times, the outer
#pH at different times, and then comparing inner v outer at the
#same times
summary(g2a)

#here are the predicted means for each of the combinations of the fixed effects (for each time, location, and
  cheese type)
newdat<-expand.grid(Time=levels(dat$Time),Cheese=levels(dat$Cheese),Location=levels(dat$Location))
newdat$pred<-predict(nlmepH4,newdat,level=0)
newdat
```

```
nlmepH5 <-lme(pH~Time*Location, random = ~1|Make/Wheel/Location, data=dat)
newdat2<-expand.grid(Time=levels(dat$Time),Location=levels(dat$Location))
newdat2$pred<-predict(nlmepH5,newdat2,level=0)
newdat2
order(newdat2$pred[newdat2$Location=="Inner"])
plot(newdat2$pred,type="h")

newdat<-expand.grid(Time=levels(dat$Time),Cheese=levels(dat$Cheese),Location=levels(dat$Location))
newdat$pred<-predict(nlmepH4,newdat,level=0)
newdat

nlmepH5 <-lme(pH~Time*Location, random = ~1|Make/Wheel/Location, data=dat)
newdat2<-expand.grid(Time=levels(dat$Time),Location=levels(dat$Location))
newdat2$pred<-predict(nlmepH5,newdat2,level=0)
newdat2

plot(newdat2$pred)
plot(newdat2$pred,type="h")
newdat2
order(newdat2$pred[newdat2$Location=="Inner"])
# 6 4 2 5 3 1
```