ABSTRACT

CAMPBELL, RACHEL E. Impact of Annatto Colorant, Different Starter Cultures, Starter Media and Starter Strains on the Flavor and Oxidative Stability of Liquid Whey and WPC. (Under the direction of Dr. MaryAnne Drake.)

Whey and dried whey ingredients are expected to have a delicate and bland flavor that is undetectable in finished goods. Factors that influence the flavor of fluid whey and whey protein include cheese type, storage conditions of fluid and dried products, variability among processing plants, and bleaching. Two separate studies were designed to provide more insight on the factors that influence the flavor of whey protein. The first study evaluated the impact of annatto color and starter culture on flavor and functionality of spray dried whey protein concentrate (WPC). Neither culture nor annatto had an effect on the functional properties of WPC (p>0.05). Sensory profiles of WPC and fresh liquid whey from products with and without annatto or starter culture were not different (p>0.05). Volatile lipid oxidation compounds were higher (p<0.05) in WPC manufactured from whey with starter culture compared to WPC from rennet-set whey. WPC with annatto had higher concentrations of p-xylene, diacetyl, pentanal, and decanal (p<0.05) compared to WPC without annatto. Interactions (p<0.05) were observed between starter and annatto for hexanal, suggesting that annatto may have an antioxidant effect when present in whey made with starter culture.

The second study investigated lipid oxidation in fresh and stored fluid whey and the influence of starter culture strain and media. Culture type greatly influenced the oxidative stability of liquid whey, with Cheddar and Mozzarella whey differing not only in flavor, but
also in volatile compound profiles. Whey from Cheddar starters had more oxidation products than Mozzarella starters. In a subsequent experiment, starter media did not have an effect on the flavor or oxidative stability of liquid whey, but strain differences were observed among mesophilic starters. Collectively, these studies demonstrate that annatto colorant has a minimal impact on fluid and dried whey flavor. In contrast, the starter culture used for cheese manufacture influences flavor and volatile compound profile of fluid whey.
Impact of Annatto Colorant, Different Starter Cultures, Starter Media and Starter Strains on the Flavor and Oxidative Stability of Liquid Whey and WPC

by
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DEDICATION

To Charlie, who kept smiling even in my darkest hour. Thank you for being the best I could ever dream of and I hope this is the best book you will ever eat.
BIOGRAPHY

Rachel Elizabeth Campbell was born on August 17, 1986. She has one brother, Jonathan, who lives in California attending Stanford University. Her parents, Jennifer and Roger Strickland, still reside in her childhood home. Rachel grew up in Chapel Hill, North Carolina and graduated from Chapel Hill High School in 2004. She began pursuing a degree in food science and obtained her BS in Food Science from North Carolina State University in 2008. During college she did 3 internships where she affirmed her love of food science and research. Rachel began her graduate studies in 2008 under Dr. MaryAnne Drake and hopes to continue many more years.
ACKNOWLEDGEMENTS

At one point, I wasn’t sure I would really ever graduate, even though I was determined to do so. I would really like to thank everyone for believing in me and all the incredible support I have received.

To my family who supported me both emotionally and financially through these times. To my parents for always being there and for watching Charlie while I had to make lots of cheese. To my grandmother for forcing me to exercise and maintain not only physical but mental health. To Elana for always listening and helping to keep me well dressed. To Dr. Drake for her guidance and support as well as many wonderful opportunities. To the MAD Lab for the early hours and the late nights in the pilot plant of tears. To the doctors at Duke Hospital for saving my life in the middle of this whole ordeal and making graduation a possibility. And, to Charlie, for sneaking into the hospital in a duffle bag to visit me. Thank you all.
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CHAPTER 1: LITERATURE REVIEW
INTRODUCTION

The discovery of whey occurred approximately 3000 years ago when the stomachs of cows were used to transport and store milk. Chymosin, which is a naturally occurring enzyme found in the stomach of cows, caused the milk to coagulate and form curds and whey (Smithers, 2008). Up until very recent history, whey has been primarily considered a waste product. This waste material was not looked upon favorably by the dairy industry as the polluting power of whey is well known and disposal methods proved tricky. Some methods used in the past were spraying whey over fields, discharging into rivers, lakes, or the ocean, discharging into the sewage system, or selling for animal feed (Smithers, 2008). None of these methods were very profitable for the dairy industry, and as environmental concerns and regulations increased, the dairy industry sought a more economical way to dispose of this waste product. With the legislative regulations about disposal strengthening and the uproar from the community demanding to recycle whenever possible, the dairy industry explored other approaches to manage the whey waste.

Nearly the same time as the environmental concerns from whey waste started rising, the production of cheese began to rise too. In 2007, the United States produced an astonishing 9.7 billion pounds of cheese, which is a 22.8% increase in cheese production since 1998 (USDA, 2008). Within the past 25 years, the demand for whey has been, and continues to steadily increase in the world market as well in the United States (Tunick, 2008). So, there is more cheese being made than ever before and due to this, there is more whey being produced than ever before.
One of the critical steps towards realizing the value of liquid whey has been the realization of the potential of whey protein. Whey proteins are an excellent way to fortify foods with protein and to help to increase the overall nutritional value of foods (Tunick, 2008). In addition, whey proteins provide various functions in foods such as gelation, thermal stability, foam formation, or emulsification (Foegeding et al., 2002).

There are two types of whey: sweet whey with a pH of at least 5.6 from the manufacture of rennet-coagulated cheeses, and acid whey with a pH no higher than 5.1 from the manufacture of acid-coagulated cheeses (Tunick, 2008). Whey contains mostly lactose (44-52g/L), proteins (6-10g/L), minerals (2.5-7.2g/L), and water that are soluble at the pH of cheese making but the composition can depend on a variety of factors including the type of whey, sweet or acid, and processing variations (Farkye, 2004; Tunick, 2008). The major whey protein fractions are β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulin, and proteose-peptone (Jayaprakasha and Brueckner, 1999). β-lactoglobulin and α-lactalbumin are the principal whey proteins, making up about 80% of the total whey proteins (Schmidt et al., 1984).

Liquid whey is typically not used as a food ingredient, so it must be further processed into a dry powder that is more commonly used in the food industry. The liquid whey is made into powder by a drying process or can be processed further by removing lipid, minerals, and lactose thus increasing the protein content and the value or price (Onwulata, 2008). Powdered whey protein concentrates (WPC) and whey protein isolates (WPI) are the two most common forms of whey protein utilized today. Whey protein concentrates can be
anywhere from 35-80% protein whereas whey protein isolates are greater than 90% protein (Tunick, 2008).

During the 17th and 18th centuries, liquid whey was used for medicinal applications and contains many biologically active proteins and peptides (Smithers, 2008). Whey protein has touted benefits such as recovery after exercise, satiety and weight management, cardiovascular health, anti-cancer effects, wound care and repair, management of infections, infant nutrition, and healthy aging (Smithers, 2008). In today’s market place, consumers are demanding more out of their foods. Whey protein offers great functionality as well as many health benefits and could prove to be a key functional ingredient in many foods in the years to come (Smithers, 2008).

While it is known that whey protein can increase the functionality and nutrition in foods, the use of whey proteins in foods has been limited due to several factors including limited knowledge of whey component properties, poor promotion, unreliable performance in food systems, views that applications should be in low-value products, and lack of economic isolation and manufacture technology (Smithers, 2008). Another short coming of dried whey has been its flavor. The desired flavor of dried whey products is a very bland or delicate flavor (Drake, 2006; Drake et al., 2008). The flavor of the dried whey and the flavor variability can carry through into the finished product and influence the consumer acceptance of this product which in turn can determine the success of the product (Russell et al., 2006, Drake, 2006; Drake et al., 2008; Wright et al., 2008).

Before whey is further processed, the liquid whey is pooled together thus creating many sources of flavor variability (Tunick, 2008). Differences in liquid whey flavors can
come from differences in milk sources, processing and handling, and starter culture blend (Carunchia Whetstine et al., 2003). Dried whey ingredients have been associated with oxidized and undesirable flavors (Carunchia Whetstine et al., 2005; Drake, 2006; Drake et al., 2008; Wright et al., 2008). These flavors may limit the use of dried whey in products that have a bland flavor or a flavor that is not strong enough to mask the flavor of whey protein (Drake, 2006; Drake et al., 2008; Wright et al., 2008). If the sources and causes of flavor variability in liquid whey were better understood, then this could lead to methods that would minimize flavor variability in dried whey (Caruncia Whetstine et al., 2003). Once whey protein concentrates and isolates are able to be produced without undesirable off-flavors and flavor variability, their usage will increase.

The food industry is large, growing, and ever demanding new, economical, and functional ingredients (Smithers, 2008). Dairy products enjoy a positive, healthy, wholesome, and desirable tasting image by the consumer. When polled, 84% of consumers felt that “cow’s milk tastes good” while 82% disagreed with the statement that “soy milk tastes good” (Russell et al., 2006). Furthermore, 20% of consumers felt that soy products delivered great taste while 63% felt that dairy products provide great taste (Russell et al., 2006). Since whey is a product of milk and cheese making, this offers a known ingredient which consumers feel confident about and could compete in the functional food market with other ingredients, such as soy protein (Smithers, 2008).

Functional foods, foods that provide a specific health benefit, are become ever more popular. The United States shows about 14% sustained growth in the category yearly and it is estimated that by 2012, this market will be over $100 billion (Smithers, 2008). Whey
products offer an array of health benefits as well as many functional properties that help it perform well in a food system. Off-flavors in whey protein are never desired, but perhaps in certain applications if a desirable flavor could be controlled, it could offer an additional flavor benefit which further enhances the food application (Mortenson et al., 2008). Once flavor and flavor variability are understood, whey protein will have an even wider appeal as it could be utilized in bland or delicately flavored foods with out undesirable flavors carrying through into the finished product (Carunchia Whetstine et al., 2003).

The market place and consumers are becoming more sophisticated, and nutrition awareness is a growing trend. The world is also growing more aware of the environment and demanding sustainability and conservation. Dried whey proteins could meet both the demands of the consumers and the producers if just a few short comings were better understood and corrected. In the past 50 years, whey protein has gone from a pesky by-product in need of cheap disposal to being a valuable ingredient in the food industry today. With emerging technologies and increasing understanding of whey, whey products will continue to climb to the top.

**WHEY PROTEINS vs. NATIVE WHEY PROTEINS**

After milk is transformed into cheese curd, the components of the milk are separated into two categories: those that are retained in the cheese and those that are lost in the whey (Farkye, 2004). This liquid whey contains approximately 0.6% protein and 93% water (Foegeding et al., 2002). After the cheese making process, the protein in the whey is referred to as whey protein (Drake et al., 2008; Varnam and Sutherland, 1994). Whey proteins that have been removed prior to the cheese making process are referred to as “native” whey...
proteins or milk serum proteins, however, since the removal was prior to cheese making, these proteins are not actually part of the whey (Drake et al., 2008; Varnam and Sutherland, 1994). It should be noted that serum proteins have been used in ice cream manufacture for some time. Arbuckle (1987) stated that the serum proteins can be used to supply 25% or less of the milk solids non fat in ice cream. In addition, often these proteins are added to ice cream to enhance some functional properties such as whipping, body and texture, resistance to heat shock, and storage and melting properties. Marshall et al. (2003) provided extensive equations and explanations for calculations.

Native whey proteins are a very valuable part of the milk and are not exposed to the cheese making process, thus leaving them free of any decreased functionality or undesirable sensory characteristics from this process. It may be advantageous to remove the milk serum proteins prior to cheese making for several reasons. If milk serum proteins were removed before the manufacture of cheese, the cheese composition would be the same since the majority of these proteins are not retained in the cheese (Nelson and Barbano, 2005a). Nelson and Barbano (2005a) developed a process using a multistage microfiltration process in order to obtain serum proteins from skim milk at a high recovery rate. Their three stage process removed 95% of the serum proteins from skim milk and yielded a high quality protein that did not contain residual coagulant, starter culture, lactic acid, or color from the cheese making process unlike traditional whey proteins. Nelson and Barbano (2005b) went on to conduct another study in which they evaluated the yield and aging of Cheddar cheese made from milk with different serum protein contents. This study showed that a high quality cheese could be made from milk with low serum protein content. It was hypothesized that
the removal of serum proteins from the milk would have little to no effect on the cheese
yield. Fat recovery and cheese yield were in fact higher when the low serum protein content
milk was used (Nelson and Barbano, 2005b). The higher fat recovery exhibited by low serum
protein milks when used in cheese production could be a crucial benefit to the dairy industry.

There is very little literature published on the functional and sensory differences
between whey protein from cheese manufacture and serum proteins (native whey protein). A
recent study conducted at the University of Helsinki in Finland compared microfiltered
native whey protein concentrate powders, from 24.1 to 37.3% protein, to traditionally
manufactured whey protein powders (Heino et al., 2007). Differences in protein content of
the powder were due to different raw materials and different processes used, such as batch
pilot plant or continuous industrial manufacture (Heino et al., 2007). Significant differences
in protein functionality were observed between the different powders. Differences in the
functional properties were not caused by the drying method. Solubility, viscosity, gelation,
foaming properties, emulsifying capacity, and water-holding capacity were measured.
Functional properties measured were slightly better in the native whey protein powders than
the traditional protein powder made from cheese whey. The gel strength, foaming, and
emulsification properties of the native whey proteins were significantly better than whey
protein powders made from cheese whey and could be explained by denaturation rate, fat
residues, and protein composition, respectively (Heino et al., 2007). Native whey protein
concentrate powders used in this study had a lower fat content and the higher fat residues
present in conventional whey protein powders led to decreased foam stability and reduced
foam volume. In addition, because the serum protein was removed before the cheese making
process, the native whey was free from other minor constituents found in cheese whey which could lead to decreased functionality in foaming stability (Heino et al., 2007).

Increased functionality is not the only benefit to native whey proteins versus conventionally processed whey proteins. Native whey proteins are purported to have a higher biological activity (Marcelo and Rizvi, 2007). A recent study showed that liquid virgin whey protein isolate may be a superior source of biologically active proteins as compared with conventionally processed whey proteins (Marcelo and Rizvi, 2007). Marcelo and Rizvi (2007) concluded that liquid whey protein isolate contained more proteins in their native form, determined by examining unique physicochemical properties, than commercial products. It was proposed that whey proteins may lose their “native conformations” during cheese making or subsequent processing, but there is little evidence supporting this theory (Marcelo and Rizvi, 2007). Whey proteins can be used not only in food industries but in other industries such as pharmaceutical and biomedical fields (Marcelo and Rizvi, 2007). Native whey proteins may be of particular interests to the pharmaceutical and biomedical industries as these proteins are still in their “native conformation” and are thought to be the only proteins that are biologically significant (Marcelo and Rizvi, 2007).

There is a lack of literature covering the sensory properties of serum (native whey) protein. Since this product was not exposed to the cheese making process and thus does not include any remnants of this process, it would be expected that serum protein (native whey) would possess a more delicate flavor and be more desirable in today’s market. In addition, the percentage of fat in native whey protein concentrate was much lower that in conventional whey protein concentrate (Heino et al, 2007). The fat content of whey products is known to
have a negative impact on flavor as fat oxidation causes off-flavors in whey products. The oxidation of lipids produces an array of compounds including aldehydes, ketones, alcohols, and alkanes (Bodyfelt et al., 1988). The flavor of dried ingredients greatly impacts the consumer acceptance of the ingredient and the willingness of the consumer to purchase a product containing that ingredient (Russell et al., 2006; Drake, 2006; Drake et al., 2008; Wright et al., 2008). Since the small amount of existing literature on the subject of native whey proteins tends to show that these proteins have increased functionality additional studies are needed to determine sensory properties of these products and to further document the benefits of serum (native whey) protein and the feasibility of removing the milk serum prior to cheese making in the current dairy industry.

**PROCESSING**

In order to produce whey protein concentrate or whey protein isolate, there are many steps between the cow’s milk and the finished functional food ingredient. In order to obtain the whey, first the milk must be made into cheese. The steps in cheese making for a typical Cheddar cheese include milk receipt, pretreatment of milk, standardization, heat treatment/pasteurization of milk, the addition of calcium chloride and/or color, acidification, addition of rennet/milk coagulant, cutting the curd, cooking/draining/curd manipulation, salting, hooping, and finally pressing (Farkye, 2004). Following the coagulation and the cutting of the cheese curd, the curds and whey are cooked to expel the whey through syneresis (Farkye, 2004). Following the cook procedure, the fluid whey is drained from the cheese curds. The curds continue through the cheese making process while the fluid whey is likewise ready for further processing. This side stream of whey is about 93% water and 0.6%
protein and must be further processed to yield whey protein concentrates or isolates (Huffman, 1996).

The process to produce WPC (whey protein concentrates) and WPI (whey protein isolates) is basically the same, however there are some minor process differences and additional steps for those products with higher protein content. The basic steps to produce a whey protein concentrate include holding, clarification, ultrafiltration, and spray drying (Huffman, 1996; Varnam and Sutherland, 1994). After the whey is produced, it is held in a large tank where holding time should be minimal. Then the whey is clarified to remove any cheese or casein fines and residual fat is removed. Traditionally centrifugation is used to clarify and this step is extremely important as any residual lipids not removed could lead to membrane fouling in the next step and off flavors in the final product (Bodyfelt et al., 1988; Varnam and Sutherland, 1994). This solution is then concentrated using ultrafiltration which separates the whey protein from lactose and other low molecular weight components. Ultrafiltration is a pressure activated membrane separation technique that physically separates whey protein and fat from lactose and minerals by using a membrane which separates these components based on molecular size (Huffman, 1996). Ultrafiltration controls the composition, enhances ingredient functionality, and enhances whey protein purity (Varnam and Sutherland, 1994; Hoffman, 1996; Onwulata, 2008). It is possible to achieve 25% to 35% solids using ultrafiltration. Depending on the protein concentration desired, the whey can be evaporated to higher solids before it is spray dried. The concentrated whey is then spray dried to produce a fine powder which can be utilized in many different food systems.
The process described above is commonly used for lower protein content products, but in order to produce >50% protein powder, another step must be added (Hoffman, 1996; Onwulata, 2008). This additional step is called diafiltration and occurs after ultrafiltration but before spray drying. Diafiltration is a membrane process that increases protein concentration, removes minerals and lactose from retentate, enhances that purity of the whey protein, and further enhances the functionality of the protein (Hoffman, 1996; Onwulata, 2008). Diafiltration is essentially a wash step (Johnson and Lucey, 2006).

WPI has a higher protein content than WPC, and thus a few more steps must be added to this process to achieve this higher protein content. Two additional steps must be added: microfiltration to remove fat and lactose hydrolysis to remove lactose. These two steps would follow after ultrafiltration and diafiltration. Another process that could be used to manufacture WPI is ion exchange which is used as a pretreatment prior to ultrafiltration. Ion exchange separates components by ionic charge rather than molecular size and permits for virtually 100% demineralization (Varnam and Sutherland, 1994; Onwulata, 2008). Cation exchange, which is common in commercial technology, involves the whey stream first taken to an acid pH so that the proteins are positively charged (Huffman, 1996). The whey is then pumped into a tank with negatively charged resin beads (Huffman, 1996). The positively charged whey proteins attach to the negatively charged resin beads and consequently, fat, lactose, and minerals are removed and carried away by the flowing stream (Huffman, 1996; Varnam and Sutherland, 1994). Once the resin is loaded with protein, the pH of the tank is made alkaline, the proteins detach, and a very dilute stream of whey protein is obtained.
(Huffman, 1996). This whey stream can be further processed and dried to obtain a high quality protein powder.

Over the past 15 years, whey processing technologies have grown more sophisticated. Emphasis has been placed on cost effectiveness and the retention of protein functionality (Smithers, 2008). With the introduction of technologies such as continuous SEParation (CSEP) chromatographic technology which has made the manufacture of unique WPIs possible, such as enriched β-lactoglobulin, the ever increasing sophistication of membrane processing which is currently used widely to concentrate and fractionate whey proteins, the use of membrane absorbers in ion exchange to isolate and fractionate proteins, many doors have been opened for the dairy industry to further process whey and add value to a product that was once simply regarded as the waste product from making cheese (Onwulata, 2008; Smithers, 2008). Johnson and Lucey (2006) wrote that “someday cheese would become the by-product of producing whey”.

**PROCESSING DIFFERENCES AND FLAVOR**

While technology has come a long way, flavor variability and off-flavors that are present in the liquid whey could translate into the powdered whey product. The extra processing steps such as pasteurization, membrane filtration, concentration, and spray drying to produce WPC or WPI can present additional off-flavors and flavor variability (Drake et al., 2008). Facilities may differ in the type of process that is run as well, for example, ultrafiltration and diafiltration versus ion exchange. Ultrafiltration physically separates whey protein and fat from lactose and minerals by using a membrane that retains molecular weights greater than 20,000 to 30,000 (Huffman, 1996; Zydney, 1998). Membrane fouling
can be a major problem and is caused by particles blocking the membrane pores, growth of microorganisms, or physicochemical reactions such as gel formations (Varnam and Sutherland, 1994). Thus, great care must be taken to ensure that cleaning and sanitizing are properly performed to prevent the loss of flux (Varnam and Sutherland, 1994). Diafiltration, which is a membrane process, further increases protein concentration by continuously adding water to the retentate stream to wash out lactose and minerals (Huffman, 1996; Zydney, 1998). In direct contrast, ion exchange separates components by ionic charge rather than molecular size and this process may be used instead of ultrafiltration and diafiltration (Onwulata, 2008). In addition to the possibility that each facility may choose to use completely different processes, they may also differ in specific storage conditions and time temperature profiles thus further contributing to variability in finished goods (Drake et al., 2008).

If the whey is from Cheddar cheese, it may need to be bleached as some annatto color carries over into the whey, imparting an undesirable color (Smith, 2004). The bleaching process oxidizes the whey to decolorize it and may result in more flavors and variability (Drake et al., 2008). There is little current research on the effects on flavor of further processing liquid whey into whey protein concentrate or isolate and the use of bleaching on flavor. In a recent study, it was found that instantizing, ion exchange, and bleaching had no effect on sensory perception flavor of either whey protein concentrate or whey protein isolate, but slight variability was found within instrumental data (Mortenson et al., 2008). Mortenson et al. (2008) noted that although instrumental data was very accurate and precise, the small differences hold little meaning as sensory panelists did not pick up differences.
However, products were manufactured using a combination of industrial and pilot scale processes. In addition, there was little control over processes for these products and no direct comparisons between products from one facility were made. As a result, facility-specific differences, which are already well established, may have masked any effects from bleaching.

ANNATTO AND BLEACHING

Annatto is a yellow/orange natural pigment that is used to color cheese. Annatto comes from the tropical tree *Bixa orellana* which is named after Francisco de Orellana who was a scientist and explorer of the upper Amazon (Giuliano et al., 2003). Clusters of the fruit, which is capsular shaped and is covered in burrs, grow on this tree and inside the fruit, there are about 10 to 50 small seeds (Ames and Hofmann, 2001). The seeds are covered in a bright red pulp, and this pulp contains the annatto pigment (Smith, 2004). The Aztecs used the annatto extract as a dye for textiles, body dye (such as in lipsticks), and also as a food colorant in the drink cacahuatl (Giuliano et al., 2003).

Latin America produces about 60% of the world’s annatto, followed by Africa (27%) and Asia (12%) (Giuliano et al., 2003). Prices for annatto seeds depend on production and are also proportional to bixin content (Giuliano et al., 2003). The United States and Europe are the two largest importers of annatto seeds, but the Japanese market is growing rapidly since the introduction of the colorant in 1963 (Ames and Hofmann, 2001).

The biggest application of annatto is in the dairy industry for coloring cheese and other dairy products. However, annatto is also commonly used in sausages, fish, margarine, snacks, dressings, sauces, and confectionary, but usage varies from country to country due to
different food cultures and legislations (Ames and Hofmann, 2001). There are two main processed products of annatto: the water-soluble norbixin and the oil-soluble ester bixin (Giuliano et al., 2003) (figures 1-4). Bixin is the major pigment of the seed coat. The stability of bixin is not affected by pH but it is unstable to light. Bixin is stable at temperatures less than 100°C but heating converts cis-bixin to trans-bixin (Smith, 2004). Norbixin is formed when the methyl group of bixin is saponified under alkaline conditions and is considered a very strong colorant (Smith, 2004; Giuliano et al., 2003). Both bixin and norbixin occur naturally in the cis form but can be converted to the trans form by light and heat (Smith, 2004). The cis form of both bixin and norbixin is more red than their trans forms (Smith, 2004).

![Figure 1: Cis-Bixin (Smith, 2004)](image1)

![Figure 2: Trans-Bixin (Smith 2004)](image2)
The chemical make up of both bixin and the derivatives of bixin make these compounds susceptible to both oxidation and reduction (Barnicoat, 1937). Barnicoat (1937) concluded that oxidation was more important in causing discoloration in colored cheeses than reduction. Oxidation is also important to the whey industry as oxidation leads to a loss of annatto color (Smith, 2004). Norbixin is able to bind with a carboxyl group of another molecule such as protein, creating a peach-red color and forming a stable complex that can help prevent oxidation and color loss (Smith, 2004). This reaction may be desirable in some products, but may make color removal in whey by bleaching more difficult.

Annatto can also produce a pink color in cheese. The exact cause of this pink discoloration and defect in cheese is unknown, however several researchers have hypothesized on the causes of pinking. Barnicoat (1950) attributed the pinking to oxidation of norbixin by sulfhydryl-type compounds however others attributed the pinking color to the reaction of norbixin with protein (Smith, 2004). In addition to causing a pink color in
cheese, annatto can cause a pink color in products containing bleached whey powders. The exact cause of this pink defect is also unknown, but important factors are believed to be pH, heat treatment of whey, and brand of annatto (Smith, 2004).

Although annatto is primarily used to color cheese, approximately 20% of the annatto will be present in the resulting fluid whey (Barincoat, 1950; Smith, 2004). The color is highly unfavorable in dried whey products and thus a decolorizing process must be performed (McDonough et al., 1968). While annatto is a large source of color in whey, there are two other sources of color that must be addressed as well: xanthophylls and Maillard reaction products. Xanthophylls are present in whey and enter in the milk as β-carotene from the food that the cow consumes (Croissant et al., 2007; Smith, 2004). The yellow color in the milk is a result from an incomplete conversion of the β-carotene to vitamin A (Smith, 2004). Croissant et al. (2007) found that milk from pasture fed Holstein and Jersey cows exhibited was more yellow in color than their similar counterparts which were fed a conventional total mixed ration diet. These findings were expected as the pasture fed cows ingested fresh forage, known to contain carotenoids, thus raising the concentration of carotenoids in the milk fat (Croissant et al., 2007). Legal bleaching agents, both hydrogen and benzoyl peroxide, will bleach xanthophylls, however due to regulations, hydrogen peroxide is not permitted to bleach xanthophylls in milk (Smith, 2004). Maillard reaction products are also a cause of color in whey and are tan to black in color. The color from this reaction, also referred to as nonenzymatic browning, is a result of interactions between amino acids and sugars, but this color cannot be removed by bleaching methods (Bodyfelt et al., 1988; Smith, 2004).
It is undesirable to have colored whey and typically bleaching is used to remove the color leftover from cheese making and to give a more uniform color to the whey powder (Chang et al., 1977). Currently in the United States there are only two compounds that are permitted to bleach whey: hydrogen peroxide (Figure 5) and benzoyl peroxide (Figure 6). While these compounds are approved, there are several restrictions to their use.

![Figure 5: Chemical Structure of Hydrogen Peroxide (USGS, 2008)](image)

Hydrogen peroxide is covered by 21CFR 184.1366 and may be used at a rate of less than 500 ppm (Smith, 2004). Residual hydrogen peroxide must be removed from the product by the addition of catalase according to FDA regulation 133.113. Catalase used must not exceed 20 ppm and be sufficient to remove any leftover hydrogen peroxide (Smith, 2004). Catalase converts hydrogen peroxide to oxygen and water as seen in Figure 7. The advantages of hydrogen peroxides are the fact that they are effective across a wide range of temperatures and total solids levels (Gilliland, 1969; Smith, 2004). In addition, hydrogen peroxide has little to no effect on the nutrients present (Teply et al., 1957). Teply et al. (1957) analyzed milks and subsequent cheese and whey when bleached by 5, 10, and 25 times the normal practice level of hydrogen peroxide ([.02] H₂O₂) and found that a strong
treatment may alter proteins and amino acids in milk but in general there was no effect on composition or nutritional value of the milk, cheese, or whey examined. The disadvantages are that hydrogen peroxide must be inactivated with catalase, could possibly cause oxidized flavors, and needs to have a long hold time to remove color. The disadvantages are that hydrogen peroxide must be inactivated with catalase, could possibly cause oxidized flavors, and needs to have a long hold time to remove color (Gilliland, 1969; Smith, 2004).

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \]

**Figure 7: Breakdown of Hydrogen Peroxide** (Smith, 2004)

The use of benzoyl peroxide is covered by 21CFR 184.1157 and unlike hydrogen peroxide, there is no rate limitation for benzoyl peroxide. The typical use rate is less than 20 ppm and commercial benzoyl peroxide products use a carrier such as starch, whey, calcium phosphate or sulfate, and magnesium carbonate (Kuramoto and Jezeski, 1954; Smith, 2004). Benzoyl peroxide is converted to benzoic acid, a natural constituent found in milk, following bleaching (Smith, 2004) (figure 8). The advantages to using benzoyl peroxide are that it is effective at lower usage levels, does not require a catalase addition to remove residues, does not pit stainless steel, and is less corrosive to equipment (Smith, 2004; Chang et al., 1977). The disadvantages to using benzoyl peroxide include the formation of oxidized flavors, the possibility that the carrier used may be considered an allergen, and concerns from other countries about this bleaching agent which has just been recently approved by CODEX (Smith, 2004). While the United States accepts the use of benzoyl peroxide readily, some countries may still be concerned about importing products that use this ingredient.
Both benzoyl peroxide and hydrogen peroxide readily bleach the annatto in liquid and have been used extensively for bleaching annatto in fluid whey (McDonough et al., 1968; Smith, 2004). Both of these bleaching agents work very well in solution, but once the whey is dried, the annatto binds to the whey proteins and this reaction cannot be reversed. As such, dried whey products are extremely resistant to bleaching (Smith, 2004). As mentioned previously, there are three sources of color in whey: xanthophylls, Maillard reaction products, and annatto. Bleaching agents will bleach xanthophylls, but regulations do not allow the bleaching of xanthophylls in milk by hydrogen peroxide (Smith, 2004). However, bleaching agents will not remove brown products that result from Maillard reactions. Hydrogen peroxide has been shown to inhibit browning, but once Maillard products are formed this agent cannot alter them (Smith, 2004).

In the processing of whey, there are two locations at which the bleaching step can occur. The peroxide can be added after the preheated whey is pumped into a storage tank or it can be added at the hot well of the evaporator (Smith, 2004). It is important to note that peroxide may not be used to control acid production by bacteria during the storage of whey or as a substitute for Good Manufacturing Practices (Smith, 2004). In order to differentiate between bleaching and preservation, the USDA has identified two specific locations in the process where the addition of peroxides would be considered for preservation and not
bleaching purposes. The addition of peroxides before the separator or at any point before preheating for the evaporator and the addition before holding the whey for more than two hours at temperatures between 7 and 63°C would be considered using peroxides for preservation purposes and is not permitted (Smith, 2004).

While bleaching can create a more desirable color, it may also alter functionality of whey proteins as well as flavor. The color may be important to consumers, but the functionality and flavor may be of greater importance. Functional properties that may be affected include solubility, viscosity, water binding, whipping, emulsification, and gelation. Oxidized flavor intensities immediately following treatment were strong but decreased during concentration and drying processes (McDonough et al., 1968). Whey proteins were denatured due to bleaching and concentration, time, temperature, and pH were all factors that determined the extent to which the proteins denatured (Cooney and Morr, 1972). In addition, different proteins were more vulnerable than others with proteose-peptones being the most susceptible to denaturation and α-lactalbumin being the most resilient (Cooney and Morr, 1972). It should be noted that much literature dealing with the bleaching of whey is quite dated. Since these studies have been conducted, milk quality, cheese making practices and whey protein processing have all greatly evolved.

Other concerns from bleaching deal with the use of benzoyl peroxide, as it has only been recently approved by CODEX and many Asian and European countries do not like the use of it (Smith, 2004). The USDEC is unclear as to why these countries are hesitant about benzoyl peroxide being used in bleaching, especially since the product of the breakdown (See Figure 8), benzoic acid, is naturally occurring in milk products. Chang et al. (1977) found
that in the decomposition of benzoyl peroxide in Edam cheese whey after it was heated to 69°C for 6 hours and cooled, 91.7% of benzoyl peroxide used was recovered as benzoic acid and minor amounts of hydroxybenzoic acids, phenylbenzoate, phenol, and benzoyl peroxide. The safety of benzoic acids and its derivative benzoates have been greatly studied, although it should be noted that benzoic acid is a normal constituent of milk and fermented products. Benzoic acid is found naturally in milk at low concentration, a few mg/kg, and in fermented dairy products at higher concentrations, about 20 mg/kg but as much as 50 mg/kg (Sieber et al., 1995). During the fermentation process, lactic acid bacteria convert hippuric acid, which is a naturally occurring component of milk, to benzoic acid (Sieber et al., 1995). Benzoic acid is also found in cheeses, but at lower concentrations than fermented dairy products (Sieber et al., 1995). Subsequently, benzoic acid naturally occurs in whey and whey protein powders. Adverse reactions to benzoic acid related compounds are rare and life-threatening reactions are extremely rare (Smith, 2004).

PROTEIN FUNCTIONALITY

One of the most appealing aspects of whey proteins is that they can serve as a functional ingredient in a food system. Protein functionality is defined by Morr and Ha (1993) as “physicochemical properties that influence the structure, appearance, texture, viscosity, mouth feel, or flavor retention of the product”. Whey protein functionalities can be influenced by many factors including the composition and processing of the whey protein as well as the composition and processing of the food system (Morr and Ha, 1993). In addition, factors that affect the cheese making process would be expected to influence the whey and
whey protein as well. These factors include heat treatment, starter culture, and cooking conditions (Schmidt et al., 1984; Farkye, 2004; Smithers, 2008;).

In order to fully utilize WPC or WPI in food formulations, the functional behavior of these ingredients must be well understood (Jayaprakasha and Brueckner, 1999). The functional behavior of these ingredients has been widely studied. Today, various technologies exist and there are many ways to further process whey. There are three main factors that influence the functionality of whey protein in regards to processing: the processing upstream prior to whey production and the control of the whey source, processing control and protein manipulation during the manufacture of concentrate or isolate, and the final composition of the whey including protein concentration as well as the composition of other non-protein components (Huffman, 1996). Although many factors appear to influence the functionality of whey protein, with better understanding, these factors can be manipulated purposefully in order to design whey proteins with enhanced functional properties and may have great potential in industry.

Although solubility may not be considered a functional property of whey protein, it is critical for other functionalities such as creating a foam, emulsion, or gel (Jayaprakasha and Brueckner, 1999). Whey proteins that have not been denatured are soluble over a wide pH range (Huffman, 1996). Heating to temperatures above 70°C can cause loss of solubility as some whey proteins will then aggregate and precipitate at their isoelectric points (Huffman, 1996). If it is necessary to heat the product, the solubility of whey proteins can be increased by adding sugar (Huffman, 1996). In addition, solubility also decreases at high salt concentrations (Jayaprakasha and Brueckner, 1999). Even if the proteins are only partially
denatured, they are still more susceptible to the effects of pH and salt concentrations (Jayaprakasha and Brueckner, 1999). It is important to note that whey proteins are soluble in acidic solutions unlike some other proteins such as casein and lactalbumin, which is imperative to applications in beverages or salad dressings (Huffman, 1996; Jayaprakasha and Brueckner, 1999). Other proteins are not able to function in acid foods making whey protein very unique and highly desirable in these applications.

As mentioned previously, when heated, whey proteins will begin to lose their solubility. Solubility and water-binding capacity are inversely related and thus as they are heated, whey proteins will begin to increase their water holding capacity (Huffman, 1996). This means that denatured proteins are insoluble but have a high water binding capacity (Jayaprakasha and Brueckner, 1999). Heating causes the protein to partially unfold, exposing more water binding sites and also increases the volume occupied by the whey protein (Huffman, 1996; Jayaprakasha and Brueckner, 1999). Heating will also cause a slight increase in viscosity (Huffman, 1996). Depending on the application, water-binding and increased viscosity may be desired. A great example of this would be in minced meat products where a denatured whey protein will increase water binding capacity and texture in the finished good. This may be highly undesirable in other applications such as beverages where low viscosity is desired so that high concentrations of whey proteins can be used thus reducing the volume of liquid required to be consumed for desired protein content (Huffman, 1996).

Whey proteins also have great gelling properties. Gels are formed when proteins interact and produce an elastic network under proper heating conditions (Huffman, 1996;
Foegeding et al., 2002). These gels are irreversible and the whey proteins start to gel when heated to about 65°C and contain about 7% protein (Huffman, 1996). The aggregation of whey protein begins with the initial swelling of the protein when exposed to heat and as the heat increases, the protein unfolds, aggregates, and forms a gel network (Rich and Foegeding, 2000; Jayaprakasha and Brueckner, 1999). Foegeding et al. (2002) found that the amount of aggregated protein forming the gel network determined gel network type and permeability. There are many factors that can affect the formation of gels which include temperature, pH, protein concentration, salt, sugars, calcium, and free sulfhydryl groups in the system (Rich and Foegeding, 2000; Jayaprakasha and Brueckner, 1999). In addition to these factors, the gelling ability of whey proteins can vary due to whey source, composition, and processing techniques and could vary greatly from one commercial whey protein concentrate to the next (Jayaprakasha and Brueckner, 1999). Gelling properties are important as they can be used to modify texture by influencing hardness, cohesiveness, and elasticity (Huffman, 1996).

Due to their gelation properties, whey proteins have the opportunity to be used in many food systems. An important application would be in meat products where up to 20% of meat proteins may be replaced with whey proteins or in processed fish products as a binding agent (Jayaprakasha and Brueckner, 1999). Whey protein products can also be used in yogurts and cheese where they improve yield and consistency (Jayaprakasha and Brueckner, 1999). Another major usage is in cakes where whey proteins could replace egg whites at a lesser cost. Huffman (1996) noted that gelation could be used to change liquid
and flowable products into a solid and Jayaprakasha and Brueckner (1999) added that this is the most important functional property of whey protein with regards to marketability.

As evidenced, there are many foods that can utilize the gelling functional property of whey protein. However, many food applications may be too sensitive to the heat needed to gel proteins. As a result, much effort has been placed on exploring cold gelling. In order to produce a cold gelling whey protein, the whey proteins are heat denatured and aggregated at conditions that do not yield a gel and then the solvent is altered to form a gel under “cold”, ambient to refrigeration, temperatures (Foegeding et al., 2002).

The production and the application of cold gelling whey proteins basically entails two steps: making the heat denatured protein solution and the gel formation at low temperatures (Bryant and McClements, 1998). When whey proteins are heat denatured, the whey proteins in their native state will unfold and aggregate but several conditions must be very carefully controlled in order to prevent gelation of the solution. First, the pH must be significantly different than the isoelectric point. In addition, the salt concentration must be low enough to prevent excessive aggregation and the protein concentration must be low enough to prevent gelling (Bryant and McClements, 1998). The tendency of protein to aggregate after heating is extremely dependent on electrostatic interactions (Bryant and McClements, 2000). In their native state, the attractive forces cannot overcome the repulsive forces and thus the protein must be unfolded to some extent before aggregation can occur (Bryant and McClements, 1998). Once this protein solution is created, it can be cooled and used directly by decreasing the electrostatic repulsion between protein molecules (Bryant and McClements, 2000). In order to form a gel, which is a three-dimensional network of aggregated proteins, the
electrostatic repulsion must be decreased between the protein molecules by increasing the mineral content or adjusting the pH (Bryant and McClements, 2000).

Bryant and McClements (2000) reported that it was possible to produce ingredients with different cold gelling characteristics simply by varying thermal treatment used in the preparation of the heat denatured whey protein solution. Being able to modify the functional properties of whey protein by simple physical techniques such as thermal treatment only adds appeal to the use of whey proteins as functional ingredients as they can be tailored for specific applications. Better understanding of whey proteins and their functional properties over recent history has led scientists to be able to modify these functional properties for specific uses in food systems.

Another functional characteristic of whey proteins is their ability to form emulsions due to the fact they contain both hydrophilic and hydrophobic regions (Huffman, 1996). The emulsion is formed because the protein is able to unfold and orient itself so that they hydrophobic groups associate with the oil phase and the hydrophilic groups associate with the water phase (Schmidt et al., 1984). By forming a protective interfacial membrane around the oil or water globules, creaming, coalescence, flocculation, and oiling off can be prevented, creating a more stable emulsion (Demetriades et al., 1997; Huffman, 1996; Jayaprakasha and Brueckner, 1999). Many food emulsions require an emulsion that is stable over a long period of time and under a variety of storage conditions (Huffman, 1996). This is considered the most important property of a protein stabilized emulsion as the protein is able to not only aid in the formation of an emulsion, but also add to the stability of the emulsion (Jayaprakasha and Brueckner, 1999). In order to determine whether the use of whey proteins
is appropriate in a particular food application, the composition, the processing, and the storage conditions that the food experiences must all be considered (Demetriades et al., 1997). It should be noted that it may be possible to alter the molecular structure, thus modifying the functional properties of whey protein in order to change the range of environmental conditions in which they could be used (Demetriades et al., 1997).

The emulsion properties of whey proteins can be utilized in many foods including comminuted or minced meats, cakes, coffee whiteners, salad dressings, and frozen desserts (Jayaprakasha and Brueckner, 1999). Since whey proteins are soluble in acidic conditions, they perform well in acidic applications such as salad dressing. In addition, whey protein can also provide emulsion stability in heated foods such as sauces. This is due to the fact that as the product is heated, the viscosity is increased and gelling can occur. The increased viscosity reduces the mobility of fat globules thus minimizing coalescence (Huffman, 1996). If gelation occurs, the gel network can totally entrap the fat (Huffman, 1996). Whey protein provides an economical solution to replacing some conventional ingredients such as eggs in cakes, caseinate in soups, gravies, or coffee whiteners, and commercial emulsifiers in ice cream (Jayaprakasha and Brueckner, 1999). The functionality of whey proteins may also be improved by using them in conjunction with other ingredients such as thickening agents or other emulsifiers (Demetriades et al., 1997).

Foams are very popular among consumers today including foods such as frozen desserts, cakes, nougat confections, meringues, soufflés, frothed drinks, and many more (Foegeding et al., 2002, 2006). Foams in foods create unique textures and are in high demand with the ever more sophisticated and demanding consumer. When a foam is formed,
the whey protein interface is formed around an air micelle promoting bubble stability (Davis and Foegeding, 2007; Huffman, 1996). Foaming and foam stability are influenced by whey protein type, protein denaturation, fat and carbohydrate concentrations, whipping method, protein concentration, pH, presence and concentration of ions such as calcium, and processing equipment used (Huffman, 1996; Jayaprakasha and Brueckner, 1999). When utilized in a food application, factors such as presence of sugar, salt, and fat, amount of whey protein, and whipping time will help determine whip volumes as well as the stability of the foam (Huffman, 1996). The true test of a great protein foam is one that obtains a desired level of air phase volume and then maintains stability when further processed by mixing, cutting, or heating (Foegeding et al., 2006).

Just as with the formation of an emulsion, a partial denaturation of the whey protein will greatly improve the ability of the whey protein to foam (Damodaran, 2005). This makes heating a very important processing step and while some heating is required to partially denature these whey proteins for improved foam formation, excessive denaturation is not desired (Damodaran, 2005). A mild heat treatment allows the proteins to partially unfold and generate a protein film which surrounds a gas bubble (Foegeding et al., 2006; Jayaprakasha and Brueckner, 1999). Foaming properties of proteins are optimal near the isoelectric points of the proteins (Foegeding et al., 2006).

Many of the products that foams are used in often contain high contents of sugar and protein (Davis and Foegeding, 2007; Foegeding et al., 2006). For this reason, it is especially important to carefully examine not only the functionality of whey protein in regards to foam, but the functionality of the foam in a food system. Sugars improve foam stability due to an
increase in viscosity of the protein solution, but it should be noted that sugars do not improve foamability (Damodaran, 2005). Sugars are able to affect the interfacial behavior of proteins by influencing the structure of the foam (Davis and Foegeding, 2007). Typically commercial products which require a foam use egg whites in their formulations (Foegeding et al., 2006). Egg whites are highly valued by those in the culinary arts for their ability to form a high volume foam which is also highly stable. The egg white is typically whipped into a meringue and then incorporated into other products, such as angel food cake, by folding and then further processing by baking (Foegeding et al., 2006). Egg whites are able to produce a foam with less protein and less whipping time than whey protein isolate (Davis and Foegeding, 2007). Much research has been done on the foaming properties of whey proteins as egg whites are very expensive and it would be highly desirable to replace eggs whites in a food system with a cheaper ingredient without losing any quality.

Foamability of a proteins solution will depend greatly on the aeration process, but egg and whey proteins have very similar abilities to incorporate air and form a foam (Foegeding et al., 2006). The slight pitfall in forming a foam with whey protein would be the fact that there may be residual fat left over in the whey protein powder. This fat may greatly hinder foam formation and even cause foam lamella to break (Jayaprakasha and Brueckner, 1999). If the residual fat were reduced, an improvement in foam capacity would be seen (Jayaprakasha and Brueckner, 1999). Once the foam is successfully formed, the problem then becomes foam stability. Foegeding et al. (2006) found that several destabilization mechanisms were occurring at the same time thus making it very difficult to pinpoint the cause of foam instability. As compared with egg white foams, whey protein foams were less
stable against gravity induced drainage but this negative effect can be decreased by increasing the viscosity of the protein solution (Davis and Foegeding, 2007; Foegeding et al., 2006).

As evidenced, whey proteins have a wide range of functionalities but no whey protein concentrate or whey protein isolate can contain all of these properties in a single ingredient (Huffman, 1996). Over the past 25 years, one of the greatest restrictions of the usage of whey protein in various food systems was their inconsistent and unreliable performance (Smithers, 2008). With the emergence of new technologies and the stricter quality requirements, whey proteins could successfully compete with other protein ingredients (Jayaprakasha and Brueckner, 1999). Research will continue to be conducted on whey protein functionality as certain functional properties can be shaped and new properties are discovered (Huffman, 1996; Foegeding et al., 2006).

**FLAVOR**

**General**

Flavor is considered the single most important factor in determining the success or failure of a food product (Morr and Ha, 1991; Drake, 2007). In general, dairy products display a wholesome and flavorful image making them well accepted with consumers, however, the flavor of whey solids has been the primary factor preventing a more widespread usage of this functional ingredient (Drake, 2007; Mortenson et al., 2008). Dairy foods can possess two types of off-flavors, those that are already present in the milk and those that develop later as a consequence of actions, such as processing (Bodyfelt et al., 1988).
Whey proteins typically exhibit a bland flavor immediately after drying, but as they age, they develop aged, stale off-flavors which are highly undesirable (Morr and Ha, 1991; Bodyfelt et al., 1988). Drake et al. (2003) developed a descriptive sensory language for dried dairy ingredients. Using this language, cardboard, raisin, fatty, soapy, cucumber and cabbage flavors have been documented in fresh and stored whey proteins (Carunchia Whetstine et al., 2005; Russell et al., 2006; Drake, 2006; Wright et al., 2006; 2008). Whey and dried whey ingredients are expected to have a delicate and bland flavor that is undetectable in finished goods (Drake, 2006). Flavors that carry through to the finished product limit the utilization of whey protein as a desired ingredient.

Many different chemical compounds contribute to the overall flavor of fluid and dried whey, making the flavor very complex (Carunchia Whetstine et al., 2003, 2005; Drake et al., 2008). In addition, these chemical compounds are very hard to define and understand how they link to specific perceived flavors (Drake, 2004). Throughout the literature, there seems to be a consensus that lipid oxidation and Maillard browning reactions are responsible for many of these off-flavors (Morr and Ha, 1991; Carunchia Whetstine et al., 2005; Mortenson et al., 2008). If the flavor variability and the off-flavors were better understood, these could be minimized by process or product changes (Drake et al., 2008).

**Liquid Whey**

The flavor of dairy products in general are derived from complex mixtures of volatile compounds and have been identified in dairy products since the mid 1950’s with the application of gas chromatography to flavor analysis (McGorrin, 2007). Volatile compounds in fluid whey can be placed into two categories, aroma-active compounds and aliphatic...
hydrocarbons (Carunchia Whetstine et al., 2003). Aroma-active compounds would include alcohols, ketones, and aldehydes and generate a perceived aroma (Carunchia Whetstine et al., 2003). Since these compounds produce an aroma, they influence and contribute to the flavors as well as the off-flavors of whey often at extremely low concentrations (Bodyfelt et al., 1988). Some of the compounds commonly found in liquid whey include but are not limited to diacetyl, hexanal, dimethyl sulfide, and 1-octen-3-one (Carunchia Whetstine et al., 2003). On the other hand, aliphatic hydrocarbons do not have an odor and thus do not contribute to the flavor of whey as aroma-active compounds (Carunchia Whetstine et al., 2003).

Flavors found in whey proteins were grouped into two categories, dairy and nondairy flavors (Carunchia Whetstine et al., 2005; Drake, 2006). Dairy flavors included sweet aromatic, cooked, and milky flavors while nondairy flavors included those not associated with fluid milk such as cardboard, animal, or cucumber (Carunchia Whetstine et al., 2005).

Every 10 units of whole milk will yield 1 unit of cheese and 9 units of liquid whey (Tunick, 2008). There are two types of liquid whey: sweet and acid. Acid whey is from the production of cottage cheese by direct acidification while sweet whey is from rennet-coagulated cheeses such as Cheddar, Swiss, or Mozzarella (Onwulata, 2008). Differences in liquid whey flavor can be caused by milk source, processing and handling, and starter culture blend (Carunchia Whetstine et al., 2003). Fresh liquid whey is susceptible to deterioration during storage and has high transportation costs, thus liquid whey is primarily dried before use (Onwulata, 2008; Varnam and Sutherland, 1994). However, chemical reactions occurring
in liquid whey prior to the drying process could prove to be detrimental to the finished whey ingredient (Tomaino et al., 2004).

Volatile lipid oxidation products are considered the major source of off-flavors in both liquid and dried whey (Carunchia Whetstine et al., 2003, 2005; Wright et al., 2008). Although there is very little lipid in both liquid and dried whey, lipid oxidation is still a prevalent problem (Carunchia Whetstine et al., 2005). The reaction of oxygen with unsaturated fatty acids causes a wide variety of volatile compounds including methyl esters, ketones, aldehydes, and free fatty acids (Morr and Ha, 1991; Carunchia Whetstine et al., 2003, 2005). Lipid oxidation products can be formed during cheese making and thus it would not be surprising to find these lipid oxidation products bound to whey proteins in liquid whey (Carunchia Whetstine et al., 2003). If oxidative reactions occur during the transportation or storage of liquid whey products, the by-products will have a chance to bind with the proteins and be carried through processing to dried whey and ultimately impact flavor (Tomaino et al., 2004). It is believed that some of these compounds formed from lipid oxidation may actually promote the Maillard browning reaction (Morr and Ha, 1991).

Some volatile compounds are found in liquid whey but not in milk. These included 1-propanol, hexanal, nonanal, and 2-nonanone and these are common volatile lipid oxidation products (Tomaino et al., 2004). In determining the oxidative stability of liquid Cheddar whey, lipid oxidation occurred during cheese making and whey processing and continued to escalate throughout storage of the liquid whey (Tomaino et al., 2004). Among whey samples from the same variety of cheese, there were also differences in the number and potency of aroma active compounds (Karagül-Yüceer et al., 2003).
There is a wide range of flavor variability in liquid whey which can be traced all the way back to the farm. Flavor variability can exist not only between wheys from different cheeses, but also in wheys produced from the same type of cheese. Within a certain type of cheese, flavor variability can be attributed to milk source and starter culture rotation (Carunchia Whetstine et al., 2003). In the United States cows typically receive a mixed ration of feed rather than being pasture fed thus leading to a more consistent milk supply (Varnam and Sutherland, 1994). However, seasonal variability still exists within milk, primarily seen in the composition of milk fat, due to feed and stage of lactation (Varnam and Sutherland, 1994). Other factors that can affect milk composition include genetics such as breed or individual and physiological factors such as age of cow and illness (Varnam and Sutherland, 1994). In addition to the milk itself, other flavor variability could be attributed to the processing time and temperature of the milk (Bodyfelt et al., 1988; Carunchia Whetstine et al., 2003). Milks are a complex biological fluid and show great flavor variability and flavor deterioration over the shelf-life of the milk due to the aforementioned causes (Bodyfelt et al., 1988; Varnam and Sutherland, 1994). These differences can carry over into liquid whey and even into dried whey.

In addition to milk source, starter cultures influence the flavor and flavor variability of liquid whey (Carunchia Whetstine et al., 2003; Karagül-Yüceer et al., 2003; Tomaino et al., 2004). *Lactococcus lactic* starter cultures, used to produce Cheddar cheese, influenced flavor and oxidative stability of liquid whey (Tomaino et al., 2004). Starter culture strains are typically rotated in production to prevent problems with bacteriophage (Varnam and Sutherland, 1994). *Lactococcus* species can differ in lipase activity, proteolytic activity, and
the production of volatile compound amount and type (Carunchia Whetstine et al., 2003). Variability within wheys produced from the same plant and from the same type of cheese was found and thus the starter culture rotation for the same type of cheese can influence overall flavor and cause flavor variability of fluid whey (Carunchia Whetstine et al., 2003; Karagül-Yüceer et al., 2003).

In addition to flavor variability in whey within a specific type of cheese, as expected, there is also variability in wheys produced from different types of cheeses (Drake et al., 2008). Wheys made from different types of cheeses have different flavors (Drake et al., 2008; Carunchia Whetstine et al., 2003). Different cheese and casein production methods resulted in wheys that differed in chemical composition (Gallardo-Escamilla et al., 2005). Gallardo-Escamilla et al. (2005) reported that wheys exhibited a more specific “cheese-like character” similar to the variety of cheese from which they were produced. Thus starter cultures would influence the flavor of whey to different degrees. Flavor from thermophilic starters will differ from the flavor from mesophilic starter cultures and the flavor of whey from acid-set curd will differ even further, although few studies have directly quantitatively addressed this topic (Drake et al., 2008). Rennet wheys were described as bland, sweet, and milky, while acid casein wheys were described as bitter, stale, rancid, and chemical (Gallardo-Escamilla et al., 2005). Within acid wheys, sensory differences were perceived in directly acidified milk and whey and those from fermentation (Gallardo-Escamilla et al., 2005).
After the liquid whey is obtained, it is sometimes pooled before it is then further processed into whey powder or further processed into whey protein concentrate or isolate (Onwulata, 2008). Sweet whey powder is made by drying fluid whey from the production of Cheddar, Swiss, Mozarella, Monterey Jack or other rennet-coagulated cheeses and is typically 70% lactose, 1.5% fat, 12% proteins, 8.5% other solids, and 4% moisture (USDA, 2004). Sweet whey has a marketed shelf life of 6 to 12 months, but due to composition, sweet whey is very susceptible to develop off-flavors and aromas.

The variability in liquid whey carries over into sweet whey powder as the composition of whey powder also depends on the composition of raw milk and processing procedures for both cheese and whey production (Sithole et al., 2006a). Not only does the variability from the liquid whey carry over into the whey powder, but the extra processing steps could further introduce more variability and limit shelf-life (Sithole et al., 2004). Sithole et al. (2006) found that evaporation and drying of whey not only introduced but intensified new flavors.

Sweet whey powders were characterized by cooked, oxidized, barny, salty, and sour flavors (Sithole et al., 2006a). Mahajan et al. (2004) analyzed aroma-active compounds in sweet whey powder using gas chromatography/olfactometry (GCO) and gas chromatography/mass spectrometry (GCMS). Important aroma-active volatiles were short-chain fatty acids, aldehydes, ketones, lactones, sulfur compounds, phenols, indoles, pyrazines, furans, and pyrroles. Some of these compounds were derived from milk or formed during the cheese making process, but others were formed during whey powder
processing (Mahajan et al., 2006). Mahajan et al. (2004) further concluded that autooxidation of lipids, caramelization of sugar, and Maillard browning explained the generation of many aroma compounds, but more sensory work would be needed to validate that the identified compounds were responsible for the characteristic aroma of sweet whey powder.

As mentioned previously, sweet whey powders contain high concentrations of lactose, and in the presence of moisture, these components may easily participate in Maillard reactions (Sithole et al., 2005). Milk proteins, and subsequently whey proteins, contain lysine residues which is the amino acid that results in the most color development during the Maillard browning reaction (Rich and Foegeding, 2000). Sithole et al. (2005) reported that the rate of deterioration in sweet whey powders from 3 different commercial processors, under both accelerated shelf-life testing and normal storage conditions, was different and the Maillard reaction played a major role in deterioration. The Maillard browning reaction is also purportedly very important in the formation of stale off-flavors in dried whey products (Morr and Ha, 1991). However, more research is needed in this area as there are many studies with conflicting results such as Sithole et al. (2005) who reported that flavor and aroma of whey remained the same over the shelf-life while Javidipour and Qian (2008) reported that concentrations of volatile compounds increased with storage time. Factors that affect browning in dried whey products include temperature, water activity, pH, and availability of reactant (Morr and Ha, 1991). The Maillard reaction can be controlled by altering the composition of these dried whey products to have unfavorable conditions for this
reaction (Morr and Ha, 1991). This could be done by removing lactose from the dried whey powder (Morr and Ha, 1991).

Sweet whey powder is just one of the many dried whey products available on the market. Whey protein concentrate and whey protein isolate are two more available dried whey ingredients on the market currently but are more expensive than sweet whey powder. As mentioned previously, sweet whey contains a high percentage of lactose which could lead to off-flavors. Whey protein isolate has a higher protein content than whey protein concentrate and contains lower levels of fat and lactose. Whey powders, including sweet whey powder, whey protein isolate, and whey protein concentrate, undergo spray drying in order to form a powder and residual lactose can react with protein to form Maillard reaction products (Carunchia Whetstine et al., 2005). Some thermally generated compounds found in these ingredients include pyrrolines, pyrazines, and furanones (Carunchia Whetstine et al., 2005). In general, these thermally generated compounds cause cooked/milky and sweet aromatic flavors that are typical of dairy products (Carunchia Whetstine et al., 2005).

While lipid oxidation and the Maillard browning reaction are considered the two biggest factors in the production of off-flavors in whey products, there are other factors that may also contribute to undesirable flavors in whey. Proteolytic enzymes, such as chymosin, may carry over into the whey after the cheese making process and promote the degradation of amino acids (Drake et al., 2008; Varnam and Sutherland, 1994). Since the amino acids vary in type and concentration from one whey to the next, this may be a source of flavor variability in whey products (Carunchia Whetstine et al., 2005). In addition, proteins may
bind volatile flavors during processing, which could result in an undesirable flavor in the finished good (Drake et al., 2008; Varnam and Sutherland, 1994).

In looking at the differences between whey protein concentrate and whey protein isolate, isolates generally have less flavor, therefore exhibiting a “better” and higher flavor quality (Mortenson et al., 2008, Morr and Ha, 1991; Carunchia Whetstine et al., 2005). Carunchia Whetstine et al. (2005) examined both whey protein isolate and whey protein concentrate from various types of cheeses as well as various manufacturers. They reported compositional differences between whey protein concentrate and whey protein isolate, and although statistically different in composition, there were no consistent differences between products made from Cheddar and Mozzarella whey (Carunchia Whetstine et al., 2005). The main compositional difference was the mineral content, and protein powders from the same manufacturing site showed similar compositions. These similarities were due to filtration and processing procedures, which may vary from plant to plant (Carunchia Whetstine et al., 2005).

While flavor variability still presents major concern, generally, whey protein isolates exhibited lower flavor intensities than whey protein concentrates and fewer aroma-active lipid oxidation compounds were found in whey protein isolates compared to whey protein concentrates (Carunchia Whetstine et al., 2005; Russell et al., 2006). The cleaner profile of whey protein isolates could be due to the fact that WPI contains less fat and less lactose than WPC, which could lead to reduced Maillard reactions and reduced lipid oxidation product formation (Mortenson et al., 2008). Mortenson et al. (2008) conducted a study comparing WPC (35%) and WPI to examine their differences in flavor by using sensory and
chromatographic data. They hypothesized that there would be differences among dried WPC and WPI due to cheese type and processing methodology. GCO and GCMS data showed that comparing the same type whey, WPC had more compounds identified than WPI possibly due to higher lactose and fat content leading to more Maillard browning and lipid oxidation product formation, respectively. In addition, since whey protein concentrate contains a higher mineral content, this could correspond to the production of more aroma active compounds (Carunchia Whetstine et al., 2005; Mortenson et al., 2008). Varying mineral content may be due to differences in type of wheys, such as Cheddar whey as compared to Mozzarella whey, or due to differences in filtration and processing procedures (Carunchia Whetstine et al., 2005).

Carunchia Whetstine et al. (2005) compared the flavor of WPC80 and WPI using instrumental and sensory techniques and found that although WPC80 contains more aroma-active compounds it did not have corresponding higher flavor intensities or more flavors than WPI. It was speculated that since proteins can bind or trap volatile compounds and the higher protein content of WPI might explain why there are more aroma-active compounds found in WPC80 than WPI (Carunchia Whetstine et al., 2005). In contrast, Mortenson et al. (2008) speculated that the volatile losses are likely due to the extra processing step required to produce whey protein isolate rather than protein binding.

General trends can be observed from looking at many studies to determine flavor and aroma characteristics of whey, but is should be noted that whey protein products have great variability and may cause inconsistent results. Overall, whey protein isolates and concentrates have similar flavor profiles with a few notable differences. Carunchia
Whetstine et al. (2005) observed key differences and similarities in WPC as opposed to WPI using both instrumental and sensory techniques and observed that whey protein isolates had soapy, animal, cucumber, and bitter characteristics while whey protein concentrates did not. Also, delicate dairy flavors such as sweet aromatic, cooked, or milky were generally not present in whey protein isolate but present in whey protein concentrates (Carunchia Whetstine et al., 2005; Mortenson et al., 2008; Wright et al., 2008). Animal flavors were not observed in WPC but were found in several of the WPI, likely caused by protein degradation (Carunchia Whetstine et al., 2005). Bitterness was also only observed in WPI and was associated with proteolysis as the potential for bitterness rises with protein content (Carunchia Whetstine et al., 2005). In contrast to previous studies (Mortenson et al., 2008; Russell et al. 2006; Morr and Ha, 1991), Carunchia Whetstine et al. (2005) reported that after sensory evaluation, WPC 80 does not have a “better flavor quality” than WPI.

**STORAGE AND Istantization**

After spray drying, whey proteins may be agglomerated to enhance functional properties (Drake et al., 2008; Varnam and Sutherland, 1994). This additional process step does require added heat to some or the entire product, such as added steam, and may cause small changes in the initial flavor of these products (Walstra et al., 1999). Agglomerated whey proteins exhibit increased dispersibility and decreased dispersion time which may be highly desirable in some applications (Walstra et al., 1999). Products that are agglomerated with lecithin are generally referred to as instantized whey protein powders (Walstra et al., 1999). Lecithin may be an additional source of flavor and instantized products may exhibit sensory and volatile changes earlier than their non- instantized counterparts thus decreasing
their shelf life (Drake et al., 2008; Wright et al., 2008). The unsaturated fatty acids in soybean oil may contribute to the formation of hexanal which is a major oxidation product in whey protein (Walstra et al., 1999).

Javidipour and Qian (2008) analyzed WPC 80, both instantized and non-instantized from a single manufacturer, under accelerated storage. The samples were stored at 35, 40, and 45°C in closed glass jars in a dark conventional oven for 15 weeks. They reported concentrations of volatile compounds increased with both storage time and temperature. In addition, instantized whey protein concentrate had higher off-flavor formation than regular whey protein concentrate across all temperatures in the study. The off-flavor formation was due to higher lipid oxidation compounds present in instantized whey protein concentrate (Javidipour and Qian, 2008). Javidipour and Qian (2008) suggested that the addition of industrial lecithin would further contribute to the formation of hexanal as it may contain soybean oil.

Wright et al. (2008) examined the impact of agglomeration on flavor and flavor stability of commercial WPC 80 and WPI. Samples were collected from different facilities and distinct sensory and instrumental volatile compound differences were documented across 18 months of storage stored at 21°C and 50% relative humidity. Within each facility, there were differences in initial flavor profiles of both instantized and non-instantized whey products. These initial differences between instantized and non-instantized products also translated to differences in shelf stability of these products. Instantized products exhibited more rapid sensory and volatile changes than non-instantized products thus indicating a
limitation in shelf life consistent with results from Javidipour and Qian (2008). Volatile analysis confirmed sensory results.
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CHAPTER 2: THE IMPACT OF STARTER CULTURE AND ANNATTO ON THE FLAVOR AND FUNCTIONALITY OF WHEY PROTEIN CONCENTRATE
The Impact of Starter Culture and Annatto on the Flavor and Functionality of Whey Protein Concentrate

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ABSTRACT

The flavor of whey protein can carry through into ingredient applications and negatively influence consumer acceptance. Understanding sources of flavors in whey protein is crucial in order to minimize flavor. The objective of this study was to evaluate the impact of annatto color and starter culture on the flavor and functionality of whey protein concentrate (WPC). Cheddar cheese whey with and without annatto (15 mL annatto /454 kg milk, annatto with 3% w/v norbixin content) was manufactured using a mesophilic lactic starter culture or by addition of lactic acid and rennet (rennet set). Pasteurized fat-separated whey was then ultrafiltered and spray dried into WPC. The experiment was replicated 4 times. Flavor of liquid wheys and WPC was evaluated by sensory and instrumental volatile analyses. In addition to the evaluation of flavor on WPC samples, color analysis ((Hunter Lab and norbixin extraction) and functionality tests (solubility and heat stability) were also performed. Both main effects (annatto, starter) and interactions were investigated. No differences in sensory properties or functionality were observed among WPC. Lipid oxidation compounds were higher in WPC manufactured from whey with starter culture compared to WPC from rennet-set whey. WPC with annatto had higher concentrations of p-xylene, diacetyl, pentanal, and decanal compared to WPC without annatto. Interactions were observed between starter and annatto for hexanal, suggesting that annatto may have an antioxidant effect when present in whey made with starter culture. Results suggest that annatto has a no impact on whey protein flavor but that the starter culture has a large influence on the oxidative stability of whey.

Key Words: whey protein, flavor, starter culture, annatto
INTRODUCTION

Whey and dried whey ingredients are expected to have a delicate and bland flavor that is undetectable in finished goods (Drake, 2006). Flavors that carry through to the finished product limit the utilization of whey protein as a desired ingredient. Factors that influence the flavor of fluid whey and whey protein include cheese type (Liaw et al., 2010), storage conditions of fluid and dried products (Drake et al., 2009; Wright et al., 2009; Liaw et al., 2010), variability among processing plants (Carunchia Whetstine et al., 2003), and bleaching (Croissant et al., 2009). A combination of consumer demand for yellow Cheddar cheese and a manufacturer ingredient demand for bland, neutral-colored WPC makes bleaching a necessary step during dried whey ingredient production (Croissant et al., 2009; Kang et al., 2010). While there is limited research on the influence of bleaching on WPC flavor, there is no research on the influence of annatto itself on WPC flavor. Annatto, comprised of the carotenoids bixin and norbixin, is a natural food colorant used to color Cheddar cheese. One previous study has been conducted on the volatile composition of annatto, but this was only conducted on the extracts themselves and not in a food matrix, such as whey (Galindo-Cuspinera et al., 2002).

Whey products made from different types of cheeses have different flavors (Drake et al., 2009; Carunchia Whetstine et al., 2003). Flavor from thermophilic starters differs from the flavor of whey mesophilic starter cultures and the flavor of whey from acid-set curd differs even further, although few studies have directly quantitatively addressed this topic (Drake et al., 2009). Whey produced from rennet-set cheese were described as bland, sweet, and milky, while whey from acid casein was described as bitter, stale, rancid, and chemical.
Within acid whey products, sensory differences were perceived in directly acidified milk and whey and those from fermentation (Gallardo-Escamilla et al., 2005). \textit{Lactococcus lactis} starter cultures, used to produce Cheddar cheese, influenced flavor and oxidative stability of liquid whey compared to whey manufactured using direct acidification (Tomaino et al., 2004). Wright et al. (2009) documented distinct flavors and volatile compounds in Mozzarella WPC 80% protein (WPC80) compared to Cheddar WPC80 from different commercial sources. Liaw et al. (2010) confirmed that distinct volatile compounds were observed between Cheddar and Mozzarella liquid whey from the same milk source. The influence of starter culture and annatto on the flavor and functionality of WPC has not been evaluated. The objective of this study was to evaluate the impact of annatto color and starter culture on flavor and functionality of whey protein concentrate (WPC).

**METHODS**

**Experimental design overview**

A 2x2 factorial design (starter, no starter, annatto, no annatto) was employed from the same lot of pasteurized milk to produce all four treatments. The order of production was randomized and production took place over 2 days. Cheddar cheese whey without and without annatto was manufactured using a mesophilic lactic starter culture or by addition of acid and rennet. Pasteurized fat-separated whey was then ultrafiltered and spray dried into WPC with 62% protein. The experiment was replicated 4 times. Flavor of liquid wheys was evaluated by sensory and instrumental volatile analysis. Retentate, prior to spray drying, was subjected to instrumental volatile compound analysis. Sensory and instrumental analyses,
color analysis (Hunter Lab and norbixin) and functionality tests (solubility and heat stability) were performed on WPC.

**Production of WPC**

Whole raw bovine milk, 390 kg, was received from the North Carolina State University Dairy Education Unit, Raleigh, NC. Milk was pasteurized at 63°C for 30 min in a pasteurization vat (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC). Milk was cooled and refrigerated at 4°C overnight for processing the next day. Four treatments of WPC (culture with annatto, culture without annatto, acid with annatto, acid without annatto) were manufactured across a 2 day period from this lot of milk.

The pasteurized milk was heated to 31°C in a 250 kg cheese vat (Model MX4, Kusel Equipment Company, Watertown, WI). For treatments with added starter culture, the milk was inoculated with a freeze dried lactic acid starter culture (50 DCU/454 kg, Choozit MA 11, Danisco, New Century, NJ). Next, calcium chloride solution (50 % (w/v), Dairy Connection Inc., Madison, WI) was added at the rate of 0.39 mL/kg of milk. The milk was agitated and allowed to ripen for 60 min. After 30 min of ripening, double strength annatto color (3% norbixin w/v, Danisco, New Century, NJ) was added at 15 mL/454 kg milk and diluted 20 times in deionized (DI) water if the treatment called for the addition of annatto. Samples were allowed to finish ripening and if the treatment did not contain culture, the pH was adjusted to 6.40 using a 1:10 dilution of lactic acid (85% w/v) (Fisher Scientific, Pittsburgh, PA) and DI water. Then, the milk was coagulated with double strength recombinant rennet (Dairy Connection Inc., Madison, WI) for 30 min at a rate of 0.09 mL/kg of milk diluted 80 times in DI water. The coagulum was cut with 0.95-cm wire knives, and
the curd and whey were allowed to rest for 5 min followed by gentle stirring for 10 min without added heat. The temperature was increased gradually from 31 to 39°C over 30 min while pH and titratable acidity were closely monitored. The curd was continuously stirred at 39°C until the target whey drain pH of 6.40 was attained. As the whey was drained, a sieve was used to remove any remaining particles and the whey was immediately pasteurized at 63°C for 30 min. The hot whey was immediately processed with a cream separator (Model FJ 125 EAR hot-bowl cream separator, Clair, Althofen, Austria) to reduce the fat content. Total percent solids and percent fat content of fluid milk and whey were analyzed using the Smart System 5 moisture/solids analyzer with SmartTrac rapid fat analysis (CEM, Matthews, NC). Percent solids measurements were also recorded with the CEM apparatus.

Pasteurized, separated whey was weighed and placed in 60 L stainless steel containers (Model 601BP Polar Ware Company, Kiel, WI). The whey was then concentrated using an ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica, MA) equipped with three polyethersulfone cartridge membrane filters (Model P2B010V05, nominal separation cutoff: 10,000 kDa, surface area: 0.5 m²). A variable speed peristaltic pump (Model 77410-10) equipped with Model 77601-00 pumpheads with silicone tubing (Model 96440-73) was used to circulate the product. Pumps, pumpheads, and tubing were obtained from Cole-Palmer (Vernon Hills, IL). Each sample was run through a separate pump head and UF assembly. Samples were maintained at 50°C using a circular immersion heater (Model 356K05K73, McMaster-Carr, Atlanta, GA) while being processed on the UF system. Permeate was collected and the UF process continued until 20-22% solids (w/v) was reached. The weight of both the permeate and the retentate was recorded. The total UF time
varied from 3 to 6 h. The final liquid UF retentate was then spray dried (Model Lab 1, Anhydro Inc., Soeberg, Denmark). The total time for drying was approximately 2 h. Powders were sealed in mylar bags and stored at -80°C. This experiment was replicated four times.

**Composition Analysis of WPC**

Total solids of WPC were determined by performing air oven drying (AOAC, 2000; method number 990.20; 33.2.44). Fat was quantified by ether extraction (AOAC, 2000; method number 989.05; 33.2.26). Protein was determined using the Kjeldahl method (AOAC, 2000; method number 991.20; 33.2.11). Mineral analysis (phosphorus, calcium, magnesium, potassium, sulfur, sodium, and iron) was determined by the NCSU Analytical Services Laboratory (Raleigh, NC) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd et al., 2009). All samples were measured in duplicate.

**Annatto Extraction**

Annatto extraction methods were modified from Croissant et al. (2009). One gram of dried whey protein was weighed into a 50 mL centrifuge tube (Nalgene, Rochester, NY) and 2 mL HPLC grade water (EMD Chemicals Inc., Gibbstown, NJ) was added. This sample was then vortexed for 30 sec. To this, 6 mL ethanol (EMD Chemicals Inc.) was added, vortexed for 30 sec and allowed to stand for 30 min. Three mL chloroform (EMD Chemicals Inc.) was added and vortexed with centrifugation at $16,500 \times g$ for 10 min at 4°C (Model RC5B, Thermo Scientific, Waltham, MA). The supernatant was removed to a separate centrifuge tube. To the remaining solids, 3 mL of chloroform was added, the sample was
vortexed to ensure mixing, and centrifuged again at $16,500 \times g$ for 10 min at 4°C. The bottom liquid layer was collected and added to the previous supernatant collected. Next, 2 mL of acetic acid (1%) were added, vortexed for 30 sec and centrifuged at $16,500 \times g$ for 10 min. The bottom chloroform layer containing the norbixin was collected and the volume was measured. The extraction procedure and measurements were performed with premium full spectrum F885 flat sheet filters covering all lights (Ergomart, Dallas, TX) to minimize norbixin isomerization and degradation (Mercadante, 2008).

In order to further filter and purify the norbixin, solid phase extraction (SPE) was utilized. The column selected was the Strata-NH$_2$ SPE column (500 mg/3 mL, Phenomenex, Torrance, CA). To condition the column, 7 mL of $n$-hexane (VWR International, West Chester, PA) was run through the column. An aliquot of the extract collected previously (1 mL) was transferred onto the conditioned SPE column. The column was rinsed with 5 mL $n$-hexane:diethyl ether (1:1 (v/v)) and 1 mL acetone (VWR International). The norbixin was eluted with 3 mL methanol:glacial acetic acid (7:3 (v/v)). Final volume was recorded.

**Spectrophotometry**

To quantify norbixin, spectrophotometry was used (UV/Vis-spectrophotometer Cary 300 Bio, Varian, Cary, NC) (Croissant et al., 2009). A 0.7 mL aliquot of each sample was measured in a 28Q10 Spectrosil quartz cuvette (Starna Cells Inc., Atascadero, CA). A standard curve was created within the concentration range of 50 ppb to 3 ppm norbixin. Norbixin powder (45% w/w, Chr Hansen, Milwaukee, WI) was rehydrated in 2.5% potassium hydroxide then diluted in methanol:glacial acetic acid (7:3 (v/v)). Carotenoids generally have three peaks of absorption maxima (Levy and Rivadeneira, 2000). The
maxima used for calculation was 458 nm. The carotenoid concentration of the SPE extract was calculated using the standard curve. Norbixin concentration was expressed as mg norbixin per kg solids, calculated by total solids and correction for dilution during the extraction and SPE processes.

**Hunter L*a*b***

Color of WPC powders was measured using a Minolta Chroma meter (CR-410, Ramsey, NJ). Ten grams of WPC was placed into the bottom of a 60 mm x 15 mm polystyrene petri dish in duplicate (Beckton Dickinson, Franklin Lakes, NJ). Each petri dish was measured in triplicate. Prior to measurements being taken, a factory-supplied calibration plate was used to calibrate the instrument. The Hunter CIE Lab color scale was used.

**Volatile compound analysis (solid phase microextraction gas chromatography mass spectrometry)**

Volatile compounds in fluid whey, retentate, and whey protein powder were extracted by solid phase microextraction (SPME). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using a modified method of Wright et al. (2006). Liquid samples were tested the day of manufacture and spray dried powders were reconstituted at 10 % solids (w/v) and evaluated within 7 days. All samples contained 10 % (w/v) sodium chloride (Fischer Scientific), and 10 ul internal standard solution (2-methyl-3-heptanone in methanol at 81 ppm (Sigma Aldrich, Milwaukee, WI)) in 20 ml autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA). Samples were injected using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890N GC with 5973 inert MSD (Agilent
Technologies Inc., Santa Clara, CA). Samples were maintained at 5°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before 30 min fiber exposure of a 1 cm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA) at 31 mm with 4 sec pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm.

The GC method used an initial temperature of 40°C for 3 min with a ramp rate of 10°C/min to 250°C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250°C. An Zb-5ms column (Zb-5ms 30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) was used for all analyses at a constant flow rate of 1 ml/min. Purge time was set at 1 min. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 250°C. Compounds were identified using the NIST 2005 library of spectra and comparison of spectra of authentic standards injected under identical conditions. Relative abundance for each compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound. Retention indices were calculated using an alkane series (Sigma Aldrich) (Van den Dool and Kratz, 1963).

Volatile free fatty acid analysis

Volatile free fatty acids in WPC were extracted and evaluated by solid phase microextraction with gas chromatography using a modified method from Tomaino et al. (2004). Samples were rehydrated at 10% (w/v) in deionized water and the pH was adjusted to 2.0 with 3.3 M hydrochloric acid (20 % v/v, VWR). Five grams of the pH adjusted sample was added to a 20 ml autosampler vial with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical). An internal standard, heptadecanoic acid (1 ppm in ether
(EMD Chemicals), was added at a rate of 5 μl to each vial. Each sample was run in triplicate. Samples were injected using a CombiPal autosampler (CTC Analytics) attached to a Varian CP-3380 gas chromatograph (Agilent Technologies). Samples were maintained at 10°C prior to fiber exposure. Samples were equilibrated at 110°C for 10 min before 40 min fiber exposure (22 mm vial penetration) of a 30 μm polydimethylsiloxane fiber (PDMS) (Supelco) with 4 sec pulsed agitation at 250 rpm. Fibers were injected for 10 min at a depth of 50 mm.

The GC method used an initial temperature of 100°C for 2 min with a ramp rate of 10°C/min to 245°C held for 13.5 min. Helium flow rate was 1ml/min and SPME fibers were introduced into the split/splitless injector at 250°C. A ZB-FFAP column (ZB-FFAP 30 m length × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex) was used for all analyses. Compounds were identified using authentic standards injected under identical conditions. Relative abundance for each compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound.

**Descriptive sensory analysis**

Sensory testing was performed using a trained descriptive sensory panel and an established dairy flavor language (Drake et al., 2003, 2009). Panelists (n = 8) each had more than 150 h of previous experience with the sensory analysis of fluid and dried whey products using the Spectrum™ descriptive analysis method (Meilgaard et al., 1999). All sensory testing was conducted in accordance with the NCSU Institutional Review Board for Human Subjects guidelines.
Liquid whey and reconstituted WPC (10% solids) were both evaluated by placing 30 mL in randomized three-digit-coded, 60 mL lidded cups (Solo Cup Company, Champaign, IL). Preparations were conducted with overhead lights off to avoid exposure to light. Liquid whey samples were evaluated immediately after fat separation on the day of production at 21 ± 2°C. Whey powders were rehydrated in deionized water (10% solids [w/v]) and evaluated within 7 days of production. Samples were evaluated by each panelist in duplicate. Sensory data were collected on paper ballots or using Compusense™ 5.0 (Compusense, Guelph, Canada).

**Solubility**

Solubility was determined by established methods (Bouaouina et al., 2006; Smith et al., 1985) with modifications. Whey protein concentrate (WPC) (10% w/v protein) was hydrated in deionized water (80% of total volume) with stirring (200 rpm) for 6 h at room temperature (22±2°C). The solutions were kept overnight at refrigeration temperature (4°C) and brought up to room temperature the next day. Once at room temperature, the solutions were adjusted to a pH of 4.6 (±.05) with 1N HCl (20% v/v, VWR). The solutions were then adjusted to their final volume. The solutions were then centrifuged at 10,000 x g for 15 min. Solutions were analyzed prior to and after centrifugation (supernatant) by the Sprint™ Protein Analyzer (CEM, Matthews, NC). Protein solubility was calculated as follows:

Protein solubility = 100-((% Protein Before - % Protein After/ % Protein Before)*100). Each sample was evaluated in duplicate and all measurements were also performed in duplicate.
Heat Stability

Heat stability was measured using a method modified from Britten et al. (1994). Whey protein concentrate (WPC) (5% w/v protein) was hydrated in deionized water (80% of total volume) with stirring (200 rpm) for 6 h at room temperature (22±2°C). The solutions were kept overnight at refrigeration temperatures (4°C) and brought up to room temperature the next day. Once at room temperature, the solutions were adjusted to a pH of 3.5 (±.05) with 1N HCl (20 % v/v, VWR). The solutions were then adjusted to their final volume. Solutions were heated to 80°C for 5 or 20 min. After heating, the solutions were centrifuged at 10,000 x g for 15 min. Solutions were analyzed prior to and after centrifugation (supernatant) by the Sprint™ Protein Analyzer (CEM, Matthews, NC). Heat stability was determined by protein solubility before and after heating. Each sample was evaluated in duplicate and all measurements were also performed in duplicate.

Statistical Analysis

All data was analyzed by a 2-way analysis of variance (ANOVA) using a general linear model (PROC GLM) in SAS (SAS Statistical Analysis Software, version 9.1, SAS Institute, Cary, NC). Both main effects and interaction effects were analyzed. Fixed effects were set type (culture vs. acid set) and annatto (with and without) and their interactions. Replication effects and all interactions with replication effects were designated random effects.
RESULTS AND DISCUSSION

Composition

WPC powders were not significantly different in composition (p>0.05) with fat averaging 4.8 ± 2.0%, protein 62.7 ± 4.0%, and moisture 3.2 ± 1.5%. Minerals were also not distinct among samples (p>0.05). Minerals measured included phosphorus (0.58±0.01%), calcium (0.83±0.02%), magnesium (0.08±0.00%), potassium (1.10±0.02%), sulfur (0.75±0.01%), sodium (2.9±0.10ppm), and iron (10.0±2.00ppm).

Annatto Extraction

Samples with annatto had higher (p<0.05) norbixin content than those which did not contain any added color (14.9 vs 1.7 ppm). WPC without annatto added also had low norbixin values, probably due to other co-extracted carotenoids that naturally occur in milk (Croissant et al., 2007). The culture set WPC had higher norbixin values (p<0.05) than WPC produced without culture (8.7 vs 7.9 ppm). This may be because rather than the pH being slowly lowered by lactic acid bacteria like the culture-set treatment, the rennet-set treatment received one single dose of diluted lactic acid to lower the pH which may have lead to the destruction of some of the annatto (Scotter, 2009). It is also possible that enzymatic activity from the culture may have protected the annatto from degradation in the whey by creating compounds that are more readily oxidized than annatto. Currently it is unknown if annatto binds to components in fluid whey.

Sensory and volatile compound analyses

Lipid oxidation is the primary reason for evolution of storage-related flavors in whey protein products, and lipid oxidation products tend to have very low flavor thresholds and are
thus easily detected in dairy products (Frankel, 1998; Wright et al., 2009; Whitson et al., 2010). Lipid oxidation products are present in freshly manufactured dried whey proteins (Javidipour and Qian, 2008; Wright et al., 2009). Tomaino et al. (2004) and Liaw et al. (2010) demonstrated that lipid oxidation products were present in fluid whey and suggested that lipid oxidation was initiated during the cheesemake procedure and that starter cultures initiated or enhanced this process. Previously, sensory profiles of fresh fluid Cheddar and Mozzarella wheys were found to be distinct from each other with Cheddar whey containing higher amounts of lipid oxidation products (Liaw et al., 2010). The sensory attributes of fresh liquid wheys in the current study with and without starter culture or with and without added annatto (less than 2 h after manufacture) were not different (p>0.05). Wheys were characterized by sweet aromatic, cooked/milky, and diacetyl flavors and sweet and sour tastes, similar to previous studies (Liaw et al., 2010, results not shown). Sensory properties of the freshly manufactured WPC were also not distinct (p>0.05). WPC were characterized by sweet aromatic and cooked/milky flavors with low cardboard flavor intensities and low astringency, also consistent with previous results (Croissant et al., 2009; results not shown).

Volatile compound analysis of the fresh liquid wheys revealed differences in only two compounds. Limonene and alpha pinene were higher in fluid wheys made with annatto compared to those made without annatto with mean values averaging 0.028 vs. 0.009 ppb and 0.021 vs 0.005 ppb, respectively (p<0.05). These two compounds are likely due to the presence of annatto and have been previously documented in commercial annatto extracts (Galindo-Cuspinera et al., 2002). Volatile compound analysis was also conducted on the liquid whey retentate prior to spray drying with solids adjusted to 10% (w/v), and no
differences were documented among the treatments (p>0.05) nor were differences detected between liquid whey and liquid whey retentate (p>0.05) except for diacetyl. Liquid whey contained higher concentrations of diacetyl (p < 0.05) than retentate regardless of annatto or starter culture with means averaging 1.26 vs. 0.603 ppb, respectively. This result may be attributed to the polarity of diacetyl, diacetyl is relatively polar and most likely washes out with the permeate during UF. Lipid oxidation products were present in all liquid wheys and retentates at very low levels (<0.5 parts per billion) consistent with previous research suggesting that lipid oxidation is initiated during the cheesemake process (Tomaino et al., 2004; Liaw et al., 2010).

In contrast to liquid whey and retentate results, differences were documented in volatile compounds of WPC (p < 0.05). Volatile lipid oxidation products were present in all WPC (Figure 1) and these products were present in higher amounts than documented in either the fluid whey or retentate prior to spray drying (p<0.05). WPC made from starter culture whey (with or without annatto) had higher concentrations of hexanal than WPC from rennet set whey (with or without annatto) (22.2 versus 11.2 ppb, p<0.05) as well as higher concentrations of 1-octen-3-ol and 2-nonanone (0.779 ppb and 2.71 ppb versus 0.543 ppb and 1.56 ppb, p<0.05). Similarly, aldehydes, such as butanal, heptanal, and decanal were also higher (p<0.05) in relative abundance in the starter culture WPC compared to the rennet-set WPC (Table 1, Figure 1). Liaw et al. (2010) suggested that lipid oxidation products were dependent on culture type with mesophilic starters producing more oxidation products than thermophilic starters.
WPC with added annatto contained higher concentrations of decanal, p-xylene, 2-butane, and pentanal than their uncolored counterparts (p>0.05) (Figure 1). Previously, p-xylene has been documented as a volatile from annatto (Galindo-Cuspinera et al., 2002). No study to our knowledge has been conducted to document annatto-related volatiles in a food matrix and it is likely that decanal, 2-butane, and pentanal, while lipid oxidation products, are present at higher concentrations in products with annatto due to the influence of annatto on the lipid oxidation process in WPC. WPC from starter culture whey with added annatto had lower concentrations (p<0.05) of lipid oxidation products than the WPC from starter culture whey without added annatto suggesting possible antioxidant activity from the annatto (Figure 1). Antioxidant effects of carotenoids are thought to be mainly from their ability to scavenge free radicals and efficiently quench singlet oxygen. Studies conducted on the antioxidant activity of annatto have been primarily conducted on bixin, with only one paper exploring norbixin (Kiokias and Gordon, 2003). Norbixin has been previously documented to retard oxidative deterioration of lipids in oil-in-water emulsions (Kiokias and Gordon, 2003).

**Volatile Free Fatty Acids**

WPC made with culture and annatto had the highest levels of volatile free fatty acids (p<0.05) (Figure 2). The other three samples were similar to each other (p>0.05) and were characterized by lower levels of volatile free fatty acids. Free fatty acids are present in most biological systems including milk in very small amounts (Cadwallader et al., 2007). Additional free fatty acids can come from triglycerides which have undergone lipolysis. Lipases can originate from a variety of sources included the milk, rennet paste, starter
bacteria, secondary starter microorganisms, non-starter lactic acid bacteria, and exogenous lipase preparations (Collins et al., 2004). Free fatty acids break down to form volatile lipid oxidation compounds such as methyl ketones, lactones, esters, alkanes, secondary alcohols and aldehydes (Collins et al., 2004). Previous studies as well as the current study suggest that the mesophilic starter culture is initiating oxidation and this process initiates with generation of free fatty acids. It is likely that the culture set sample made with annatto had higher levels of free fatty acids because they have yet to break down into other volatiles due to the antioxidant effect of annatto.

**Functionality**

No significant differences due to either addition of culture or annatto (p>0.05) were found in the solubility or the heat stability of WPC. Solubility is a prerequisite for other functional properties, such as foaming, emulsifying, and gelling (deWit and Klarenbeek, 1984) and complete solubility is necessary for optimum functionality. All WPC had excellent solubility (>95%) at the pH (4.6) tested. This pH is near the isoelectric point, where the net charge of the protein is minimal, thus protein interactions are favored resulting in minimum solubility (Jayaprakasha and Brueckner, 1999). When whey proteins are heated above 70°C, they are susceptible to thermal denaturation. This can cause partial loss of solubility between pH 3 and 5 due to protein aggregation and precipitation (Jayaprakasha and Brueckner, 1999). Neither culture nor annatto had any effect on the heat stability of the WPC in this study (p>0.05).
CONCLUSIONS

Dried whey protein, such as whey protein concentrate (WPC) or whey protein isolate (WPI) are desired to be flavorless, however this is rarely the case. Flavor and flavor variability can influence not only the quality of the product, but also consumer acceptance. This study investigated whether culture or annatto had an effect on functional or sensory properties of WPC and investigated lipid oxidation in the cheesemake process. Neither culture nor annatto had an effect on the functional properties of WPC (p>0.05). Descriptive analysis of both WPC and fresh liquid whey did not indicate differences between samples (p>0.05). Volatile compound analysis results confirmed that lipid oxidation products were present in all samples, even the fresh liquid whey, indicating that lipid oxidation begins during the cheese make process. Lipid oxidation compounds were higher (p<0.05) in WPC manufactured from whey with starter culture compared to WPC from rennet-set whey. WPC with annatto had higher concentrations of p-xylene, diacetyl, pentanal, and decanal (p<0.05) compared to WPC without annatto. Interactions (p<0.05) were observed between starter and annatto for hexanal, suggesting that annatto may have an antioxidant effect when present in whey made with starter culture. Further studies are needed to fully understand how to minimize lipid oxidation products in liquid whey.

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Barbano and Brandon Nelson are gratefully acknowledged. The use of tradenames does not imply endorsement nor lack of endorsement by those not mentioned.
REFERENCES


### TABLES AND FIGURES

**Table 1. Relative abundance (PPB) of selected aldehydes in WPC62**

<table>
<thead>
<tr>
<th>Category</th>
<th>Acid*Annatto</th>
<th>Acid* No Annatto</th>
<th>Culture*Annatto</th>
<th>Culture* No Annatto</th>
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<tr>
<td>Butanal</td>
<td>0.668&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.362&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.29&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.21&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>Pentanal</td>
<td>0.215&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.236&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.164&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.659&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Hexanal</td>
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<td>9.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heptanal</td>
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<td>1.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nonanal</td>
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<td>0.254&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a–b</sup>Means in a row not sharing a common superscript are different (<i>P</i> < 0.05).
Figure 1. Principle component biplot of selected volatiles in WPC extracted by solid phase microextraction (1: Acid No Annatto, 2: Acid With Annatto, 3: Culture With Annatto, 4: Culture No Annatto)
Figure 2. Principle component biplot of volatile free fatty acids from WPC (1: Acid No Annatto, 2: Acid With Annatto, 3: Culture With Annatto, 4: Culture No Annatto)
CHAPTER 3: THE EFFECT OF STARTER CULTURE AND STORAGE ON THE FLAVOR OF LIQUID WHEY
The Effect of Starter Culture and Storage on the Flavor of Liquid Whey

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ABSTRACT

Dried whey proteins are a multi-category food ingredient with a desired bland and delicate flavor. The primary off flavors found in dried whey proteins have been attributed to lipid oxidation products. A deeper understanding of what or when lipid oxidation initiates in fluid whey is crucial to understand how to minimize off flavors in dried whey protein. The objectives of this study were to determine when lipid oxidation initiated during the cheese make process and to further elucidate the role of starter cultures as sources of lipid oxidation in whey. Fluid Cheddar, Mozzarella and rennet-set wheys were manufactured from skim and whole milk. Liquid wheys and milks that wheys were manufactured from were evaluated by descriptive sensory and volatile instrumental analysis within 2 h of manufacture and following storage for 3 days at 4°C in the dark. Culture type greatly influenced the oxidative stability of liquid whey, with Cheddar and Mozzarella whey differing not only in sensory profile, but also in volatile compounds. The type of starter culture (Mozzarella vs. Cheddar) had more influence on flavor than the set type (acid vs. culture). Milks had lower relative abundances of volatile free fatty acids than their liquid whey counterparts and consequently higher relative abundances of lipid oxidation products than their whey counterparts. Whole and skim milks had more fat than their fluid wheys and whey from whole milk had more fat than whey from skim milk. Volatile lipid oxidation products in wheys were higher than in their respective milks, but oxidation in both milks and wheys proceeded with storage time. Wheys from Cheddar starters displayed more oxidation products than wheys Mozzarella starters. Starter media did not have an effect on the flavor and oxidative stability of liquid whey, however, culture strain influenced lipid oxidation of fluid whey.
Key Words: whey, flavor, lipid oxidation, culture
INTRODUCTION

Whey proteins are an excellent way to fortify foods with protein and to add nutritional value to foods (Tunik, 2008). In addition, whey proteins provide various functions in foods such as gelation, thermal stability, foam formation, and emulsification (Foegeding et al., 2002). A limitation in the usage of whey protein is its flavor and flavor variability. Whey and dried whey ingredients are expected to have a delicate and bland flavor that is undetectable in finished goods (Drake, 2009). Flavors that carry through to the finished product limit the utilization of whey protein as a desired ingredient.

Liquid whey is typically not used as a food ingredient, so it must be further processed into a dry powder. Differences in liquid whey flavors can be derived from differences in milk sources (Croissant et al., 2007), processing and handling (Carunchia Whetstine et al., 2005), and starter culture blend (Carunchia Whetstine et al., 2003, Tomaino et al., 2004). Croissant and others (2009) demonstrated that off flavors identified in liquid whey carried through to dried whey protein, suggesting that liquid whey may be studied in order to identify means to minimize undesirable flavors in dried whey.

Wheys made from different types of cheeses have different flavors (Drake et al., 2008; Gallardo-Escamilla et al., 2005; Carunchia Whetstine et al., 2003). Flavor from thermophilic starters differed from the flavor of fluid whey from mesophilic starter cultures (Liaw et al., 2010) and the flavor of whey from acid-set curd will differ even further, although few studies have directly quantitatively addressed this topic (Drake et al., 2008). Rennet wheys have been described as bland, sweet, and milky, while acid casein wheys were described as bitter, stale, rancid, and chemical (Gallardo-Escamilla et al., 2005). Within acid
wheys, sensory differences were perceived between directly acidified milk and whey and those from fermentation (Gallardo-Escamilla et al., 2005).

In the United States, the two main sources of liquid whey are Mozzarella and Cheddar. Liaw et al. (2010), reported that Mozzarella and Cheddar liquid whey were distinct in flavor and volatile compounds initially but that Cheddar whey was also more prone to lipid oxidation than Mozzarella whey. Mesophilic Lactococcus lactis starter cultures, used to produce Cheddar cheese, influenced flavor and oxidative stability of liquid whey (Carunchia Whetstine et al., 2003; Tomaino et al., 2004). Campbell et al. (2010) recently demonstrated that lipid oxidation products were higher in concentration in dried whey protein concentrate (WPC) manufactured from mesophilic starter culture (Cheddar) whey compared to WPC from rennet-set whey. These results in conjunction with those from Liaw et al. (2010) suggested that starter culture played a critical role in the lipid oxidation of fluid whey. Few studies have compared flavor and volatile compounds of whey protein from different starter culture types or whey with and without starter cultures. This study investigated fluid whey manufactured from different types of starter cultures to further elucidate the role of starter culture as a source of lipid oxidation in whey. All liquid wheys and the milks that the wheys were manufactured from were subjected to descriptive sensory and volatile instrumental analysis.

METHODS

Experimental Design

The same lot of pasteurized whole milk (protein 3.39±0.01%, fat 3.80±0.01%) was used to produce all samples within each replicate for each experiment. The order of
production was randomized. All experiments were replicated 3 times. This study was divided into two parts. Experiment 1 consisted of evaluation of wheys from both a mesophilic and thermophilic starter culture from skim or whole milk. Since Mozzarella and Cheddar cheese not only differ in culture type, but in the way they are made, milk controls exposed to the same time/temperature profile and rennet-set wheys were also included in this experimental design (Figure 1A). Based on the findings of experiment 1, experiment 2 compared a series of whole milk wheys manufactured from different mesophilic starters to further investigate the source and onset of lipid oxidation (Figure 1B).

**Production of Liquid Whey**

Whole raw bovine milk (180 kg) was received from the North Carolina State University Dairy Education Unit, Raleigh, NC. Milk was heated to 63°C and held for 30 min in a pasteurization vat (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC). Milk was separated with a hot bowl cream separator after pasteurization (experiment 1) to obtain skim milk (model DD275NT GEA Westfalia Separator, Inc., Northvale, NJ). Immediately following pasteurization and separation, milk was heated to temperature (31°C – Cheddar (Ched); 35°C – Mozzarella (Mozz)) in a water bath and assigned to various treatments. If the sample was made with culture, the milk was inoculated with starter culture (see Table 1). Next, calcium chloride solution (50% w/v, Dairy Connection Inc., Madison, WI) was added at the rate of 0.39 mL/kg of milk. The milk was agitated and allowed to ripen for 60 min. If the treatment did not contain culture, the pH was adjusted to 6.40 using a 1:10 dilution of lactic acid (85% w/v) (Fisher Scientific, Pittsburgh, PA) and deionized water. Then, the milk was coagulated with double strength recombinant
rennet (Dairy Connection Inc., Madison, WI) for 30 min at a rate of 0.09 mL/kg of milk
diluted 80 times in deionized water. The coagulum was cut and the curd and whey was
allowed to rest for 5 min followed by gentle stirring for 10 min without added heat. The
temperature was increased gradually (39°C for Ched and 40°C for Mozz) over 30 min while
pH and titratable acidity were closely monitored. The curd was continuously stirred until the
target whey drain pH of 6.40 was attained. As the whey was drained, a sieve was used to
remove fines and the whey was immediately pasteurized at 63°C for 30 min. After
pasteurization, the whey was cooled to 25°C within 20 min by placing it in an ice water bath
with stirring followed by time zero analysis. Additional sample was cooled to 8°C and then
placed at 3°C in the dark for 72 h.

**Compositional analysis**

Total solids, fat, and protein (total nitrogen) were determined. Total solids were
determined by air oven drying (AOAC, 2000; method number 990.20; 33.2.44). Fat was
quantified using the Pennsylvania modified Babcock method (AOAC, 2000; method number
989.04). Protein was determined using the Sprint™ Rapid Protein Analyzer (CEM,
Matthews, NC). Each sample replication was evaluated in duplicate.

**Descriptive sensory analysis**

Sensory analysis of milk and fluid wheys was performed using a trained descriptive
sensory panel and an established sensory lexicon (Drake et al., 2003, 2009; Liaw et al.,
2010). Panelists (n = 8) each had more than 150 h of previous experience with the sensory
analysis of fluid and dried whey products using the Spectrum™ descriptive analysis method
(Meilgard et al., 2007). All sensory testing was conducted in accordance with the NCSU Institutional Review Board for Human Subjects guidelines.

Liquid milk or whey was evaluated by placing 30 mL of sample in random three-digit-coded, 60-mL lidded cups (Solo Cup Company, Champaign, IL). Preparations were conducted with overhead lights off to minimize exposure to light. Liquid wheys or milks were evaluated fresh (within 2 h of production) and after 3 days of storage at 3C in the dark.

**Instrumental Volatile Compound Analysis**

Volatile compounds were extracted by head space-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME GC-MS). Methods were modified from Liaw et al. (2010). All injections were made on an Agilent 6890N GC with 5973 inert MSD with a ZB-5ms (30m x 0.25mm ID x 0.25µm) column (Phenomenex, Torrance, CA). Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics, Alexandria, VA). For volatile analysis, 5 ml of liquid whey was added to 20 ml SPME vials (MicroLiter Analytical Supplies, Inc., Suwanee, GA) and 10% salt was added to each vial which was prepared in triplicate. Internal standard (10 ul of 81 ppm 2-methyl-3-heptanone in methanol) was added to each vial. Vials were equilibrated for 25 min at 40°C with 4 sec pulsed 250 rpm agitation. A single DVB/Carboxen/PDMS 1 cm fiber (Supelco, Bellefonte, PA) was used for all analysis. The SPME fiber was exposed to the samples for 40 min at depth 3.1 cm. The fiber was retracted and injected at 5.0 cm in the GC inlet for 5 min. Initial analysis, scanning from 35-350 m/z, was performed to identify compounds of interest. Single ion monitoring (SIM) was employed subsequently to resolve compounds from background interference and increase sensitivity.
The GC method used an initial temperature of 40°C for 3 min with a ramp rate of 10°C/min to 250°C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250°C. An ZB-5ms column (ZB-5ms 30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex, Torrance, CA) was used for all analyses at a constant flow rate of 1 ml/min. Purge time was set at 1 min. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 250°C. Compounds were identified using the NIST 2005 library of spectra and comparison of spectra of authentic standards injected under identical conditions. Relative abundance for each compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound. Retention indices were calculated using an alkane series (Sigma Aldrich, Milwaukee, WI) (Van den Dool and Kratz, 1963).

Data Analysis

Compounds were identified using mass spectra and retention index comparisons to the NIST 2005 mass spectral library. Authentic standards were run using the same methods to positively identify compounds. Relative abundances of each compound were compiled from total ion counts from samples evaluated in SIM mode for each peak of interest compared to the peak area of the internal standard. Average values (ppb relative to internal standard) for the compounds identified were compiled.

Volatile Free Fatty Acid Analysis

The method used was described by Campbell et al. (2010). Samples were rehydrated at 10% (w/v) in deionized water and the pH was adjusted to 2.0 with 3.3M HCl (20% v/v, VWR). Five grams of the pH adjusted sample was added to a 20 ml autosampler vial with
steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA). An internal standard, heptadecanoic acid (C17 1ppm in ether (EMD Chemicals)), was added at a rate of 5 μl to each vial. Each sample was run in triplicate. Samples were injected using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) attached to a Varian CP-3380 gas chromatograph equipped with a flame ionization detector (FID). Samples were maintained at 10°C prior to fiber exposure. Samples were equilibrated at 110°C for 10 min before 40 min fiber exposure (22mm vial penetration) of a 30μm polydimethylsiloxane fiber (PDMS) with 4 sec on-off pulsed agitation at 250 rpm. Fibers were injected for 10 min at a depth of 50 mm. The GC method used an initial temperature of 100°C for 2 min with a ramp rate of 10°C/min to 245°C held for 13.5 min. Helium flow rate was 1ml/min and SPME fibers were introduced into the split/splitless injector at 250°C. A ZB-5ms column (ZB-FFAP 30 m length × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex, Torrance, CA) was used for all analyses.

Compounds were identified using authentic standards injected under identical conditions. Relative abundance for each compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound.

Statistical Analysis

Sensory and volatile compounds were analyzed by 3-way analysis of variance (ANOVA) using a mixed model (PROC MIXED) in SAS (SAS Statistical Analysis Software, version 9.1, SAS Institute, Cary, NC). Following a significant F statistic and p value (p<0.05), statistical lettering was obtained on the least squared means values by PROC
GLIMMIX (SAS Statistical Analysis Software, version 9.2, SAS Institute, Cary, NC). Both main effects and interaction effects were analyzed. Fixed effects were milk type (skim vs. whole milk), cheese (Mozzarella vs. Cheddar and rennet set vs. control (milk) vs. culture set), and time (fresh (<2 h) vs. stored (3 days)) and their interactions. Milk type was the main plot factor and cheese was the sub plot factor in a split plot arrangement of fixed effect factors. Replication effects and all interactions with replication effects were viewed as random effects.

RESULTS

Compositional Analysis

Whole milk contained 3.39±0.01% protein and 3.80±0.01% fat while skim milk contained 0.04±0.01% fat. In experiment 1, liquid wheys did not differ significantly (p>0.05) in protein or total solids, averaging 0.92±0.06% and 6.75±0.21% respectively. Liquid wheys made with skim milk versus whole milk differed significantly (p<0.05) in fat content averaging 0.01±0.01% and 0.19±0.02% respectively. In experiment 2, in which all the wheys were manufactured from whole milk, wheys did not differ significantly (p>0.05) in protein, fat, or total solids averaging 0.87±0.02%, 0.21±0.03%, and 6.30±0.34% respectively.

Descriptive Analysis

Main effects included milk type (skim vs. whole), cheese (Mozzarella vs. Cheddar and rennet vs. culture), and time (day 0 vs. day 3) (Table 2). Significant interactions included milk type*cheese, milk type*time, cheese*time, and milk type*cheese*time. Milk type, cheese, time, milk type*time, and cheese*time were significant factors (p<0.05) (Table 2) which merited further investigation. By evaluating each factor broken down by attribute, statistical
lettering was assigned making comparisons more clear (Table 3). Wheys made without culture (rennet set) were lower in overall aroma intensity, sweet aromatic, buttery, and sour aromatic flavors (p<0.05). Wheys made from whole milk had greater cardboard flavor than those made from skim milk (p<0.05). Over time, aroma intensity, sour aromatic, sweet aromatic, cooked, and sweet taste all decreased (p<0.05) while cardboard flavors increased (p<0.05).

**Instrumental Analysis**

The main effects, milk type (skim vs. whole), cheese (Mozzarella vs. Cheddar and rennet vs. culture) and time (day 0 vs. day 3) were all significant (Tables 5, 6). Only one interaction, milktype*time, was significant (p<0.05). Cheddar wheys were higher in diacetyl, decanal, heptanal, nonanal, and acetic acid (p<0.05) compared to Mozzarella wheys. Those made from whole milk were lower in 2-pentylfuran and 2-nonanone than those from skim (p<0.05). Over time, 2,6 nonadienal, decanal, heptanal, and octanal all decreased (p<0.05). Similarly, diacetyl, 2,6 nonadienal, decanal, 2.4 nonadienal, hexanal, and nonanal all decreased over time in milks (p<0.05).

**DISCUSSION**

Previous studies have established that wheys made from different types of cheeses have different flavors (Liaw et al., 2010; Drake et al., 2008; Gallardo-Escamilla et al., 2005; Carunchia Whetstine et al., 2003). Liaw et al. (2010) also compared fresh and stored liquid Mozzarella and Cheddar wheys. Initially, Mozzarella whey demonstrated lower flavor intensities than Cheddar whey, however, both liquid wheys aged similarly with increased cardboard and oxidized flavors over time with concurrent decreased sweet aromatic and
cooked notes. Similar results were noted in the current study. The flavor of Mozzarella and Cheddar wheys were distinct with Mozzarella whey having higher cooked flavors and Cheddar whey having higher sour aromatic and cardboard notes (p<0.05) (Table 3). Wheys made without culture (rennet set) were lower in sweet aromatic, buttery, and sour aromatic flavors (p<0.05). Wheys made from whole milk had greater cardboard flavor than those made from skim (p<0.05). Wheys made from whole milk were expected to have higher cardboard flavor due higher fat content and lipid oxidation. Previously, differences in the flavor of fluid whey have been attributed to different starter cultures and different processing techniques used to produce these cheeses (Liaw et al., 2010; Carunchia-Whetstine et al., 2003; Karagul-Yuceer et al., 2003; Gallardo-Escamilla et al., 2005). The current study demonstrated that fat content, culture and storage time all contributed to flavor differences in whey.

Volatile compound analysis also documented differences between Mozzarella and Cheddar liquid wheys. Cheddar whey was significantly higher in many volatile compounds such as diacetyl, heptanal, nonanal, and acetic acid (p<0.05) compared to Mozzarella whey (Table 4). Wheys made from skim milk did not differ greatly from those made from whole milk with the exception of 2-pentylfuran and 2-nonanone, which were found in greater concentrations in skim whey (p<0.05) than in whole milk whey. These two compounds are a product of lipid oxidation, which can occur due to storage temperatures, oxygen levels, heat treatments, or homogenizations (Frankel, 1988). This is most likely due to the extra processing step (separation) that skim milk must endure which requires both high heat and high shear, which in turn incorporates air, in order to separate the fat.
The amount of straight chain aldehydes in a product increase as a result of lipid oxidation reactions. Lipid oxidation is a complex, chain reaction which is separated into three distinct phases: initiation, propagation, and termination (Coupland and McClements, 1996). The oxidation of fat can be influenced by light, trace metals, antioxidants, temperature, and fatty acid composition (Frankel, 1998). Many important dairy volatile compounds such as esters, aldehydes, alcohols, ketones, lactones, and hydrocarbons are formed via lipid oxidation (Frankel, 1998). Aldehydes and ketones are generally responsible for the undesirable off-flavors formed by lipid oxidation due to their low thresholds (Frankel, 1998; Whitson et al., 2010). Aldehydes are derived from either hydroperoxides of polyunsaturated fatty acid components or from further oxidation of polyunsaturated aldehydes (Frankel, 1998). In the present study, 2,6 nonadienal, decanal, heptanal, and octanal decreased after 3 days storage (p<0.05) (Table 4). Liaw et al. (2010) observed a similar pattern with fluid wheys, lipid oxidation products decreased for up to 6 days when wheys were stored at 3C. Aldehydes may bind to proteins and appear to decrease in concentration. In addition, aldehydes may react with amines to produce Schiff bases. Frankel (1988) also addressed the complexity of reaction of oxidation products with proteins in model systems and indicated that such interactions would be very complex and difficult to pinpoint in actual foods. Liaw et al. (2010) speculated that the aldehydes were further degrading into other compounds or reacting with sulfur compounds.

Skim milk and wheys from skim milk were higher in free fatty acids compared to whole milk and whey from whole milk (p<0.05) (Table 7). Milk type was the only significant effect on free fatty acids. Free fatty acids break down and form various aldehydes
and lipid oxidation compounds. Skim milk and wheys from skim milk are higher in phospholipids as a proportion of fat content than whole milk or whey from whole milk, which have been considered a possible off-flavor source in wheys (Morr and Ha, 1993). Milks were included in this study to determine if oxidation in wheys was due to oxidation that occurred naturally with milk storage. Like liquid wheys, lipid oxidation occurred in milks with storage. Oxidation occurred in whole and skim milks at similar rates (milk type * time; p>0.05). Whole milks had higher oxidation products (2,6 nonadienal, decanal, 2,4 nonadienal, hexanal, 1-hexen-3-one, heptanal, 3-methyl butanal, and 2-methyl butanal) than skim milks. Lipid oxidation occurred in all culture-set wheys as well and rennet-set wheys. Both set-types aged similarly (cheese*time; p>0.05). These results demonstrate that lipid oxidation occurs with storage of milk or whey. Milk fat will oxidize in fluid milk with storage due to the breakdown of free fatty acids into volatile compounds, such as aldehydes. Milk fat in liquid whey behaves in a similar manner with cold storage. The presence or absence of starter culture also had an impact on lipid oxidation products in fresh whey with oxidation products higher initially in wheys with starter culture. Oxidation in skim milk and skim milk wheys may proceed differently than their whole milk counterparts due to the higher fraction of phospholipids in the fat. 2-pentylfuran and 2-nonanone, both lipid oxidation products, were higher in skim wheys suggesting that fat content is not the sole cause of lipid oxidation but perhaps fat type (phospholipids) also played a key role. In this study, storage, total fat content, fraction of phospholipids, and starter culture all impacted lipid oxidation of fluid wheys. Previous studies have demonstrated that flavor and volatile compound differences observed in fluid wheys carry-through into dried whey proteins.
If the wheys in the current study were processed into a higher protein product, such as WPC, it is likely that these effects would be more pronounced.

In the follow-up experiment, Cheddar whey was examined further examined to determine the role of the starter culture in whey flavor and oxidation. Our first experiment, as well as others (Liaw et al., 2010), demonstrated that Cheddar whey was more likely to experience higher levels of oxidation than Mozzarella whey so Cheddar starter cultures and starter medias were examined. Previously, strain differences were hypothesized to influence the flavor and oxidative stability of liquid Cheddar whey (Tomaino et al., 2004; Carunchia-Whetstine et al., 2003). Wheys from freeze dried and frozen starters as well as from different starter strains were explored in fluid whey over time. In addition to investigating these two factors (strain, culture media), autoclaved strain 3 was evaluated to determine if the culture media played a role in flavor. Whey without culture and only set by rennet was produced as a control. Buttery flavor was only detected in those samples with active culture (MA11, M58, M60) and cardboard flavor increased over time in all samples, regardless of culture (p<0.05) (Table 8). In addition, cooked/milky flavors decreased over time in both whey and milk (p<0.05). Volatile compound results were consistent with sensory results (Table 9, 10). Diacetyl, which contributes to buttery flavor, was significantly higher in wheys containing live culture (p<0.05). Volatile compound changes over time were not extensive, with the exception of wheys from MA11 and M58 which showed significant increases in hexanal over time (p<0.05). Culture strain did have an impact on flavor; however media format (freeze dried vs. frozen) did not have a significant effect. Not many differences between strains were depicted via descriptive analysis. Strain 1 (M58) and strain 3 (MA11) were most
similar in volatile compound profiles. Both of these strains caused more oxidation, shown by increases in lipid oxidation compounds (such as hexanal) than strain 2 (M60). The whey with added autoclaved media was almost identical in flavor profile to the rennet set whey, indicating that media has no impact on the oxidative stability of liquid whey. Results of this study are in agreement with previous research conducted by Tomaino et al., (2004) which demonstrated that *Lactococcus lactis* starter cultures used alone and in conjunction with each other influenced the flavor and oxidative stability of whey. Previous studies have demonstrated that mesophilic cultures have an effect on whey flavor, but they did not determine if this effect was specific to starter culture, specific starter culture strains, starter media or the processing time/temperature profile of the different cheeses.

**CONCLUSIONS**

Culture type greatly influenced the oxidative stability of liquid whey, with Cheddar and Mozzarella whey differing not only in flavor, but also in volatile compound profiles. Whey from Cheddar starters had more oxidation products than Mozzarella starters. In a subsequent experiment, starter media did not have an effect on the flavor or oxidative stability of liquid whey however, culture strain does have an effect. Sensory profiles of wheys from all 3 strains of *Lactococcus lactis* starter cultures used in this experiment were similar; however differences between strains were depicted in volatile compound analysis.

**ACKNOWLEDGEMENTS**

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North Carolina State University, Raleigh, NC. The advice and assistance of Dave Potter is gratefully acknowledged. The use of tradenames does not imply endorsement nor lack of endorsement by those not mentioned.
REFERENCES


FIGURES AND TABLES

Table 1: Types of Starter Cultures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starter Culture</th>
</tr>
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<tbody>
<tr>
<td>Cheddar</td>
<td>Choozit™ MA 11, Danisco, New Century, NJ</td>
</tr>
<tr>
<td>Mozzarella</td>
<td>Choozit™ LH100, Danisco, New Century, NJ; Choozit™ TA61, Danisco, New Century, NJ</td>
</tr>
<tr>
<td>Strain 1</td>
<td>Choozit™ FRO M58, Danisco, New Century, NJ</td>
</tr>
<tr>
<td>Strain 2</td>
<td>Choozit™ FRO M60, Danisco, New Century, NJ</td>
</tr>
<tr>
<td>Strain 3</td>
<td>Choozit™ MA 11, Danisco, New Century, NJ</td>
</tr>
<tr>
<td>Inactivated Strain 3 (Autoclaved)</td>
<td>Choozit™ MA 11, Danisco, New Century, NJ diluted 1:20 with DI water, then placed in autoclave (45 min/121°C) to kill all live culture</td>
</tr>
<tr>
<td>Rennet-set</td>
<td>pH was adjusted to 6.40 using a 1:10 dilution of Lactic Acid (85%) (Fisher Scientific, Pittsburgh, PA) and DI water</td>
</tr>
</tbody>
</table>
Table 2: F Statistics (gray line) and probability values (white line) for the ANOVA analysis of the impact of milk type, cheese, and time on the flavor of liquid whey.

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>Aroma Intensity</th>
<th>Sweet Aromatic</th>
<th>Cooked</th>
<th>Buttery</th>
<th>Sweet Taste</th>
<th>Cardboard</th>
<th>Sour Aromatic</th>
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<td>0.012</td>
<td>&lt;.0001</td>
<td>0.336</td>
<td>0.002</td>
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<td><strong>Milk Type * Cheese</strong></td>
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<td>3.07</td>
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<td>7.56</td>
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Statistically significant interactions are highlighted in bold type
Table 3: Means separation for attributes with significant F statistics and interactions from descriptive sensory analysis of liquid whey (Experiment 1)

<table>
<thead>
<tr>
<th>Milk Type</th>
<th>Aroma Intensity</th>
<th>Sweet Aromatic</th>
<th>Cooked</th>
<th>Buttery</th>
<th>Sweet Taste</th>
<th>Cardboard</th>
<th>Sour Aromatic</th>
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<tbody>
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<td>Skim</td>
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<td>Whole</td>
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<tr>
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<td></td>
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<tr>
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<td>1.9</td>
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<tr>
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<td>2.5</td>
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<td>1.7</td>
<td>1.7</td>
<td>3.0</td>
<td>0.9^</td>
<td>2.3</td>
<td>ND</td>
<td>1.4</td>
</tr>
<tr>
<td>Skim * MozzarellaRennet * 0 day</td>
<td>1.5</td>
<td>1.2</td>
<td>2.9</td>
<td>ND</td>
<td>1.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Skim * CheddarCulture * 3 day</td>
<td>1.8</td>
<td>1.5</td>
<td>2.3</td>
<td>1.2^</td>
<td>1.8</td>
<td>ND</td>
<td>1.0</td>
</tr>
<tr>
<td>Skim * CheddarRennet * 3 day</td>
<td>1.5</td>
<td>1.2</td>
<td>1.9</td>
<td>ND</td>
<td>1.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Skim * MozzarellaCulture * 3 day</td>
<td>1.8</td>
<td>1.6</td>
<td>2.2</td>
<td>1.1^</td>
<td>1.9</td>
<td>ND</td>
<td>1.3</td>
</tr>
<tr>
<td>Skim * MozzarellaRennet * 3 day</td>
<td>1.5</td>
<td>1.5</td>
<td>2.4</td>
<td>ND</td>
<td>2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Whole * CheddarCulture * 0 day</td>
<td>1.9</td>
<td>1.8</td>
<td>2.1</td>
<td>0.8^</td>
<td>2.1</td>
<td>ND</td>
<td>1.3</td>
</tr>
<tr>
<td>Whole * CheddarRennet * 0 day</td>
<td>1.6</td>
<td>1.2</td>
<td>2.5</td>
<td>ND</td>
<td>2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Whole * MozzarellaCulture * 0 day</td>
<td>2.0</td>
<td>1.7</td>
<td>3.0</td>
<td>1.1^</td>
<td>2.3</td>
<td>ND</td>
<td>1.4</td>
</tr>
<tr>
<td>Whole * MozzarellaRennet * 0 day</td>
<td>1.4</td>
<td>1.2</td>
<td>2.6</td>
<td>0.1^</td>
<td>2.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Whole * CheddarCulture * 3 day</td>
<td>1.8</td>
<td>1.2</td>
<td>1.8</td>
<td>0.7^</td>
<td>1.5</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Whole * CheddarRennet * 3 day</td>
<td>1.2</td>
<td>1.1</td>
<td>1.7</td>
<td>ND</td>
<td>1.6</td>
<td>0.7</td>
<td>ND</td>
</tr>
<tr>
<td>Whole * MozzarellaCulture * 3 day</td>
<td>1.3</td>
<td>1.3</td>
<td>2.0</td>
<td>0.7^</td>
<td>1.9</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Whole * MozzarellaRennet * 3 day</td>
<td>1.4</td>
<td>1.7</td>
<td>1.7</td>
<td>0.2^</td>
<td>1.9</td>
<td>0.6</td>
<td>ND</td>
</tr>
</tbody>
</table>

^Means in the same column not sharing a common superscript are different (P < 0.05) within each factor.

Means shaded in grey are not statistically significant (P>0.05); Attributes were scored using a 0 to 15 point universal Spectrum scale (Meilgaard et al., 1999). Most fluid whey attributes fall between 0 and 3 on this scale (Liaw et al., 2010).
Table 4: Means separation for attributes with significant F statistics and interactions from descriptive sensory analysis of milks (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>Aroma Intensity</th>
<th>Sweet Aromatic</th>
<th>Cooked</th>
<th>Milkfat</th>
<th>Sweet Taste</th>
<th>Feedy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milk Type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skim</td>
<td>1.5</td>
<td>1.9</td>
<td>2.7</td>
<td>ND</td>
<td>2.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Whole</td>
<td>1.6</td>
<td>1.6</td>
<td>2.5</td>
<td>3.0</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 day</td>
<td>1.4</td>
<td>1.8</td>
<td>2.8+a</td>
<td>1.5</td>
<td>2.3+a</td>
<td>1.7b</td>
</tr>
<tr>
<td>3 day</td>
<td>1.6</td>
<td>1.7</td>
<td>2.4+c</td>
<td>1.5</td>
<td>2.0+c</td>
<td>1.0a</td>
</tr>
</tbody>
</table>

*a,b* Means in the same column not sharing a common superscript are different (*P < 0.05*) within each factor.

Means shaded in grey are not statistically significant (*P > 0.05*).

Attributes were scored using a 0 to 15 point universal Spectrum scale (Meilgaard et al., 1999). Most fluid whey attributes fall between 0 and 3 on this scale (Liaw et al., 2010).
Table 5: Means separation for attributes with significant F statistics and interactions from volatile analysis of wheys (Experiment 1) in ppb

<table>
<thead>
<tr>
<th></th>
<th>Diacetyl</th>
<th>2,6-nonenal</th>
<th>Decanal</th>
<th>Heptanal</th>
<th>2-pentylfuran</th>
<th>Octanal</th>
<th>2-nonanone</th>
<th>Nonanal</th>
<th>Acetic Acid</th>
<th>2-methyl butanal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milk Type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skim</td>
<td>0.020</td>
<td>0.008</td>
<td>0.015</td>
<td>0.134</td>
<td>0.139a</td>
<td>0.060</td>
<td>0.309a</td>
<td>0.154</td>
<td>0.057</td>
<td>0.020</td>
</tr>
<tr>
<td>Whole</td>
<td>0.020</td>
<td>0.006</td>
<td>0.008</td>
<td>0.115</td>
<td>0.074b</td>
<td>0.049</td>
<td>0.139b</td>
<td>0.113</td>
<td>0.075</td>
<td>0.023</td>
</tr>
<tr>
<td><strong>Cheese</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheddar Culture</td>
<td>0.049a</td>
<td>0.008</td>
<td>0.013ab</td>
<td>0.149a</td>
<td>0.128</td>
<td>0.062</td>
<td>0.214</td>
<td>0.161a</td>
<td>0.152a</td>
<td>0.020</td>
</tr>
<tr>
<td>Cheddar Rennet</td>
<td>0.004b</td>
<td>0.010</td>
<td>0.016a</td>
<td>0.165a</td>
<td>0.132</td>
<td>0.068</td>
<td>0.202</td>
<td>0.175a</td>
<td>0.039b</td>
<td>0.024</td>
</tr>
<tr>
<td>Mozzarella Culture</td>
<td>0.014b</td>
<td>0.005</td>
<td>0.007b</td>
<td>0.069b</td>
<td>0.069</td>
<td>0.038</td>
<td>0.243</td>
<td>0.127ab</td>
<td>0.027b</td>
<td>0.019</td>
</tr>
<tr>
<td>Mozzarella Rennet</td>
<td>0.003b</td>
<td>0.007</td>
<td>0.010b</td>
<td>0.113ab</td>
<td>0.097</td>
<td>0.051</td>
<td>0.239</td>
<td>0.071b</td>
<td>0.046b</td>
<td>0.024</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 day</td>
<td>0.021</td>
<td>0.009a</td>
<td>0.015a</td>
<td>0.147a</td>
<td>0.114</td>
<td>0.062a</td>
<td>0.239</td>
<td>0.167a</td>
<td>0.072</td>
<td>0.022</td>
</tr>
<tr>
<td>3 day</td>
<td>0.015</td>
<td>0.006b</td>
<td>0.008b</td>
<td>0.100b</td>
<td>0.099</td>
<td>0.047b</td>
<td>0.208</td>
<td>0.097b</td>
<td>0.060</td>
<td>0.022</td>
</tr>
<tr>
<td><strong>Milk Type * Time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 day * whole</td>
<td>0.017</td>
<td>0.008</td>
<td>0.009</td>
<td>0.133</td>
<td>0.077b</td>
<td>0.056</td>
<td>0.135</td>
<td>0.141</td>
<td>0.078</td>
<td>0.019ab</td>
</tr>
<tr>
<td>0 day * skim</td>
<td>0.024</td>
<td>0.009</td>
<td>0.020</td>
<td>0.162</td>
<td>0.151a</td>
<td>0.069</td>
<td>0.344</td>
<td>0.199</td>
<td>0.073</td>
<td>0.025ab</td>
</tr>
<tr>
<td>3 day * whole</td>
<td>0.015</td>
<td>0.005</td>
<td>0.006</td>
<td>0.096</td>
<td>0.072b</td>
<td>0.042</td>
<td>0.143</td>
<td>0.086</td>
<td>0.072</td>
<td>0.028a</td>
</tr>
<tr>
<td>3 day * skim</td>
<td>0.015</td>
<td>0.007</td>
<td>0.010</td>
<td>0.105</td>
<td>0.126a</td>
<td>0.052</td>
<td>0.274</td>
<td>0.108</td>
<td>0.042</td>
<td>0.015b</td>
</tr>
</tbody>
</table>

*Means in the same column not sharing a common superscript are different (P < 0.05) within each factor.
Those shaded in grey are not statistically significant (P>0.05)
Table 6: Means separation for attributes with significant F statistics and interactions from volatile analysis of milks (Experiment 1) in ppb

<table>
<thead>
<tr>
<th>Milk Type</th>
<th>Diacetyl</th>
<th>2,6-Nonenal</th>
<th>Decanal</th>
<th>2,4-Nonadienal</th>
<th>Hexanal</th>
<th>1-hexen-3-one</th>
<th>Heptanal</th>
<th>Nonanal</th>
<th>3-methyl butanal</th>
<th>2-methyl butanal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim</td>
<td>0.002</td>
<td>0.002b</td>
<td>0.008b</td>
<td>0.003b</td>
<td>0.122b</td>
<td>0.015b</td>
<td>0.036b</td>
<td>0.056</td>
<td>0.007b</td>
<td>0.023b</td>
</tr>
<tr>
<td>Whole</td>
<td>0.005</td>
<td>0.007a</td>
<td>0.017a</td>
<td>0.013a</td>
<td>1.020a</td>
<td>0.172a</td>
<td>0.063a</td>
<td>0.094</td>
<td>0.063a</td>
<td>0.175a</td>
</tr>
</tbody>
</table>

Cheese

<table>
<thead>
<tr>
<th>Milk Type</th>
<th>Butyric</th>
<th>Hexanoic</th>
<th>Octanoic</th>
<th>Decanoic</th>
<th>Dodecanoic</th>
<th>Tetradecanoic</th>
<th>Hexadecanoic</th>
<th>Octadecanoic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Linolenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheddar Milk</td>
<td>0.005</td>
<td>0.005</td>
<td>0.015a</td>
<td>0.009</td>
<td>0.677a</td>
<td>0.107a</td>
<td>0.040</td>
<td>0.084</td>
<td>0.040</td>
<td>0.110</td>
<td></td>
</tr>
<tr>
<td>Mozzarella Milk</td>
<td>0.003</td>
<td>0.004</td>
<td>0.010b</td>
<td>0.007</td>
<td>0.470b</td>
<td>0.079b</td>
<td>0.029</td>
<td>0.066</td>
<td>0.029</td>
<td>0.089</td>
<td></td>
</tr>
</tbody>
</table>

Time

<table>
<thead>
<tr>
<th>Milk Type</th>
<th>Butyric</th>
<th>Hexanoic</th>
<th>Octanoic</th>
<th>Decanoic</th>
<th>Dodecanoic</th>
<th>Tetradecanoic</th>
<th>Hexadecanoic</th>
<th>Octadecanoic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Linolenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim</td>
<td>0.002</td>
<td>0.018a</td>
<td>0.012a</td>
<td>0.051a</td>
<td>0.120a</td>
<td>0.430a</td>
<td>1.58a</td>
<td>0.726</td>
<td>1.272</td>
<td>0.571</td>
<td>0.023</td>
</tr>
<tr>
<td>Whole</td>
<td>0.003</td>
<td>0.006a</td>
<td>0.009b</td>
<td>0.027b</td>
<td>0.040a</td>
<td>0.117a</td>
<td>0.352a</td>
<td>0.197</td>
<td>0.287</td>
<td>0.111</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Table 7: Milk Effect on the Volatile Free Fatty Acids in Liquid Whey and Milk in ppm

Liquid Whey

<table>
<thead>
<tr>
<th>Milk Type</th>
<th>Butyric</th>
<th>Hexanoic</th>
<th>Octanoic</th>
<th>Decanoic</th>
<th>Dodecanoic</th>
<th>Tetradecanoic</th>
<th>Hexadecanoic</th>
<th>Octadecanoic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Linolenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim</td>
<td>0.003</td>
<td>0.015</td>
<td>0.013a</td>
<td>0.045a</td>
<td>0.056</td>
<td>0.345a</td>
<td>1.180a</td>
<td>0.666</td>
<td>1.041</td>
<td>0.455</td>
<td>0.019</td>
</tr>
<tr>
<td>Whole</td>
<td>0.003</td>
<td>0.004</td>
<td>0.007b</td>
<td>0.013a</td>
<td>0.054</td>
<td>0.050a</td>
<td>0.156a</td>
<td>0.073</td>
<td>0.113</td>
<td>0.026</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Milk

<table>
<thead>
<tr>
<th>Milk Type</th>
<th>Butyric</th>
<th>Hexanoic</th>
<th>Octanoic</th>
<th>Decanoic</th>
<th>Dodecanoic</th>
<th>Tetradecanoic</th>
<th>Hexadecanoic</th>
<th>Octadecanoic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Linolenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim</td>
<td>0.003</td>
<td>0.015</td>
<td>0.013a</td>
<td>0.045a</td>
<td>0.056</td>
<td>0.345a</td>
<td>1.180a</td>
<td>0.666</td>
<td>1.041</td>
<td>0.455</td>
<td>0.019</td>
</tr>
<tr>
<td>Whole</td>
<td>0.003</td>
<td>0.004</td>
<td>0.007b</td>
<td>0.013a</td>
<td>0.054</td>
<td>0.050a</td>
<td>0.156a</td>
<td>0.073</td>
<td>0.113</td>
<td>0.026</td>
<td>0.009</td>
</tr>
</tbody>
</table>

a–bMeans in the same column not sharing a common superscript are different ($P < 0.05$)
Those shaded in grey are not statistically significant ($P>0.05$)
Table 8: Experiment 2: Sensory attributes of liquid whey and milk

<table>
<thead>
<tr>
<th>Liquid Whey</th>
<th>Aroma Intensity</th>
<th>Sweet Aromatic</th>
<th>Cooked/Milky</th>
<th>Buttery</th>
<th>Cardboard</th>
<th>Sweet Taste</th>
<th>Sour Aromatic</th>
<th>Astringency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rennet Set Day 0</td>
<td>1.7abc</td>
<td>1.2a</td>
<td>2.5ab</td>
<td>ND</td>
<td>ND</td>
<td>2.0a</td>
<td>0.8a</td>
<td>1.2a</td>
</tr>
<tr>
<td>Rennet Set Day 3</td>
<td>1.9bc</td>
<td>1.3a</td>
<td>2.2a</td>
<td>ND</td>
<td>0.7ab</td>
<td>2.0a</td>
<td>0.8a</td>
<td>1.0a</td>
</tr>
<tr>
<td>Autoclaved Strain 3 Day 0</td>
<td>1.1a</td>
<td>1.3a</td>
<td>2.6ab</td>
<td>ND</td>
<td>ND</td>
<td>1.8a</td>
<td>0.6a</td>
<td>1.2a</td>
</tr>
<tr>
<td>Autoclaved Strain 3 Day 3</td>
<td>1.4a</td>
<td>1.4a</td>
<td>2.1a</td>
<td>ND</td>
<td>0.8ab</td>
<td>2.0a</td>
<td>0.4a</td>
<td>1.0a</td>
</tr>
<tr>
<td>Strain 2 Day 0</td>
<td>2.2c</td>
<td>1.4a</td>
<td>3.2bc</td>
<td>1.2cd</td>
<td>ND</td>
<td>1.8a</td>
<td>0.7a</td>
<td>1.2a</td>
</tr>
<tr>
<td>Strain 2 Day 3</td>
<td>1.7abc</td>
<td>1.4a</td>
<td>2.2a</td>
<td>0.2a</td>
<td>1.1b</td>
<td>2.1a</td>
<td>0.4a</td>
<td>1.0a</td>
</tr>
<tr>
<td>Strain 3 Day 0</td>
<td>2.2c</td>
<td>1.5a</td>
<td>3.4c</td>
<td>1.4d</td>
<td>ND</td>
<td>1.8a</td>
<td>0.7a</td>
<td>1.0a</td>
</tr>
<tr>
<td>Strain 3 Day 3</td>
<td>2.0bc</td>
<td>2.1b</td>
<td>2.4a</td>
<td>1.2cd</td>
<td>0.5a</td>
<td>2.1a</td>
<td>0.7a</td>
<td>1.0a</td>
</tr>
<tr>
<td>Strain 1 Day 0</td>
<td>2.1bc</td>
<td>2.3b</td>
<td>3.3bc</td>
<td>0.8bc</td>
<td>ND</td>
<td>1.8a</td>
<td>0.7a</td>
<td>1.2a</td>
</tr>
<tr>
<td>Strain 1 Day 3</td>
<td>1.9bc</td>
<td>2.3b</td>
<td>2.3a</td>
<td>0.6ab</td>
<td>0.9ab</td>
<td>2.1a</td>
<td>0.5a</td>
<td>1.0a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Milk</th>
<th>Aroma Intensity</th>
<th>Sweet Aromatic</th>
<th>Cooked/Milky</th>
<th>Milk Fat</th>
<th>Sweet Taste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Milk Day 0</td>
<td>2.0a</td>
<td>1.9b</td>
<td>3.4b</td>
<td>3.0a</td>
<td>2.0a</td>
</tr>
<tr>
<td>Whole Milk Day 3</td>
<td>2.2a</td>
<td>1.5a</td>
<td>3.0a</td>
<td>3.1a</td>
<td>2.0a</td>
</tr>
</tbody>
</table>

* a–dMeans in the same column not sharing a common superscript are different ($P < 0.05$).
* Attributes were scored using a 0 to 15 point universal Sepctrum scale (Meilgaard et al., 1999).
* Most fluid whey attributes fall between 0 and 3 on this scale (Liaw et al., 2010).
Table 9: Experiment 2: Selected aldehydes in liquid whey and milk in ppb

<table>
<thead>
<tr>
<th>Category</th>
<th>3 Methyl Butanal</th>
<th>2 Methyl Butanal</th>
<th>Pentanal</th>
<th>Hexanal</th>
<th>Heptanal</th>
<th>Octanal</th>
<th>Nonanal</th>
<th>Decanal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liquid Whey</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rennet Set 0d</td>
<td>0.003ab</td>
<td>0.003a</td>
<td>0.001b</td>
<td>0.412c</td>
<td>0.106b</td>
<td>0.042ab</td>
<td>0.242a</td>
<td>0.008a</td>
</tr>
<tr>
<td>Rennet Set 3d</td>
<td>0.005ab</td>
<td>0.006a</td>
<td>0.006ab</td>
<td>0.853bc</td>
<td>0.165ab</td>
<td>0.052ab</td>
<td>0.307a</td>
<td>0.009a</td>
</tr>
<tr>
<td>Inactivated Strain 3 0d</td>
<td>0.004ab</td>
<td>0.005a</td>
<td>0.003ab</td>
<td>0.501c</td>
<td>0.174ab</td>
<td>0.041ab</td>
<td>0.226a</td>
<td>0.009a</td>
</tr>
<tr>
<td>Inactivated Strain 3 3d</td>
<td>0.004ab</td>
<td>0.004a</td>
<td>0.003ab</td>
<td>0.954bc</td>
<td>0.108b</td>
<td>0.042ab</td>
<td>0.197a</td>
<td>0.008a</td>
</tr>
<tr>
<td>Strain 1 0d</td>
<td>0.005ab</td>
<td>0.005a</td>
<td>0.003ab</td>
<td>0.420c</td>
<td>0.129b</td>
<td>0.042ab</td>
<td>0.247a</td>
<td>0.009a</td>
</tr>
<tr>
<td>Strain 1 3d</td>
<td>0.006ab</td>
<td>0.005a</td>
<td>0.005ab</td>
<td>2.976a</td>
<td>0.209ab</td>
<td>0.055ab</td>
<td>0.339a</td>
<td>0.008a</td>
</tr>
<tr>
<td>Strain 2 0d</td>
<td>0.003b</td>
<td>0.005a</td>
<td>0.004ab</td>
<td>0.260c</td>
<td>0.125b</td>
<td>0.042ab</td>
<td>0.248a</td>
<td>0.010a</td>
</tr>
<tr>
<td>Strain 2 3d</td>
<td>0.006a</td>
<td>0.006a</td>
<td>0.005ab</td>
<td>0.756c</td>
<td>0.174ab</td>
<td>0.053ab</td>
<td>0.391a</td>
<td>0.011a</td>
</tr>
<tr>
<td>Strain 3 0d</td>
<td>0.003ab</td>
<td>0.010a</td>
<td>0.003ab</td>
<td>0.421c</td>
<td>0.129b</td>
<td>0.040b</td>
<td>0.236a</td>
<td>0.009a</td>
</tr>
<tr>
<td>Strain 3 3d</td>
<td>0.006ab</td>
<td>0.016a</td>
<td>0.003ab</td>
<td>2.481ab</td>
<td>0.272a</td>
<td>0.075a</td>
<td>0.494a</td>
<td>0.016a</td>
</tr>
<tr>
<td><strong>Milk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Milk 0d</td>
<td>0.003a</td>
<td>0.003b</td>
<td>0.001b</td>
<td>0.056b</td>
<td>0.022b</td>
<td>0.010b</td>
<td>0.013b</td>
<td>0.001b</td>
</tr>
<tr>
<td>Whole Milk 3d</td>
<td>0.003a</td>
<td>0.005a</td>
<td>0.005a</td>
<td>0.158a</td>
<td>0.070a</td>
<td>0.034a</td>
<td>0.048a</td>
<td>0.003a</td>
</tr>
</tbody>
</table>

*Means in the same column not sharing a common superscript are different (P < 0.05)
Table 10: Experiment 2: Volatile compound means of liquid whey and milk in ppb

<table>
<thead>
<tr>
<th></th>
<th>Diacetyl</th>
<th>2,6 Nonadienal</th>
<th>E 2 nonanal</th>
<th>2,4 Nonadienal</th>
<th>1-Hexen-3-one</th>
<th>Z 1,5 Octadien-3-one</th>
<th>2 Pentyl Furan</th>
<th>1-octen-3-one</th>
<th>Benzencetaldehyde</th>
<th>2-nonanone</th>
<th>Acetic Acid</th>
<th>DMDS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liquid Whey</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rennet Set 0d</td>
<td>0.002d</td>
<td>0.004b</td>
<td>0.007a</td>
<td>0.003b</td>
<td>0.020b</td>
<td>0.044c</td>
<td>0.055bc</td>
<td>0.041ab</td>
<td>0.006a</td>
<td>0.052c</td>
<td>0.014a</td>
<td>0.002bc</td>
</tr>
<tr>
<td>Rennet Set 3d</td>
<td>0.004d</td>
<td>0.009ab</td>
<td>0.016a</td>
<td>0.003b</td>
<td>0.055a</td>
<td>0.093ab</td>
<td>0.098ab</td>
<td>0.058ab</td>
<td>0.017a</td>
<td>0.093abc</td>
<td>0.022a</td>
<td>0.009abc</td>
</tr>
<tr>
<td>Dead MA 11 0d</td>
<td>0.004d</td>
<td>0.007ab</td>
<td>0.006a</td>
<td>0.003b</td>
<td>0.020b</td>
<td>0.058bc</td>
<td>0.031c</td>
<td>0.026b</td>
<td>0.019a</td>
<td>0.078bc</td>
<td>0.009a</td>
<td>0.003bc</td>
</tr>
<tr>
<td>Dead MA 11 3d</td>
<td>0.003d</td>
<td>0.005b</td>
<td>0.003a</td>
<td>0.003b</td>
<td>0.029ab</td>
<td>0.055bc</td>
<td>0.044bc</td>
<td>0.026b</td>
<td>0.012a</td>
<td>0.059bc</td>
<td>0.016a</td>
<td>0.005abc</td>
</tr>
<tr>
<td>M 58 0d</td>
<td>0.062abc</td>
<td>0.007ab</td>
<td>0.011a</td>
<td>0.003b</td>
<td>0.020b</td>
<td>0.050bc</td>
<td>0.047bc</td>
<td>0.034ab</td>
<td>0.013a</td>
<td>0.106abc</td>
<td>0.073a</td>
<td>0.003bc</td>
</tr>
<tr>
<td>M 58 3d</td>
<td>0.043bc</td>
<td>0.010ab</td>
<td>0.014a</td>
<td>0.005ab</td>
<td>0.055a</td>
<td>0.075abc</td>
<td>0.116a</td>
<td>0.055ab</td>
<td>0.019a</td>
<td>0.115abc</td>
<td>0.021a</td>
<td>0.010abc</td>
</tr>
<tr>
<td>M 60 0d</td>
<td>0.071abc</td>
<td>0.007ab</td>
<td>0.015a</td>
<td>0.002b</td>
<td>0.017b</td>
<td>0.048c</td>
<td>0.040c</td>
<td>0.081a</td>
<td>0.020a</td>
<td>0.113abc</td>
<td>0.076a</td>
<td>0.002c</td>
</tr>
<tr>
<td>M 60 3d</td>
<td>0.088a</td>
<td>0.009ab</td>
<td>0.018a</td>
<td>0.004ab</td>
<td>0.028ab</td>
<td>0.076abc</td>
<td>0.084abc</td>
<td>0.043ab</td>
<td>0.024a</td>
<td>0.162abc</td>
<td>0.074a</td>
<td>0.018a</td>
</tr>
<tr>
<td>MA11 0d</td>
<td>0.040c</td>
<td>0.006b</td>
<td>0.009a</td>
<td>0.003b</td>
<td>0.022b</td>
<td>0.047c</td>
<td>0.047bc</td>
<td>0.057ab</td>
<td>0.019a</td>
<td>0.097abc</td>
<td>0.026a</td>
<td>0.004bc</td>
</tr>
<tr>
<td>MA11 3d</td>
<td>0.055abc</td>
<td>0.014a</td>
<td>0.023a</td>
<td>0.006a</td>
<td>0.053a</td>
<td>0.112a</td>
<td>0.130a</td>
<td>0.044ab</td>
<td>0.025a</td>
<td>0.188a</td>
<td>0.062a</td>
<td>0.014ab</td>
</tr>
<tr>
<td><strong>Milk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Milk 0d</td>
<td>0.002a</td>
<td>0.001b</td>
<td>0.001a</td>
<td>0.001b</td>
<td>0.014b</td>
<td>0.013a</td>
<td>0.004b</td>
<td>0.022a</td>
<td>0.003b</td>
<td>0.025b</td>
<td>0.021a</td>
<td>0.003a</td>
</tr>
<tr>
<td>Whole Milk 3d</td>
<td>0.002a</td>
<td>0.004a</td>
<td>0.005a</td>
<td>0.003a</td>
<td>0.021a</td>
<td>0.022a</td>
<td>0.009a</td>
<td>0.029a</td>
<td>0.016a</td>
<td>0.050a</td>
<td>0.008a</td>
<td>0.003a</td>
</tr>
</tbody>
</table>

* Means in the same column not sharing a common superscript are different (P < 0.05)
Figure 1A: Experimental Design: Comparing the impact of mesophilic and thermophilic starter cultures on liquid whey flavor

Figure 1B: Experimental Design: Comparison of the impact of different mesophilic starter cultures on liquid whey flavor