Effects of Mineral Content in Bovine Drinking Water: Does Mineral Content Affect Milk Quality?

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ABSTRACT

Implications of water chemistry on milk synthesis are not well described yet water is an important nutrient for dairy cattle. High mineral concentrations (>0.3 mg/kg Fe and others) may be associated with natural levels in ground water, contaminating sources, drought conditions, or storage systems. This study evaluated effects of added iron in bovine drinking water on milk composition (Ca, Cu, Fe, P) measured by inductively coupled plasma mass spectrometry and oxidative stability measured by thiobarbituric acid reactive substances assay for malondialdehyde (MDA), volatile chemistry and sensory analysis (triangle test). Prepared ferrous lactate treatments, corresponding to 0, 2, 5, and 12.5 mg/kg drinking water levels were given abomasally (10 L/d) to 4 lactating dairy cows over 4 periods (1 wk infusion/period) in a Latin square design. Milk was collected (d6 of infusion), processed (homogenized, pasteurized), and analyzed within 72 h of processing and 7 d of refrigerated storage. No differences in MDA (1.46±0.04 mg/kg) or iron (0.22±0.01 mg/kg) were observed in processed milk. Cross effects analysis (treatment*cow) showed significant differences in calcium, copper and iron ($P < 0.05$). Sensory differences ($P < 0.05$), in treatment vs. control, suggested iron from water sources contributes to milk flavor changes. A case study with high and low (0.99; 0.014 mg/kg) iron treatments revealed no significant differences ($P > 0.05$) in mineral composition (0.23±0.06 mg/kg Fe) or MDA (0.77±0.03 mg/kg) of raw milk. Iron added to milk causes changes in oxidation; high levels of iron in bovine drinking water may not have observed effects.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Water is often overlooked as one of the most important nutrients for dairy cattle yet cows consume a high amount, ca. 0.0946 m^3 (25 gallons), of water per day. In addition, water usage on the dairy farm to clean and cool cows and irrigate the land increases water demand to nearly 5.41 x 10^{-8} km^3/cow/yr (14,300 gallons) which is equivalent to ca. 4.51 x 10^{-9} km^3/cow/mo (1,191 gallons) (Chase, 2006). Global water demand for livestock (animals used for food production) is projected in 2025 at 235.7 km^3, an increase of over 630% compared to the 37 km^3 utilized in 1995. The dairy industry will be affected by the increased demand for water resources (Rosegrant and Cai, 2002).

Dairy farmers must be resourceful to overcome the challenges of water shortages. Recycling or reuse of water for dairy cattle consumption may be necessary during drought or in severely restricted fresh water regions. Though these methods may provide the needed water to produce milk, understanding possible implications of the mineral characteristics of these water sources on cow health and milk quality is imperative (Collignon, 2009). An excess of any heavy metal, particularly iron and copper, may cause adverse effects on milk quality (Hegenauer et al., 1979a).

High concentrations of iron and other heavy metals may be associated with natural levels in ground water, run-off from mining or other contaminating sources, drought conditions, or even from the watering systems used for storing water for animal consumption (McNeill, 2006; Bury et al., 2011). Iron concentrations in groundwater sources are variable. In a study of mineral composition of well water, iron concentration ranged between less than ten µg/kg to greater than 300 µg/kg. The iron concentration in water in the southwest portion of Virginia generally ranges

between 10 to over 300 µg/kg. Areas of the United States with lower concentrations include the mid-west and the Great Plains (Figure 1.1). The northern United States tends to have higher concentrations of iron in comparison to the south. However, the greatest concentrations of iron seem to be most prevalent in the mid Atlantic to north eastern United States (Ayotte et al., 2011). Most dairy production in the United States is located in the west, which has less risk of high iron concentrations in ground water. Dairy production is also higher in the upper Midwest and northeast where there is greater incidence of high iron-contaminated water (MacDonald et al., September 2007).

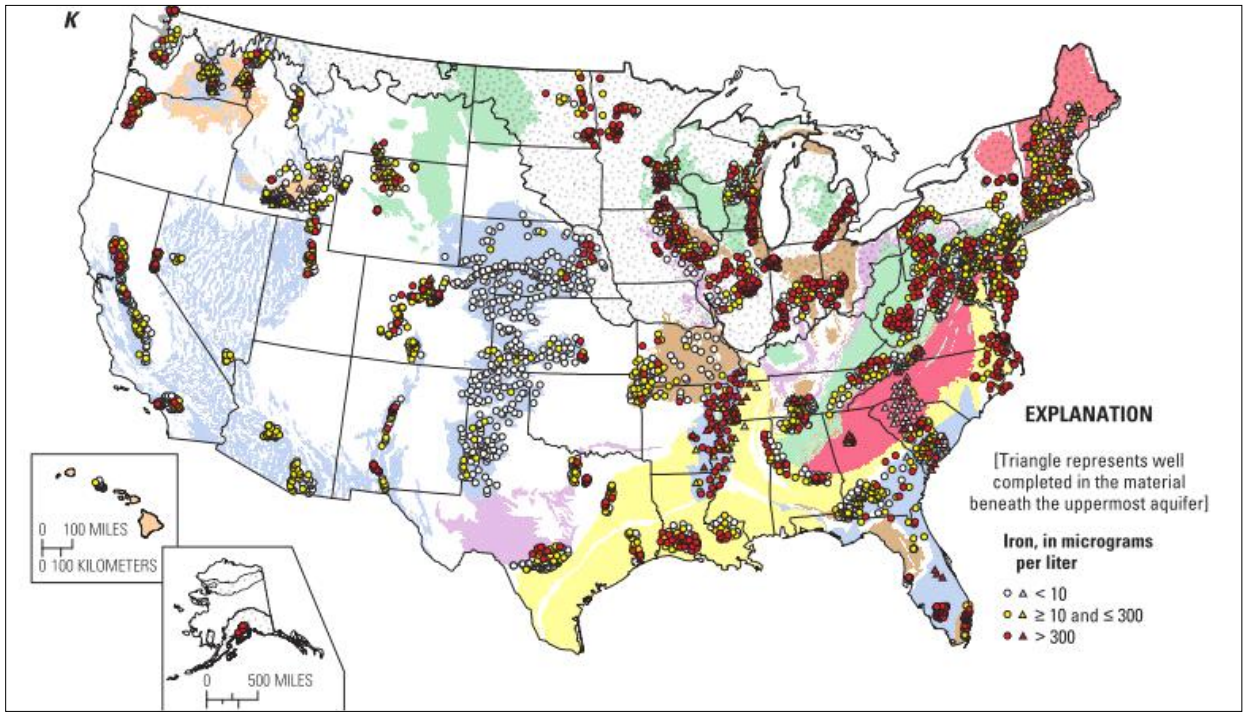

Figure 1.1: Groundwater Iron Concentrations. Groundwater iron concentrations of the USA courtesy of the U.S. Geological Survey (Ayotte et al., 2011). Color gradients on map show major aquifer groups.

High iron concentration in bovine drinking water, at levels higher than the USEPA secondary maximum contaminant level (SMCL) of 0.3 mg/kg, can affect copper and zinc absorption and, thus may affect cattle health and performance (Murthy et al., 1972; National Primary Drinking Water Regulations, 2011). Elevated levels of iron in the water given to cattle

could have a potential effect on milk synthesis, too. Though it is known that iron acts as a pro-oxidant, little research has been identified that examines the specific role of iron in the water given to dairy cattle (Sugiarto et al., 2010). It is known that iron can affect palatability of the water provided to cattle, which may cause the animals to consume less water, leading to less milk production (National Primary Drinking Water Regulations, 2011). This study seeks to answer the possibility that excess iron in the water provided to cattle may alter milk composition and affect processed milk properties and oxidative stability.

It is known that iron added directly to milk can affect oxidation rates and, consequently, the chemical composition of milk (Hegenauer et al., 1979a; Gaucheron et al., 1996; Gaucheron, 2000; Raouche et al., 2009). Outside of the elevated oxidative effects that added iron has on milk, the effect of elevated iron on milk proteins has also been studied. Lactoferrin, an iron-binding protein present in milk, may be affected by additional iron in milk. Lactoferrin concentration increased in human breast milk when mothers were given elevated dietary iron (Zapata et al., 1994). A similar increase in lactoferrin levels in bovine milk in response to increased iron in their dietary water may occur although there are no studies to document this hypothesis Another study based on lactoferrin in infant formula has shown that lactoferrin was able to inhibit oxidation acceleration by binding to the iron added to the infant formula. A suggestion that has been made is to supplement infant formula with lactoferrin to control the effects of iron on oxidation rates (Satue-Gracia et al., 2000).

Supplementation of milk with iron has shown effects, not only on oxidation rates, but also on the mineral distribution in the milk. The addition of ferrous chloride ($FeCl_2$) to skim milk significantly decreased the pH of milk from 6.73 to 6.63. This, in turn, also displaces calcium and inorganic phosphate in the milk, which could potentially affect processes such as cheese

making (Chaplin, 1984; Gaucheron et al., 1996).

Milk used for cheese making must have the proper calcium to phosphorus ratio and pH to allow for a successful process (Upreti and Metzger, 2007). With a lower milk pH, the calcium is more soluble, altering colloidal casein needed for cheese making. If the calcium concentrations in the casein are lower, cheese yield is reduced or calcium chloride must be added. Cheese manufacturers incur economic costs associated with altered milk mineral and protein composition. Potential issues are not limited to cheese making and could extend to other milk products such as a phenomena known as "feathering" in individual coffee creamer cups (Hutkins, 2006). The direct effects that iron in drinking water has on milk synthesis and composition and subsequent milk and dairy product processing and quality are not known.

Excess iron intake may affect milk synthesis, with subsequent effects on milk composition, flavor and oxidation rates. It is possible that an increase in iron can lead to spontaneous oxidation in the final milk product (Hegenauer et al., 1979b). Spontaneous oxidation is defined as the oxidation of milk/milkfat due to a number of factors rather than any recognized cause in particular. Current studies suggest that this spontaneous oxidation may be due to low concentrations of antioxidants but this notion is not fully explored (Nicholson and Charmley, 1993; Timmons et al., 2001). This can cause significant problems since it often occurs in herds that tend to be well-managed and often have no other problems. Many times only a few cows will produce milk that readily undergoes spontaneous oxidation rather than the entire herd, furthering difficulties in finding a solution to the problem (Barrefors et al., 1995). This spontaneous oxidation flavor oven develops without addition of other oxidants like heavy metals and is seemingly unexplained (Frankel, 1991). The susceptibility of milk to spontaneous oxidation often varies and in some milk can occur very quickly. Many commercial dairies have

very few cattle displaying these qualities in their milk (<10%), but subsequent oxidation of commingled milk in the bulk tank can progress rapidly (Timmons et al., 2001). This spontaneous oxidized flavor of milk renders it unsuitable for human consumption in many cases (Nicholson and Charmley, 1993).

Oxidation of milk, and consequently, milk products causes unacceptable off-flavors that can result in substantial economic losses. These impacts in profit loss may not only occur in milk, but also in foods using oxidized dairy ingredients. If ignored, this chemical reaction can create an undesirable and unacceptable product from an otherwise sound food source, affecting consumer satisfaction and product integrity. Research has revealed that consumers can readily perceive oxidized off-flavors, such as those induced by light, and do not prefer these flavors when compared to flavor of untainted products (White and Bulthaus, 1982).

Oxidation of homogenized, pasteurized milk is less susceptible to metal-induced oxidation and when inaccurately stored can quickly become subject to light-induced off-flavor. These two processes, each resulting in oxidation, create distinct flavor profiles. The metal-induced oxidation often causes a cardboard-type off-flavor while the latter produces a burnt feathers characteristic. The metal-induced, or termed generic by some studies, flavors from oxidation also can offer an astringent (pucker) mouthfeel sensation (Alvarez, 2009). These negative sensory characteristics from the milk subsequently can affect dairy product (cream, butter, yogurt, ice cream, milk powder) quality and shelf-life as well as other food products in which these are incorporated.

This study is part of an interdisciplinary project that examines the implications of water quality on cattle health and milk. The goal of this project was to evaluate the relationship between iron content in the bovine water supply and resulting processed milk quality. Milk was

collected from cows under controlled experimental conditions of iron exposure, represented by abomasal infusion, as well as a case study from a dairy farm with two water sources delivering low and high iron concentrations to the lactating dairy cows. Milk quality, with specific attention to mineral composition and milk oxidation, was assessed. This study will offer more information on how iron affects milk synthesis, composition, and resulting product quality and functionality.

The objectives of this study were to:

Characterize the effect of low, moderate, and high levels of abomasally-infused iron on milk composition, specifically iron, copper, phosphorus, calcium, protein and ash content of processed fluid milk;

Determine the effect of iron concentration on oxidative stability of fluid whole processed milk over 7 days of storage;

Identify if low and high iron concentrations from two water sources provided to a lactating dairy herd affected mineral content and oxidative stability of raw milk.

The results of this multidisciplinary study could potentially impact the consideration necessary by dairy farmers to ensure an appropriate water supply for their herd. Water quality is carefully monitored for human consumption but the water supply for the dairy herd producing Grade A milk does not have EPA defined SMCL. These results could potentially have national and international implications pertaining to water use, reuse or recycling in dairy production and processing operations.

CHAPTER II

LITERATURE REVIEW

Water Availability and Quality Needed for Dairy Production and Processing

Water Use in Dairy Production. Water withdrawal is defined as water use that takes water from its source and is no longer available due to evaporation, incorporation into products, consumption or removed from the immediate water environment (Vickers, 2010). In the 2005 U.S. Geographical Survey, a total of 0.008 km^3/d (2,140 M gal) of water was withdrawn and used for livestock (livestock, feed lots, dairy operations) and aquaculture, totaling 3 percent of the water withdrawn in the United States. Of this, 60 percent was supplied by groundwater rather than surface water (Barber, 2009). Standards for water sources on dairy farms are separated into farm operations and milking operations. Farm operation water regulations require that ground water must be a safe distance (depending on the nature of the contaminant and water source) from contamination sources (salts, detergents and other substances that dissolve in water). Milking operations require potable water and some can be reclaimed from heat exchangers for milking operations one time (Grade "A" Pasteurized Milk Ordinance, 2011).

Dairy producers are making the shift to fewer cows producing higher volumes of milk, resulting in lower costs and resources. Since 1944 the annual production of milk per cow has increased fourfold in the United States (53 B kg milk/25.6 M cattle in 1944; 84 B kg milk/9.2 M cattle in 2007) which reduces the overall water requirements but increase the importance of the individual cow (Capper et al., 2009; MacDonald et al., September 2007).

Water Use in Dairy Processing. Water needs for dairy, including processing, are high. Water used in processing fluid milk is for heating, cooling, washing, and clean-up. To avoid contamination, potable water used in milking operations on the farm usually cannot be reused as

reclaimed water must fulfill strict regulations to avoid product contamination (Grade "A" Pasteurized Milk Ordinance, 2011). Dairy production, compared to beef, consumes a relatively high amount of water (Simmons, 2011). Approximately four gallons of water is needed to produce one gallon of milk; some processing plants have effectively implemented conservation strategies to reduce the ratio of water to milk (1:1). Water use has become more efficient as in 2007, as 65 percent less water was being used per gallon of milk produced than in 1944 (Raouche et al., 2009; Simmons, 2011).

Implications of Drought in Dairy Production and Processing. The sparse rain in 2012 and record high temperatures provided for one of the most devastating and widely known droughts in the United States in the last 25 years (Sutter, 2012; U.S. Drought 2012: Farm and Food Impacts, 2013). Drought, as defined by soil moisture depleted below levels for healthy crops (Gleick et al., 2012), has forced farmers to ration feed and water among dairy cows as feed prices increased.

Cows require approximately 0.0946 m^3 (25 gallons) of water per day (Chase, 2006); in response to the drought conditions many dairy cows were culled, lowering milk production as feed and water sources became scarcer. Milk production in 2013 is projected to have no increase due to the high feed costs from the 2012 drought (U.S. Drought 2012: Farm and Food Impacts, 2013). In some cases these extreme conditions have forced producers to sell their stock and consider bankruptcy. Not only are these pressing environmental hazards devastating, but they are also very costly to farmers, consumers, and government (Sutter, 2012). In response, farmers and producers are increasing their water conservation strategies and implementing recycling strategies (Gleick et al., 2012).

Water conservation, recycling and reuse must be used with caution to keep water quality

acceptable (Collignon, 2009). Water that is physically separated from milk products such as water used in heating and cooling for milk operations or in the processing plant can also be reclaimed with limited numbers of reuse and specific applications (culinary steam, cleaning solution, pre-rinse, heating). Reclaimed water in processing cannot be carried over day to day (Grade "A" Pasteurized Milk Ordinance, 2011).

When water conservation simply is not enough and water must be reused or recycled, other strategies like reverse osmosis are employed to ensure water safety and quality in effluent streams by reverse osmosis. This method, while excellent for filtering out impurities, can be very costly (Milani et al., 2011). Large corporate entities like the McDonald's Corporation are striving to conserve by using less water (e.g. beef processing facilities) for example (Muirhead, 2012).

Water Quality Implications in Dairy Production and Processing. Agricultural, industrial and residential influences contribute to water contamination. Water contaminants include microorganisms, disinfectants (and byproducts), chemicals (organic and inorganic), radionucleotides, minerals and metals (National Primary Drinking Water Regulations, 2011). These factors not only play a role in the effects on water at the biological level, but water chemistry is also affected. Such contaminants result in undesired chemicals in the water, alterations of the natural pH balance and increased costs for water treatment to ensure its safety for consumers (Gleick et al., 2012). The Environmental Protection Agency (EPA) in cooperation with the United States Department of Agriculture (USDA) have established effluent limitations as well as restrictions on feeding to limit nitrogen and phosphorus produced by the cows to limit the impacts on water resources.

Water composition widely varies across the United States, particularly in groundwater (Ayotte et al., 2011). Water can carry many microminerals including heavy metals. The most

pertinent heavy metal of concern for this study is iron. Iron is notably one of the most important metals mined in the world and is imperative for steel production, with about 98 percent of mining efforts channeled into steelmaking. This mining process is vital for manufacturing, transport and construction industries but water can become easily contaminated (Bury et al., 2011). Even abandoned mines from years past continue to contaminate streams in the United States (Gleick et al., 2012). Pollution of groundwater and surface water alike have not been restricted to mining effects; municipal and agricultural practices have also contributed (Theron et al., 2008).

Due to the negative sensory aspects and discoloration that iron imparts on drinking water, the United States Environmental Protection Agency (EPA) has assimilated secondary standards, which are non-enforceable guidelines to regulate contaminants. These specific contaminants have aesthetic effects or changes on the appearance of water that may be displeasing to consumers. For iron the secondary standard is 0.3 mg/kg (National Primary Drinking Water Regulations, 2011). Data gathered at Virginia Polytechnic Institute and State University has revealed a groundwater iron concentrations of ca. 0.2 mg/kg, near the secondary standard set by the EPA (Dietrich, 2007).

Iron in water systems can be found in various chemical forms. Ferrous iron found in groundwater (Fe^{2+}) can oxidize to become ferric iron (Fe^{3+}), forming precipitates. This oxidation process can be affected by pH, temperature and the presence or absence of several catalysts. When there are large amounts of ferrous iron present in water the dissolved oxygen in the water will be used to oxidize the ferrous iron to ferric iron, depleting the oxygen in the water and steadily lowering the pH of the water, affecting water quality (Stumm and Lee, 1961).

Despite the negative effects iron can cause, secondary standards for iron are not

enforceable and iron levels are unregulated. In some cases, groundwater levels have been recorded to be as high as 240 mg/kg, far above the secondary standard set by the EPA. Iron also varies among diel periods (24 hour blocks of time) with the cycle of ferrous to ferric iron and back (Kay et al., 2009). Iron may bind to phosphorus, a vital nutrient for crop growth. With an increase in iron, and in some cases aluminum, there is less available phosphorus for plants, causing problems for agriculture (Sims and Sharpley, 2005). If these excess nutrients, particularly metals, are not controlled they can ultimately create pollution problems by building up in soil, surface water, and in groundwater (MacDonald et al., September 2007). The effects of iron from bovine drinking water on milk phosphorus concentrations have not been studied.

Implication of Iron in Bovine Nutrition. Water and dairy farming share a close relationship since the major component of milk is water (87 %). The influence of water on milk production is demonstrated by higher levels of milk production in areas of high rainfall (Tait et al., 2005). During a water shortage or water contamination, milk production can be affected by alterations in milk quality or lower production (Chase, 2006). Due to high levels of water needed per cow, it is likely that high (above EPA secondary standards) iron content in water (>0.3 mg/kg) could influence milk composition and oxidative stability (National Primary Drinking Water Regulations, 2011). Genther and Beede demonstrated that metallic flavors in milk, as occurs at the EPA SMCL, may cause cattle to consume less water and produce less milk (Gary et al., 2007; Genther and Beede, 2013).

Minerals may also contaminate milk in post-processing situations if water high in minerals is used for cleaning purposes. Water composition, varying by location, can be affected by plumbing materials. Copper, iron, and stainless steel are all metals used for piping. Iron pipes can leech iron into the water being carried (Gidi et al., 2004). Iron corrosion in plumbing is

affected by a number of factors, many of which are common in water distribution systems (McNeill, 2006). Due to the metal leeching problems many metal pipes have been replaced with galvanized piping and plastics (McNeill, 2006).

Milk Composition and Mineral Stabilization

General Milk Composition Holstein cattle produce milk containing ca. 3.5% milk fat and generally produce 10-40 kilograms per day of milk, which is composed of ca. 8.7-34.8 kilograms of water (2.3-9.21 gallons). Whole processed milk is comprised of about 87% water, 3.25% fat and 4% protein. The remaining constituents in milk are lactose, minerals and other solids (Hunt and Nielsen, 2009). As a high percent of milk is water, it is important to consider the dietary water of the cow and how this could affect milk quality.

Milk is also rich in bioavailable minerals used by the human body, including phosphorous and calcium. Other minerals of interest are iron and copper, however both are pro-oxidants, which have biological significance as well (Hegenauer et al., 1979a; Hegenauer et al., 1979b). Copper is present in levels of 0.1-0.6 mg/kg in raw milk (Goff and Hill, 1993; Hunt and Nielsen, 2009). Raw milk generally contains 930 mg/kg of phosphorus (900-1000 mg/kg) where calcium content is often higher (1180 mg/kg) (White and Davies, 1958; Goff and Hill, 1993; Jensen, 1995). Calcium is present in milk in the form of primarily calcium citrate as well as calcium phosphate (in casein) (Jensen, 1995). Iron averages 0.5 mg/kg with a range of 0.3-0.6 mg/kg in raw milk (Hunt and Nielsen, 2009). Similar concentrations of minerals have been found in whole processing (pasteurized, homogenized) milk (1130 mg/kg Ca, 0.11 mg/kg Cu, 0.3 mg/kg Fe, 910 mg/kg P) (Milk Facts, 2013).

Iron and phosphorous bind to milk proteins (Jenness, 1974; Walstra et al., 1984; Sims and Sharpley, 2005). About 10 percent of iron in milk is bound to casein proteins, with 20-30 percent

bound to iron-binding proteins like lactoferrin and transferrin and is in the ferric (Fe^{3+}) form (Jenness, 1974; Fransson and Lonnerdal, 1983; Jensen, 1995).

Iron content of bovine milk tends to vary with location, stage of lactation, time of the year and breed. A 0.4 mg/kg fluctuation of iron content in milk has been recorded during a bovine lactation period. Higher levels of iron are expected in early stages of milk production and highest (2-3 times that of normal milk) in milk containing colostrum (1-2 mg/kg) (Underwood, 1971; Murthy et al., 1972; Jensen, 1995). Iron content of milk is not affected by diet but studies on the effects of drinking water are minimal (Murthy et al., 1972; Fransson and Lonnerdal, 1983).

Proteins. About 20 percent of iron is located in the fat fraction of milk, with 30-60 percent bound to transferrin or lactoferrin in the aqueous phase, and another 10 percent bound to the casein. Cow milk contains 0.019-0.194 mg/kg each transferrin and lactoferrin (Jenness, 1974; Fransson and Lonnerdal, 1983; Jensen, 1995; Lönnerdal, 2009). Milk proteins include whey (20 percent) and casein (80 percent). The casein micelles occur in milk as colloidal complexes of protein subunits (α_{s1}, α_{s2}, β, κ) and salts (calcium phosphate) (Jensen, 1995; Hutkins, 2006). These proteins not only act as carriers for minerals but they contribute to the physical characteristics like flavor, color, gelling and foaming properties (Jenness, 1974).

Lactoferrin is the primary whey protein in milk and has a high affinity for ferric iron (Jenness, 1974; Fransson and Lonnerdal, 1983). Lactoferrin has two binding sites for iron. Manganese and zinc also bind to the same site (Lönnerdal et al., 1985). Lactoferrin levels have been shown to decrease throughout milk production but are higher in cattle with infections, suggesting a role in infection control (Rainard et al., 1982; Satue-Gracia et al., 2000). Antimicrobial activity of lactoferrin has been studied and it may aid in nutritional uptake in

infants (Lönnerdal, 2009). Aside from antimicrobial properties, lactoferrin has demonstrated the ability to act as an antioxidant by binding iron (Satue-Gracia et al., 2000).

The relationship between increased dietary lactoferrin and the amount of iron in the resulting human breast milk is unknown (Satue-Gracia et al., 2000). Human infants are not able to readily consume bovine milk due their inability to fully digest the proteins and minerals (Jensen, 1995). For infants unable to consume breast milk fortified infant formula is often given, supplemented with iron. Iron as a pro-oxidant often makes infant formulas susceptible to oxidation. Lactoferrin, a metal chelator, inhibited the prooxidant effects of iron in infant formula, attributed to its high iron affinity (Jensen, 1995; Satue-Gracia et al., 2000; Decker et al., 2010a). Transferrin inhibits lipid oxidation in foods by binding to iron; affinity is strongest at a neutral pH (Mancuso et al., 1999; Decker et al., 2010a).

Lipids. Milk contains antioxidant properties including the saturation of milk lipids and the presence of vitamin E (tocopherol) (Varnam and Sutherland, 1994). Light, temperature, and metals contribute to an increased risk autooxidation, leading to milk oxidation despite the natural protection (Clark et al., 2009).

Pasteurized milk, unlike raw milk, does not tend to exhibit auto oxidation but light-induced reactions are a cause of concern. Milkfat globules in raw milk are enveloped by the milkfat globule membrane (MFGM), which provides protection of milkfat against oxidative processes until disruption, such as homogenization, occurs. When homogenized, the milkfat globule membrane is disrupted and contact of trace minerals (Fe, Cu, Zn) with lipids greatly increases. Homogenization increases the surface area of the fats by ten times or more (Decker et al., 2010b). Churning of milk disrupts the MFGM, increasing the chances of lipids coming into contact with minerals.

During homogenization, emulsification of milkfat with the protein fraction occurs, exposing lipids to iron binding proteins. A stable phosphoprotein protective barrier might prevent the iron from affecting the lipids by staying bound to the proteins like lactoferrin (Hegenauer et al., 1979b). Lactoferrin and transferrin may prevent the interaction of minerals with milk lipids (Allen and Hamilton, 1994). If iron fortification can be carried out such that the iron binds to the proteins, the oxidation process slows by a significant degree (Sugiarto et al., 2010; Guzun-Cojocaru et al., 2011).

Other than post-processing effects, research supports the notion that different milking periods can have an effect on oxidation of milk. Winter milk, higher in acidity than summer milk, is more susceptible to oxidized flavors (Brown and Thurston, 1940).

Explorations on the oxidative stability of milk as determined by feed types have been considered. In particular the types of fatty acids in the milk produced by the bovine as a result of varying feeds and additives have been observed (Havemose et al., 2006). This alteration of the milk fatty acid profile is thought to have a health benefit, though it comes at a cost. With more unsaturated fatty acids comes a higher risk of oxidation. Specifically, the polyunsaturated fats (PUFAs) are at an increased loss risk from the oxidation reactions (Gonzalez et al., 2003). Alternatively, modification of fatty acid profiles of milk has been used to suppress oxidative processes in milk. Focant et al. supplemented cattle feed with antioxidants to change the fatty acid profiles of milk. This was relatively effective and resulted in less oxidation of the final milk product (Focant et al., 1998).

Role of Iron in Milk Shelf Life

Effect of Iron Induced Oxidation on Milk Flavor. Milk, naturally low in iron, has been supplemented with iron to increase nutritive value (Kurtz et al., 1973). Iron-supplemented milk was used in chelation (with proteins) studies to prevent the iron from increasing oxidation rates (Hegenauer et al., 1979b; Guzun-Cojocaru et al., 2011). Elevated levels of ferrous iron causes increased lipid peroxidation (Hegenauer et al., 1979b). When iron is added in the ferric form less lipid peroxidation results (Kay et al., 2009). Though there is less lipid peroxidation, the addition of the ferric compounds to milk can still impart metallic flavors (Gaucheron et al., 1996). Heavy metals (iron) can decrease the stability of fats even at low levels and trace amounts of metals can cause further oxidation problems (Dobarganes and Velasco, 2002). Iron supplementation of milk can cause various undesirable effects in the final product contributing to a decreased shelf life (Gaucheron et al., 1996).

Oxidation of milk can have economic and product quality impacts, as milk plays a major role as a product as well as a food ingredient. Due to the relatively high fat content of whole milk (3.25 percent) it is particularly susceptible to chemical changes as a response to time, light, and pro-oxidants.

Milk is susceptible to oxidation, altering the protein structure, fatty acid composition, nutrient value, and sensory quality (Decker et al., 2010b). Oxidation alone can contribute to the poor acceptability of many dairy products and shorten product shelf life. During oxidation, peroxides are formed due to the reaction between the unsaturated fatty acids and oxygen. More specifically, the esters in these unsaturated fatty acids reaction with the oxygen which in turn yields carbonyl compounds. These carbonyl compounds then provide the “oxidized” flavor that is unpleasant to many consumers and are detectable at very low levels (Brown and Thurston,

1940). Milk has a mild and mellow flavor, oxidative defects can be readily noted and are not easily masked. The “oxidized” flavor can often be detected by consumers, especially in fluid milk (Frankel, 2005). Flavor profiles of milk affected by metal-induced oxidation include cardboard, papery, metallic, painty, cappy, oily, and fishy (Havemose et al., 2006; Clark et al., 2009). Metal-induced flavor is characterized by a rapid taste reaction when the product is placed in the mouth. The flavor also has a tendency to linger even after the sample is expectorated (Bodyfelt et al., 1988; Clark et al., 2009).

Off-flavors associated with oxidation and poor quality can carry over into other products including milk, butter, yogurt, and cream (Decker et al., 2010b). Metallic flavors, originally pinpointed to metals used in pipes in dairy processing plants, have caused serious metallic off-flavors with high frequency. Maintenance of product production without metallic off-flavors is a challenge, as water supplies must be constantly controlled against the exposure to copper, iron and manganese. Butter and cheese have a water rinse processing step and water quality must be considered. Hard water can relay metals to the final dairy product. Metallic or oxidized flavors in butter may be initiated by rinsing water; the oxidized flavors are readily detected during the classification process are often given a “below grade” rating (USDA, 1989; Clark et al., 2009). While many off-flavors are attributed to light-oxidized flavors, metals such as copper are also responsible for the formation of off-flavors (Jenq et al., 1988; Cadwallader et al., 2007).

Oxidation can be the cause of significant losses. Various techniques have been tested and are used in order to incorporate antioxidants into milk products. Several of these methods are by injecting antioxidants into the cows, adding antioxidants to feeds or the direct addition of antioxidants into final dairy products (Nicholson and Charmley, 1993; Jung et al., 1998; van Aardt et al., 2005a). The bioavailability is greatly decreased when antioxidants are ingested and

antioxidant levels will not remain constant as storage of the milk will decrease the antioxidant level (Jensen, 1995; Nielsen et al., 2001). Currently antioxidants are added directly to milk and are considered an additive and must be labeled as such (Food and Drug Administration; van Aardt et al., 2005a).

Assessment of metal induced oxidation. Oxidation is widely categorized into two types: photooxidation and autooxidation, the latter of which is more pertinent to this study. Lipid oxidation yields free radicals, lipid hydroperoxides, and a number of secondary oxidation products (Decker et al., 2010a).

The process of oxidation can be characterized by three stages: initiation, propagation, and termination (Fennema, 1996). Hydroperoxides, primary products of oxidation, are readily formed and can cause further degradation to the lipids and destruction of flavor quality. Hydroperoxides, intermediate compounds, are decomposed into alkoxy radicals and hydroxy radicals by homolysis of the peroxide bond to further the oxidation process (Henry et al., 1992).

The secondary, and sometimes tertiary, oxidation products include aldehydes, ketones, alcohols, hydrocarbons and core aldehydes. Secondary products are those that are detectible at generally low levels and can provide negative sensory aspects: off-flavors and aromas (Gonzalez et al., 2003; Decker et al., 2010b). Many different molecular species are responsible for the volatiles produced from the oxidation reactions and make quantification of oxidation difficult.

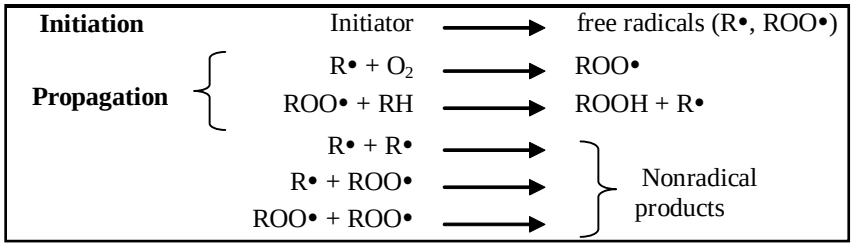

Figure 2.1: Stages of Autooxidation (Fennema, 1996)

Many tests can be run on oxidized products to test for certain volatiles, indicating differing degrees of oxidation. Analytical tests include peroxide value (PV), anisidine value (AV), thiobarbituric acid reactive substances (TBARS), carbonyl value, and total polar compounds (Fritsch, 1981). TBARS assessment is a widely known and accepted way to detect lipid peroxidation by measuring a secondary product of oxidation, malondialdehyde (MDA) (Moore and Roberts, 1998).

Conjugated diene analysis can also be used to measure hydroperoxide, primary oxidation products, development (Frankel, 2005). Measured at 234 nm using a spectrophotometer will allow the detection of conjugated dienes (Moore and Roberts, 1998). This method is a rapid method that requires minimal reagents and no reaction time (Moore, 2009). Peroxide value is another method for determining degree of oxidation, though low peroxide values do not necessarily note that no oxidation has taken place, making this method somewhat incomplete (Gonzalez et al., 2003).

Other methods for assessing degrees of oxidation include chromatographic methods. Gas chromatography (GC) is more useful for quantification of certain oxidation products which allows a greater understanding of the reaction taking place. These methods include high performance liquid chromatography (HPLC), high performance size exclusion spectroscopy (HPSEC), gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS). Hexanal, octanal, and nonanal, all products from oxidation reactions, were consistently and significantly present in all samples that had off-flavors (Barrefors et al., 1995). GC used by van Aardt et al. was used to detect hexanal, heptanal and 1-octene-3-one as responsible for oxidative flavors (van Aardt et al., 2005b). The GC-MS method is used analyze the volatile secondary products of oxidation, providing an indication of oxidation.

Sensory analysis is another way to assess the degree of oxidation in a product. Analytical methods are sometimes unable to detect very low levels of volatiles responsible for oxidation, whereas human response may be much more sensitive to the volatiles (Ogden, 1993). Sensory methods can bridge a gap that the analytical methods cannot. Comparisons between the tested analytical methods and sensory results are common in research as the two methods often agree on results (Decker et al., 2010b). Significant sensory differences have been shown to agree with analytical methods reveling effects of light oxidation (van Aardt et al., 2005b). In particular the following compounds; hexanal, 2-hexanol, heptanol, 2-octenal, and 2,4-decadienal correlate with oxidation as detected by sensory methods and the TBARS analysis (Gordon et al., 2007).

CHAPTER III
EFFECTS OF MINERAL CONTENT OF BOVINE DRINKING WATER: DOES IRON AFFECT MILK QUALITY?

ABSTRACT

Water is an important nutrient for dairy cattle; however, influences of water chemistry on milk synthesis are not well described. High mineral concentrations (>0.3 mg/kg Fe and other metals) may be from natural sources in ground water, run-off from contaminating sources, drought, or water storage systems. This study evaluated the effects of added iron in bovine drinking water on milk composition and oxidative stability. Ferrous lactate treatments corresponding to 0, 2, 5, and 12.5 mg/kg drinking water concentrations were delivered through the abomasum at 10 L/d to 4 lactating dairy cows over 4 periods (1 wk infusion/period), in a Latin square design. On d6 of infusion milk was collected, processed (homogenized, pasteurized), and analyzed. Mineral content (Fe, Cu, P, Ca) was measured by inductively coupled plasma mass spectrometry. Oxidative stability of whole processed milk was measured by thiobarbituric acid reactive substances (TBARS) assay for malondialdehyde (MDA) and sensory analysis (triangle test) within 72 h of processing and after 7 d of storage (4^{o}C). Significant sensory differences ($P < 0.05$) between processed milks from cows receiving iron and the control infusion were observed. No differences in TBARS (1.46±0.04 mg/kg MDA) or mineral content (0.22±0.01 mg/kg Fe) were noted. Ca, Cu, and Fe concentrations had significant differences for the 2-way interaction of iron treatment by cow ($P < 0.05$). A case study of raw milk from cows receiving water with naturally high (0.99 mg/kg) and low (0.014 mg/kg) iron content revealed no significant differences ($P > 0.05$) in mineral composition (0.23±0.06 mg/kg Fe) or analytical measures of oxidation (0.77±0.03 mg/kg MDA). While iron added directly to milk causes changes in oxidation of milk, high levels of iron given to cattle may not have an observed effect.

INTRODUCTION

Water is often overlooked as one of the most important nutrients for dairy cattle yet cows consume a high amount, ca. 0.095 m^3 (95 L; 25 gallons), of water per day, plus the water used to irrigate land and cool cattle (Chase, 2006). Global water demand for livestock (animals used for food production) is projected in 2025 at 235.7 km^3, an increase of over 630% compared to the water utilized in 1995. The dairy industry will be affected by the increased demand for water resources (Rosegrant and Cai, 2002).

Dairy farmers must be resourceful to overcome the challenges of water shortages. Recycling or reuse of water for dairy cattle consumption may become necessary for augmenting ground and surface water resources. Understanding possible implications of the mineral characteristics of water sources on cow health and milk quality is imperative (Collignon, 2009). An excess of any heavy metal, particularly iron and copper, may cause adverse effects on milk quality (Hegenauer et al., 1979a).

High concentrations of iron and other heavy metals may be associated with natural levels in ground water, run-off from mining or other contaminating sources, drought conditions, or even from the watering systems used for storing water for animal consumption (McNeill, 2006; Bury et al., 2011). Iron concentrations in groundwater sources are variable. In a study of mineral composition of well water across the U.S., iron concentration ranged between less than ten µg/kg to greater than 300 µg/kg. The iron concentration in well water in the southwest portion of Virginia displays that broad range (10 to 300+ µg/kg) (Ayotte et al., 2011).

High iron concentration in bovine drinking water, at levels higher than the USEPA secondary maximum contaminant level (SMCL) of 0.3 mg/kg (300 µg/kg), can affect copper and zinc absorption, thus, may affect cattle health and performance (Murthy et al., 1972; National

Primary Drinking Water Regulations, 2011). Elevated levels of iron in the water given to cattle could have a potential effect on milk synthesis, too. Though it is known that iron acts as a pro-oxidant, little research has been identified that examines the specific role of iron in the water given to dairy cattle (Sugiarto et al., 2010). It is known that iron can affect palatability of the water provided to cattle, which may cause the animals to consume less water, leading to less milk production (National Primary Drinking Water Regulations, 2011). This study seeks to answer the possibility that excess iron in the water provided to cattle may alter milk composition and affect processed milk properties and oxidative stability (Jensen, 1995). As iron added directly to milk affects oxidative stability, it also affects the chemical composition of milk. Addition of ferrous chloride to skim milk has shown a decrease in pH and displacement of minerals such as calcium and inorganic phosphate from colloidal phase to the aqueous phase in the β-casein (Hegenauer et al., 1979a; Chaplin, 1984; Gaucheron et al., 1996; Gaucheron, 2000; Raouche et al., 2009).

Oxidation of milk and, consequently, milk products causes unacceptable off-flavors that can affect milk quality, contributing to decreased product sales. Off-flavor impacts may not only occur in milk but also in foods using oxidized dairy ingredients. Oxidation reactions can create an undesirable and unacceptable product from an otherwise sound food source, affecting consumer satisfaction and product integrity. Research has revealed that consumers can readily perceive oxidized off-flavors and do not prefer these flavors when compared to flavor of untainted products (White and Bulthaus, 1982).

This experimental study is part of an interdisciplinary project that examines the implications of water quality on cattle health and milk quality. The goal of this project was to evaluate the relationship between iron content in the bovine water supply and resulting processed

milk quality This study will offer more information on how iron affects milk synthesis, composition, and resulting product quality and functionality.

The objectives of this study, as addressed through four experiments, were:

Experiment 1: Effect of Direct Addition of Iron on Oxidative Stability of Milk.

Evaluate the effect of direct addition of iron, up to 30 mg/kg ferrous sulfate, on changes in oxidative characteristics (sensory differences, malondialdehyde concentration) of commercially processed milk.

Experiment 2: Validation of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and TBARS Extraction Protocols on Processed Milk

Evaluate the reproducibility of mineral analyses used in the experimental and case studies.

Experiment 3: Influence of Abomasal Infusion of Iron on Oxidative Stability of Processed Milk

Characterize the effect of low (200 mg/kg), moderate (500 mg/kg), and high (1250 mg/kg) levels of abomasally-infused iron on milk composition, specifically iron, copper, phosphorus, calcium, protein and ash content of processed fluid milk. Determine the effect of iron concentration on oxidative stability of fluid whole processed milk over 7 days of storage.

Experiment 4 (Case Study): Effect of Low and High Iron Sources in Bovine Drinking Water on Mineral Composition and Oxidative Stability of Raw Milk

Identify if low and high iron concentrations from two water sources provided to a lactating dairy herd affected mineral content and oxidative stability of raw milk.

The results of this multidisciplinary study could potentially impact the consideration necessary by dairy farmers to ensure an appropriate water supply for their herd. Water quality is carefully monitored for human consumption but the water supply for the dairy herd producing Grade A milk does not have EPA-defined SMCL. These results could potentially have national and international implications pertaining to water use, reuse or recycling in dairy production and processing operations.

MATERIALS AND METHODS

Sample collection/preparation steps and experimental design for each experiment are described first. Analytical methods used in more than one study are subsequently described.

Experiment 1: Effect of Direct Addition of Iron on Oxidative Stability of Milk.

Preparation of Iron Stock Solutions. Four iron stock solutions (0 mg/kg (control), 0.3 mg/kg, 3 mg/kg, 30 mg/kg) were made with food grade ferrous sulfate (FeSO4; Sigma-Aldrich, PA, CAS 13463-43-g). Solutions were prepared by adding ferrous sulfate (control (0 mg/kg): no addition; low: 0.010 g; medium: 0.10g; high: 1.0g) into distilled water (30 mL; wt/wt) with agitation. Stock solutions were made within one hour of application into the milk.

Preparation of Iron-Contaminated Processed Milk Samples. Whole milk (3.25% milkfat; Kroger brand, Cincinnati, OH) was purchased from the local supermarket in five one gallon packages (high density polyethylene, no visible light protection additives). All packages had the same code date and were selected from the front row display on neighboring levels. Each gallon of milk was prepared such that approximately 3 L remained in the original packaging by means of removing and discarding 785 mL of the commercial milk. Four treatments (control 0mg/kg, low 0.3 mg/kg, medium 3.0 mg/kg, high 30 mg/kg) were prepared by adding 30 mL of each stock solution to 3L of milk (one package per iron concentration; two packages per control). Calculated ferrous sulfate concentrations in the milk, based on stock solution addition, were 0 mg/kg (control), 0.0027 mg/kg (low), 0.027 mg/kg (medium) and 0.27 mg/kg (high). Milk was stored in a dark walk-in cooler (Tonka, Hopkins, MN) at 4^{o}C for 3 days before analyses were completed. Oxidative stability was determined by sensory triangle test analyses and thiobarbituric acid reactive substances (TBARS) assay (see Analytical Methods section).

Sensory and Statistical Analyses. Duplicate TBARS assays were completed in each

replicate (n=3). An ANOVA was run to determine differences between treatments. Sensory analysis testing was approved by the Virginia Tech Institutional Review Board (IRB 12-158, Appendix A). Statistical parameters for the sensory testing by triangle tests were $\alpha = 0.05$, $\beta = 0.20$, and proportion of discriminators (p_d) of 30%, requiring 43 sensory participants (43 observations per test comparison). A critical number of 21 correct observations per test comparison was needed to claim that the two samples were different (Meilgaard et al., 2007).

Experiment 2: Validation of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and TBARS Extraction Protocols on Processed Milk.

Two commercially processed milk products (3.25% milkfat; Simple Truth Organic, Kroger, Cincinnati, OH; Kroger brand, Kroger, Cincinnati, OH) were purchased locally and stored in a dark walk-in cooler at 4°C for one day. A nitric acid digestion extraction procedure for infant formula was applied to extraction of minerals in both milk products with subsequent analyses of extracted minerals by ICP-MS (Cook and Suddendorf, 1984). The degree of variability of the extraction process and ICP-MS analysis was estimated based on standard deviation of quadruplicate extractions from each product. Reliability of the mineral composition from the extraction was compared to literature values for processed (homogenized, pasteurized) milk (Murthy et al., 1972; McKinstry et al., 1999; Brescia et al., 2003). A TBARS assay also was completed on the commercial milk samples (quadruplicate extractions) to verify reliability of the extraction and to estimate degree of variation over 7 days (days 1, 8) of refrigerated storage (4°C; no light exposure). An ANOVA was used to determine differences between days of storage and milk type (organic, nonorganic). Data was compared to previous data from our laboratory and to published data, as available.

Experiment 3: Influence of Abomasal Infusion of Iron on Oxidative Stability of Processed Milk

Milk from this study was collected from cows receiving abomasal infusion as previously described in the literature in press (Feng et al., 2013).

*Preparation of Ferrous Lactate Solutions***.** Ferrous iron was chosen as it has high bioavailability for the dairy cow (Nutrient Requirements of Dairy Cattle, 2001). Ferrous lactate ($C_6H_{10}FeO_6$) treatments were prepared at four concentrations: control (0 mg/kg), low (200 mg/kg), medium (500 mg/kg) and high (1250 mg/kg). These levels corresponded to drinking water levels of 0 mg/kg, 2 mg/kg, 5 mg/kg, and 12.5 mg/kg, assuming each cow drinks 100 L/day. Cows were infused with 10 L/day. Ferrous lactate solutions were made using double distilled water and the iron content of each solution was monitored each day. Infusion bags were changed nightly and immediately taken to lab for iron analysis to ensure that content was 95% or greater of the intended amount. Spectrophotometry was used to ascertain iron content (DU-460 Spectrophotometer, Beckman, Miami, FL) (Feng et al., 2013).

Preharvest Experimental Conditions and Raw Milk Collection. Before the beginning of the study, approval for the study from the Virginia Tech Institutional Animal Care and Use Committee (IACUC) was obtained (12-027-DASC). During the treatment period, four ruminally-cannulated early lactation (second lactation) cows (2 Holstein and 2 Holstein x Jersey cross) were isolated from external water sources to eliminate outside variables, housed in individual stalls, and given a standard basal diet and a standardized water source (Table D1).

The treatments given to each cow were randomly assigned during each of four 2-week periods so that each cow served as her own control (Table 3.1). Each cow each received all iron water treatments in a 4x4 Latin square design. Each period, lasting two weeks, consisted of a wash out period during the first 7 days when the cattle were individually fed in Calan doors

(American Calan, Northwood, NH) once each day, had consistent access to feed and water except during milking, and received no abomasal infusion. Milk collected during this time was discarded. From day 8 to 14, each cow was fed twice daily (0600 and 1800 h) in individual stalls. Individual cows were infused with 10 L of ferrous lactate water solution of the assigned treatment via the abomasum. Each cow had continuous access to water; feed was offered at 5-10%, in wet basis, excess of the previous day's intake. Milk that was not analyzed from the study was decanted in an appropriate manner.

On day 13 of each period, milk from the evening (1800 hour) milking of each cow was collected, allowing for 6 days of total infusion time before milk collection. Raw milk was collected in 5-gallon stainless steel milk cans, transported immediately to the Food Science and Technology Dairy Processing Laboratory, and stored in a walk in cooler (Tonka, Hopkins, MN) at 4°C. Milk from each cow was processed within 18 hrs of collection. For a more detailed outline of the design see Table D2.

Table 3.1: Experiment 3, Latin Square Design. 4 x 4 Latin square design illustrating randomization (between cows; within cow) assignments of iron concentration[1] for abomasal infusion for each period[2].

Cow Number[3]	Period 1	Period 2	Period 3	Period 4
4541	Control	Low	Medium	High
4543	Low	High	Control	Medium
4558	Medium	Control	High	Low
4559	High	Medium	Low	Control

[1] Each 10 L solution contained control (0 mg/kg), low (200 mg/kg), medium (500 mg/kg) or high (1250 mg/kg) levels of ferrous lactate.
[2] Period of infusion: 7 days post wash out period of 7 days; each cow infused with 10 L/day of the assigned iron treatment.
[3] Jersey x Holstein (4541,4543); Holstein (4558, 4559)

Milk Processing. Milk from each cow was processed separately. Milk was pre-warmed to 55-60°C and separated into cream and skim milk using a pilot plant separator (Elecrem separator, model IG, 6400rpm, Bonanza Industries Inc., Calgary, Alberta, Canada). Cream was added to

skim milk to achieve 3.18%±0.04% milkfat, as verified by the Babcock procedure using AOAC 989.04 (Bradley, 2000). Milk was homogenized using a laboratory 2-stage homogenizer (13.8 Mpa (2000psi)—first stage; 5.52 Mpa (800 psi)—second stage)(model 15MR, 55.2 Mpa (8000psi), APV Gaulin, Inc., Everett, Massachusetts, U.S.A.).

Standardized milk was vat pasteurized at 66°C for 30 minutes. Microbial quality was assessed by aerobic plate count standard methods using aerobic count petrifilm (3M Petrifilm, Microbiology Products 3M Health Care, ST Paul MN), following standard methods (Laird DT, 2004). Processed milk, two gallons per cow (treatment), was packaged in translucent food grade high density polyethylene gallon packages. Packages were sanitized with a chlorine (100 mg/kg) rinse (dH_2O). Milk packages were stored inside a large portable cooler to prevent light penetration and the cooler was placed in a larger walk-in cooler (Tonka, Hopkins, MN) at 4°C case for 11 days. This holding period allowed for adequate time for all analyses to be run, as it was not possible to run all analyses within 24 hours, effectively showing a difference in week one and week two analyses (day 1 and day 8). Each analysis procedure was repeated on the same day of each week. Each period sequence was as follows: processing (d0), TBARS (d1, d8), GC-MS and sensory (d2, d9), protein, ash and ICP-MS preparation (d3, d10). Week one analyses were run within 72 h of milk processing (subsequently designated d1) and week two analyses were run within 7 days of week one analyses (subsequently designated d8).

Virginia Tech IRB approval was received prior to recruitment of human subject participants (IRB 12-227, Appendix E). Participants completed three sensory triangle tests (d1 and d8), comparing milk from the control cow to each treatment. The order of the three triangle tests and within each 3-sample test was balanced across panelists. Statistical parameters of α = 0.05, β=0.3, and p_d of 30% required a minimum of 36 observations per comparison on each day

of testing (Meilgaard et al., 2007). A critical number of 18 correct observations per test comparison was needed to determine that the treatment milk was different from the control milk on each testing day of each period when n=36 participants (Meilgaard et al., 2007). A critical number of 58 correct observations per overall test comparison over the four periods (n = 144 total observations) was needed to establish that there was a significant difference between control and treatments for day 2 and day 9. The combined number of observations (n=288) per comparison required a critical number of 96 to establish if there was a significant difference between control and each treatment.

Statistical Analyses. A Latin square design was used for assigning iron-infusion treatments to each cow (n=4 replications). A random effects analysis of variance (ANOVA) was used to determine the effects of infused iron on the dependent variable of composition (total protein, ash, concentration of each mineral) using JMP vs. 10.0.0 Pro Statistical Discovery Software (SAS Institute Inc, Cary, NC). Effects of time (2 levels: d1, d8) and treatment (4 levels of iron) on oxidative stability parameters (TBARS, total volatiles) were analyzed by Tukey-Kramer analysis. If no time effects were observed, values were treated as duplicates to increase the power of the test. A cross effects analysis was completed to see the effects of 2-way and 3-way interactions (cow by treatment, treatment by period, period by cow, and cow by period by treatment) for gross composition dependent variables (protein, ash) and TBARS. An effects test of week nested within treatment was also run for TBARS. Mean values and standard errors for each dependent variable were calculated for each iron-infusion treatment by week (day). All statistical measures were carried out with a predetermined alpha of 0.05. Virginia Tech's Laboratory for Interdisciplinary Statistical Analysis (LISA) was consulted to ensure appropriate statistical analyses were used.

Experiment 4 (Case Study): Effect of Low and High Iron Sources in Bovine Drinking Water on Mineral Composition and Oxidative Stability of Raw Milk

A dairy farm in Franklin County, Virginia was identified with two water sources having different iron concentrations. 204 cows had *ad libitum* access to the water source to the low iron (mean±SD; 0.014±0.005 mg/kg Fe) water source (free stall) and the other portion of the herd (136 cows) had *ad libitum* access to the high iron (0.99±0.61 mg/kg Fe) water source (outside water hydrant). Water iron levels were determined by ICP-MS. Feed, milking and other herd management conditions were consistent for all milking cows. The influence of extended exposure of high iron water consumption on composition and oxidative stability of raw milk was determined.

Dairy Herd Information Association (DHIA) Analyses. Samples from 340 cows from the dairy farm were submitted to the Virginia Tech DHIA lab for sample analysis. Samples were obtained by a DHIA technician on the farm site. The technician recorded milk weights from cows and took milk samples in vials. Preservative treatments, bronopol to control bacteria and natamycin to suppress yeast growth, were in sample vials before sample addition. Samples were shipped via UPS, no refrigeration, and delivered day after collection. Testing was done promptly by the DHIA lab for fat composition, protein, nonfat solids and somatic cell count. Samples were analyzed using flowcytometry technology to determine somatic cell count and Fourier Transform Infared analysis for milk composition (CombiFoss^{TM}, Foss, Denmark).

During the testing each sample was heated a minimal amount and returned to room temperature. DHIA-tested samples were retrieved and taken to the Food Science and Technology building within 12 hours of receipt by the DHIA lab where they were stored in a dark cooler at 4^{o}C to prevent further oxidation for 12 hours. Individual cows were separated into the two treatments based on herd information provided by the farm owner. Each racking number on the

sample correlated to a cow number that identified which water source was provided to the cow. Pooled test samples were created by mixing 10 mL each from 17 individual cows. The pooled samples (n=8 samples per water treatment) were transferred into plastic LDPE bottles, labeled appropriately, and stored in a dark cooler at 4^{o}C for 4 hours. Due to DHIA container failures some individual cow samples had to be discarded so not all 372 cows were represented in the pooled samples. In order to make sample sizes equal for both treatments 272 cow samples were used in the study.

Pooled samples were extracted and TBARS analyses completed on the same day that pooled samples were created. Each pooled sample was analyzed in duplicate. A nitric acid digestion was performed on each sample within 5 days of being pooled; the digested samples were analyzed for mineral content using ICP-MS after 15 days of storage.

Statistical Analyses. Means and standard error were determined. Differences between iron treatments in reference to MDA concentration (TBARS), mineral content (Ca, Cu, Fe, P), and lab analyses from the DHIA laboratories (milk volume, percent fat, percent protein) were determined by using ANOVA. All statistical measures were carried out with a predetermined alpha of 0.05.

Analytical Methods

Milk Composition Analyses. In addition to the fat analysis by Babcock method, milk was analyzed for gross composition (total protein, ash) and mineral composition (day 1 and day 8 for experiment 3). Protein content was determined following the instructions using a commercial protein analysis kit (2-D Quant Kit, General Electric, Fairfield, Connecticut, USA). Percent ash was measured using the gravimetric method (AOAC 945.46) (Bradley, 2000). Ash was calculated with the following formula (Siddique et al., 2010).

$$\text{Ash (\%)} = \frac{ash\ weight\ (grams)}{sample\ weight\ (grams)} \times 100\%$$

Calcium, copper, iron and inorganic phosphorus concentrations was measured on each sample (experiment 2, 3, 4) by ICP-MS (Thermo Electronic Corporation, X-Series ICP-MS, Waltham, MA). Before the milk was analyzed by ICP-MS, the milk was prepared using a modified nitric acid digestion procedure based on a method described for infant formula (Cook and Suddendorf, 1984). A 10% nitric acid (HNO_3) solution was made from 67-70% HNO_3 (EMD, Canada, CAS 7697-37-2). Glassware was cleaned by soaking overnight in 10% HNO_3 and thoroughly rinsed with nanopure water. Milk was vigorously shaken to ensure mixing before allocating 50mL into a 250 mL beaker. HNO_3 (3 mL) was added to milk and placed on a hotplate to evaporate to less than 10 mL, but not to dryness. The sample was then removed from the hotplate, cooled, and 5 mL of HNO_3 added. The beaker was covered with a watch glass to allow a gentle reflux action to occur until the mixture was again evaporated to less than 10mL; it was then removed to let cool. Hydrochloric acid (EMD, Canada, CAS 7647-01-0)-H_2O (5 mL of 1:1 solution) and 5 mL of nanopure water were added to the cooled mixture and heated for 15 minutes. After the final heating, the mixture was cooled and filtered using Whatman^{TM} 41 paper filters to remove lipids (GE Healthcare, Buckinghamshire, UK). The filtered liquid was stored in 60 mL low density polyethylene sealed plastic bottles and stored at room temperature until ICP-MS analysis using an inductively coupled plasma emission spectrometer using Standard Method 3125B (Rice et al., 2012).

Oxidation Analyses. Oxidative stability of milk was estimated based on assessment of malondialdehyde by means of the TBARS test and changes in volatile chemistry using gas chromatography-mass spectroscopy (GC-MS). Sensory evaluation was used to determine if overall differences in flavor and odor existed between milk from control and iron-infused cows

for each treatment level. Analyses were completed on days 1 and day 8 post-processing and all analyses for oxidation were completed within 24 hrs of each respective day.

TBARS. TBARS analyses were completed to measure secondary metabolites of oxidation (aldehydes), specifically malondialdeyde (MDA), which can indicate oxidation (Spanier and Traylor, 1991). Each sample (1 mL) was homogenized in a 15 mL plastic centrifuge tube by means of a vortex prior to the analysis and weighed for accuracy. Samples were diluted by adding 4 mL dH_2O and recovering 1 mL of the diluted sample for analysis. Then 2 mL of solution I (0.375% thiobarbituric acid, 0.506% SDS, 9.370% acetic acid) were added with 0.1 mL of solution III (antioxidant and chelator solution). Samples were vortexed to ensure proper mixing. Samples were capped and kept at 95°C in a water bath for 60 minutes. Cooled samples were placed under a fume hood and 2.5 mL of solution II (15:1 mixture of n-butanol and pyridine) were added. Samples were mixed by vortexing for 10 seconds each then centrifuged at 25°C at 3000 rpm for 15 minutes. Approximately 2.5-3 mL of the top-layer organic solution were pipetted into a cuvette and the absorbance was read at 532 nm under a fume hood (Milton Roy Spectronic 21D Spectrophotomer, Milton Roy Company, Rochester, NY). The amount of MDA per sample was determined by using a standard curve made by means of a linear regression on absorbance values and mg/kg malondialdehyde of the 4 standard tetramethoxypropane (TMP) solutions. Standard solutions, one per concentration, were 0, 2.5, 5, and 7 micromol TMP. Samples of milk were tested in duplicate.

Volatile Chemistry. GC-MS analysis was used to determine changes in the volatile straight chain aldehydes in the milk (experiment 3) as a result of the oxidation process (Barrefors et al., 1995). Volatile compounds were adsorbed and concentrated onto an 85-μm carboxenpolydimethyl siloxane solid phase micro-extraction (SPME) fiber (Supelco, Bellefonte,

PA) that had been conditioned at manufacturer recommended temperature conditions (Supelco, Bellefonte, PA). The fiber was conditioned using an empty sealed vial to calibrate the machine and to ensure desorption of extraneous compounds on the SPME fiber prior to sample analysis. Samples of milk (8 mL) were pipetted into 20 mL amber vials. All samples were kept, by means of a tray cooler, at 4^{o}C (MC 03-01, PAL System CTC Analytics, Lake Elmo, MN). The SPME fiber was injected (4 mm) into the sample headspace through the septum using an autosampler (CombiPAL, CTC Analytics / Leap Technologies, San Diego, CA). The sample was heated to 45°C on an RCT basic heater with an ETS-D4 Fuzzy Controller (IKA Werke, Wilmington, NC) while being agitated at 250rpm, 5 seconds on and 2 seconds off,. The extraction time, allowing adsorption, was 20 minutes long. An HP5890A along with a HP 5972 series mass selective detector (Hewlett Packard, Palo Alto, CA) was used to analyze the volatile oxidation products that were adsorbed onto the fiber. Desorption occurred onto a DB-5 capillary column (30m x0.25 mm I.D. x 0.25µm film thickness, J&W Scientific, Folsom, CA). Volatiles were separated on the column by the following conditions: helium gas flow 1.8 mL/min; injector temperature of 280^{o}C. Initial run temperature was 35^{o}C for 15 seconds. The temperature then increased at a rate of 15^{o}C per minute until 180^{o}C was reached and was held for 30 seconds. The temperature was then increased at 20^{o}C/minute to reach a final detector temperature of 260^{o}C and held for 30 seconds. The program analysis was run in splitless mode. Total run time was approximately 25 minutes. Eternal standards of hexanal (Acros Organics, New Jersey, USA, CAS 66-25-1) and pentanal (Acros Organics, New Jersey, USA, CAS 110-62-3) were diluted in dH2O and used to identify and quantitate volatile oxidation products (Li, 2011). The chromatograms, both for samples and standards, were analyzed and plotted in HP ChemStation software (Hewlett Packard, Palo Alto, CA). Standards were evaluated and identified by peak times and areas under their respective

curves. For each period (four total) in the experimental study, milk was analyzed for volatiles within 3 days of processing and again within 10 days of processing. Each treatment was tested in duplicate (n=8).

Sensory Evaluation. Sensory discrimination testing by triangle tests was used to determine if samples were perceptibly different, possibly as a function of oxidation. Sensory testing in experiment 1 and 3 occurred in the sensory laboratory of the Food Science and Technology Department. Panelists were seated in individual booths under white lighting. Testing was managed using the Sensory Information Management System (SIMS) software (Sensory Computer System LLC, Morristown, NJ). Data were collected using touchscreen monitors, downloaded to the server, and data compilation for each triangle test completed by the software.

Milk samples (ca. 30 mL) were poured into portion cups (60 mL), capped with lids, and refrigerated (4°C) until testing. Samples were coded with 3-digit numbers. Triangle test samples (control versus iron treatment) were presented in a balanced order as determined by the SIMS software. Panelists were asked to taste each sample, comparing the milk aroma and flavor, and identify which sample was different from the other two.

RESULTS AND DISCUSSION

Experiment 1: Effect of Direct Addition of Iron on Oxidative Stability of Milk.

The addition of water to raw or processed fluid milk is not legal (FDA, 2011); potable water may be added as an ingredient in reconstituted fluid products or in dairy products. Incidental water addition occurs indirectly, in very small volumes, through drops of rinse water on equipment or packaging. Such minor amounts of water, when contaminated with iron or copper, may be sufficient to contribute to oxidation of milk and degradation of milk quality. It is even possible that iron remains in the form of salts such as ferrous chloride on the processing equipment and contaminates milk processed on the machinery.

Malondialdehyde (MDA), a secondary oxidative product measured by the TBARS assay, provides an indication of the total aldehydes in a product. This measurement technique tests primarily for MDA levels, though other aldehydes are included. A low concentration of aldehydes is natural in milk (0.55±0.08 mg/kg MDA) but when oxidation occurs, aldehyde concentrations increase and can markedly alter milk flavor and aroma. In this study, MDA was low (0.28±0.00 mg MDA /kg milk) in the control sample (no iron addition), whereas milk with the high iron addition reached 1.68±0.07 mg MDA /kg (Figure 3.1). Johnson (2012) identified that an MDA of 1.3 mg/kg milk (2% fat) was typically sufficient to distinguish light oxidized milk from non-oxidized milk (Johnson, 2012). There were no significant differences ($P > 0.05$) in MDA concentrations between the control milk and milk contaminated with low levels of iron (0.3 mg $FeSO_4$/kg water; EPA SMCL for iron in water) added to the milk (0.0028 mg $FeSO_4$/kg milk) (Figure 3.1; Table G1). However, water containing 3 mg $FeSO_4$/kg did increase the MDA concentrations significantly ($P < 0.05$) in milk with the medium (0.028 mg $FeSO_4$/kg milk) level compared to the control (0 mg $FeSO_4$/kg milk) and low (0.0028 mg $FeSO_4$ /kg milk) iron-

containing water treatments. MDA concentration in milk with the high iron treatment (1.68 mg MDA /kg ±0.07) was significantly different from the MDA in milk with the medium treatment (1.09 mg MDA /kg±0.04) and above the 1.3 mg MDA/kg concentration recognized by Johnson (2012) as a sensory threshold for perceptible oxidation. Sensory results confirmed that the addition of iron contributed to sensory differences, even at the low level of iron addition (EPA SMCL concentration; Table G2), which suggests that the sensory difference between a non-oxidized and a slightly oxidized milk sample may be more sensitive than the TBARS assay and that the incidental contamination of milk with water containing iron can affect milk sensory quality. Although not specifically tested, the just noticeable sensory difference between a non-oxidized sample and a slightly oxidized sample (control vs. low iron treated sample) may correspond to as little as 0.2 mg/kg MDA, based on the TBARS assay. This estimate is based on a direct sensory comparison of samples, using a triangle test method, and may not be sufficient to identify oxidation in an independent sample by an untrained consumer. These subtle differences did not seem to have the same effect in experiment 3, but the milk used in experiment was from individual cows and processed differently than in a commercial setting.

It is well known that the addition of iron to milk by direct addition (Gaucheron et al., 1996; Gaucheron, 2000; McKie et al., 2001; Raouche et al., 2009) and to various milk products (unstandardized, skim milk, infant formula) causes oxidation (Hegenauer et al., 1979a; Gaucheron et al., 1996; Satue-Gracia et al., 2000; Raouche et al., 2009). Iron is quite unstable, particularly in the ferrous form (McKie et al., 2001) and rapidly initiates auto-oxidation of unsaturated fatty acids. However, this study documented that even a small amount of iron in the water source used within the dairy farm or processing plant for cleaning of cows, equipment, or packaging can affect quality of the final product.

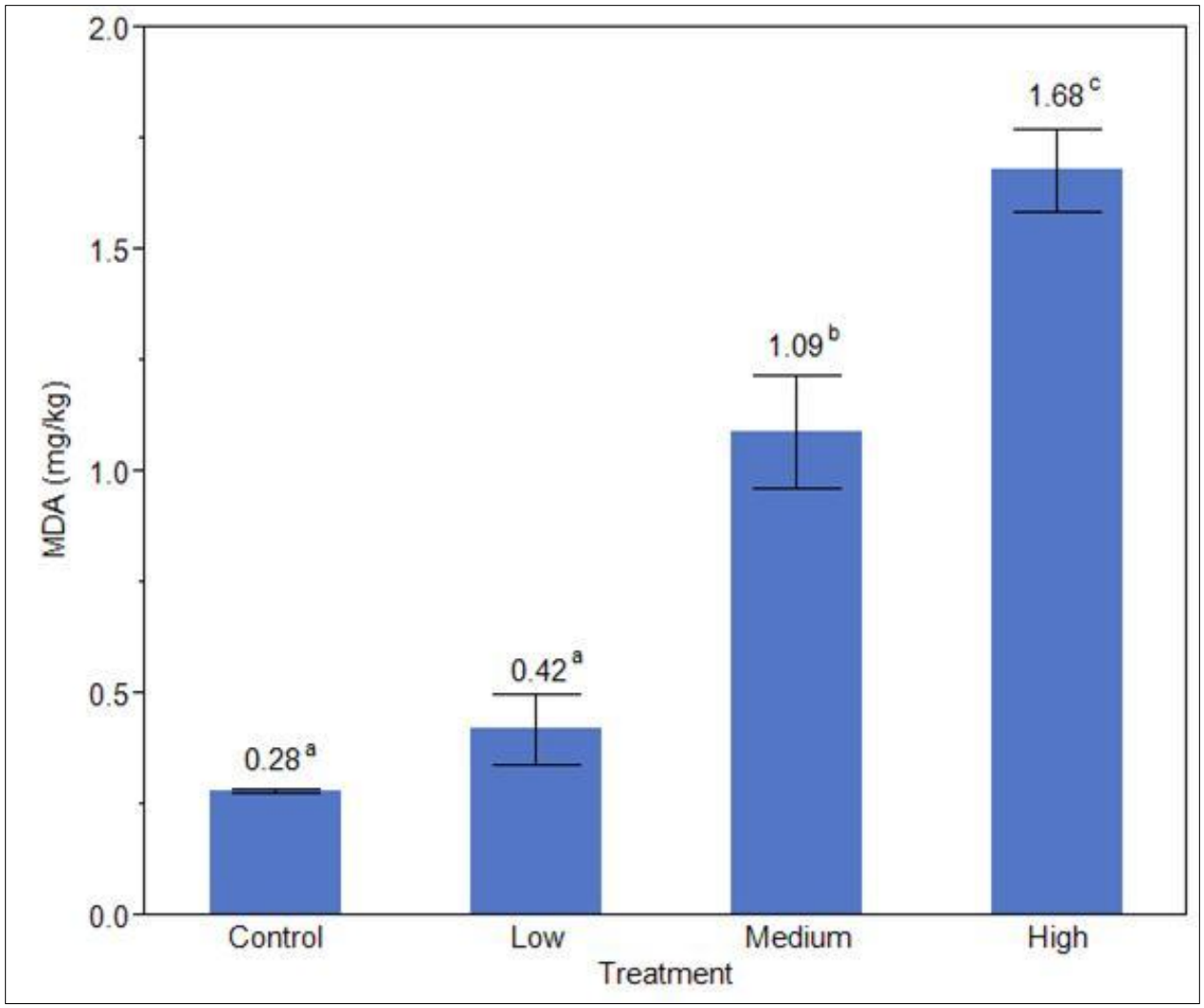

Figure 3.1: Experiment 1, Treatment vs. MDA. Malondialdehyde (MDA) concentration (mg/kg; mean ± SE; n=3), as indication of oxidation in commercially processed milk treated with iron (ferrous sulfate) solutions at four levels [control (0 mg/kg low (0.3 mg/kg; EPA SMCL level), medium (3 mg/kg) and high (30 mg/kg)]. Final (added) iron concentrations in milk were control (0 mg/kg); low (0.0028 mg/kg); medium (0.028 mg/kg); and high (0.28 mg/kg). Milk was stored for 3 days (4°C; no light exposure). Error bars display standard error from the mean. Statistically significant differences ($P < 0.05$) are represented by different superscript letters, determined by ANOVA.

Experiment 2: Validation of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and TBARS Extraction Protocols on Processed Milk

This purpose of this validation study was to ensure that both the nitric acid digestion methods and ICP-MS use could be replicated with low variation. It was also imperative to ensure that the analyses obtained results that were within normal range as compared to published data for milk mineral composition.

Published concentrations of calcium, copper, iron and phosphorus in processed whole milk, based on USDA Nutrient Database (Milk Facts, 2013), are 1130 mg/kg 0.11 mg/kg, 0.30 mg/kg, and 910 mg/kg respectively. Mineral (Ca, Cu, Fe, P) concentration between milk and organic processed milk (900±13.3 mg/kg Ca, 0.029±0.002 mg/kg Cu, 0.215±0.008 mg/kg Fe, and 650±10.3 mg/kg P) were not statistically different ($P > 0.05$) (Table H1). Based on the published values, the extraction and ICP-MS analysis of each mineral was 80% for calcium, 27% for copper, 72% for iron and 71% for phosphorus. However, the phosphorus analyzed is inorganic. While mineral levels are lower than reported by USDA (Milk Facts, 2013), observations for calcium, iron and phosphorus fall within the ranges for each mineral found within published literature. Previous studies on commercially processed milk have shown that mineral content varies, perhaps based on analytical procedure as well as milk source (Murthy et al., 1972; Hunt and Meacham, 2001; Birghila et al., 2008; Hunt and Nielsen, 2009). Mineral concentration in processed milk range between 1097-1495 mg/kg Ca, 0.042-0.18 mg/kg Cu, 0.17-1.45 Fe, and 854-930 mg/kg P (Murthy et al., 1972; Goff and Hill, 1993; Rodríguez Rodríguez et al., 1999; Hunt and Meacham, 2001; Hunt and Nielsen, 2009). Atomic absorption spectroscopy of mineral composition in commercially processed milk showed broad ranges in copper (0.042-0.18 mg/kg Cu) and iron concentrations (0.20-1.45 mg/kg Fe) (Murthy et al., 1972). Birghila et al. (2008) reported iron concentrations in pasteurized milk between 0.8 mg/kg

to as high as 11.84 mg/kg, displaying that iron levels in milk vary widely (Birghila et al., 2008). It is known that copper precipitates easily at a moderate pH, ca. pH 6 and that iron has a tendency to lower pH (Chaplin, 1984; Gaucheron et al., 1996; Balintova and Petrilakova, 2011). Method of analysis does seem to have an effect on mineral recovery, contributing to a wide range of mineral reports. Studies on mineral waters have shown lower recovery rates for Fe and Cu in comparison to Al, Ni or Mo (Haraguchi et al., 2004).

ICP-MS analysis for each mineral provided low variance, with standard deviations less than 10%, with the exception of iron (16%). Low variation in the present study shows that mineral analysis using ICP-MS provides a way to compare relationships among treatments and consequential effects on mineral levels. The low degree of variation may still allow conclusions to be drawn about mineral level relationships among milk samples (Goff and Hill, 1993).

Experiment 3: Influence of Abomasal Infusion of Iron on Oxidative Stability of Processed Milk

Milk Composition. Compositional analyses of the raw milk from this study was reported in Feng et al. (Feng et al., 2013). Protein (3.8%), fat (3.2%) and ash (0.6%) content of the processed milk were not statistically different ($P > 0.05$) with respect to cow, week, nor treatment (Table I2 and I3) and were within normal ranges for processed milk (Murthy et al., 1972; Jenness, 1974; Goff and Hill, 1993; Moreno-Rojas et al., 1993; Moreno-Rojas et al., 1994). However, processing period did have a significant effect on percent protein (data not shown) and there was a statistically significant ($P=0.01$) 3-way interaction (treatment*cow*period) (Table I3). Variations in composition may be attributed to small variations in processing conditions, differences in cow breed, stage of lactation, as progression of the season (Jensen, 1995).

Iron, copper, phosphorus and calcium concentrations in the processed milk did not differ significantly ($P > 0.05$) among the different iron infusion treatments (935±26 mg/kg Ca,

0.040±0.001 mg/kg Cu, 0.220±0.010 mg/kg Fe, 847±27 mg/kg P) based on ANOVA analysis. Main effects tests did show significant interactions for calcium, copper, and iron for 2-way interaction of cow x treatment ($P < 0.05$), suggesting that treatment may have affected mineral composition of milk differently among cows (Table I4). Concentrations for calcium, copper, iron and phosphorus were 82%, 36%, 74%, and 93% of the USDA reported values for whole (3.25%) milk (Milk Facts, 2013) and were similar to the results from the ICP-MS study on milk and organic milk. These minerals were specifically reported as indicators of pro-oxidants (copper, iron) in the milk and to determine effects of dietary iron on milk calcium and phosphorus, which are important for casein stability and dairy product processing. The pro-oxidants, copper and iron, revealed significant interactions for the 3-way interaction between treatment x cow x period (Table I4).

Colloidal Ca and P are strongly associated with the casein proteins of milk (Goff and Hill, 1993). The Ca:P ratio (ca. 4:3) is important to note as it plays a vital role in cheese making and balancing the pH for proper ripening (Upreti and Metzger, 2007). Cheese curd, mostly casein, contains calcium phosphate ($Ca_3(PO_4)_2$). Cheese making potential for milk can be influenced by the Ca:P ratio as calcium phosphate plays an integral part is curd formation and stabilization (Cerbulis and Farrell, 1976; Hutkins, 2006). The Ca:P ratio of the means in this study was about 3.3:3. However, the wide variation in the experimental study suggests that this may not be accurate for each cow (Figure 3.2).

Mineral composition of milk has been linked to bovine feed. Copper levels in milk are attributed to copper concentrations in feed (Havemose et al., 2006) whereas there is very little information about the relationship of iron concentrations in feed or water associated with changes in iron concentrations in the milk (Underwood, 1971; Murthy et al., 1972). Copper and

iron content in the milk are affected by lactation stage; iron content is also dependent on breed, season of the year, and other handling methods (Moreno-Rojas et al., 1993; Sikiric et al., 2003). Post-milking iron contamination historically was also associated with cans, pipes, or other storage methods (Underwood, 1971; Murthy et al., 1972). However, this is less common than seen in years past at the dairy industry has shifted the use of copper pipes to other materials (Clark et al., 2009; Anonymous, 2010).

The experimental study showed more variation in mineral composition than in the direct iron addition study on processed commercial milk. The direct iron addition study was based on five replicate analyses from two different samples and illustrated that the protocols employed were reproducible. In this study, cow to cow variation contributed substantially to larger standard errors. While a Latin square is an appropriate experimental design, treatment replications (n=4 per treatment) were obtained from different cows. The following factors may have contributed to the variation observed: limited number of cattle in the study, number of breeds (Holsteins (n=2) and crossbreeds (n=2)), order of treatment received in reference to stage of lactation and multiple processing steps for each treatment within each period (Murthy et al., 1972; Moreno-Rojas et al., 1993; Moreno-Rojas et al., 1994). Figures 3.2 and 3.3 (Table I1) illustrate the variation in concentration of these minerals in processed milk by treatment as well as individual cow-to-cow variation. Calcium, copper and iron all showed significant differences for the 2-way interaction of treatment x cow ($P < 0.05$).

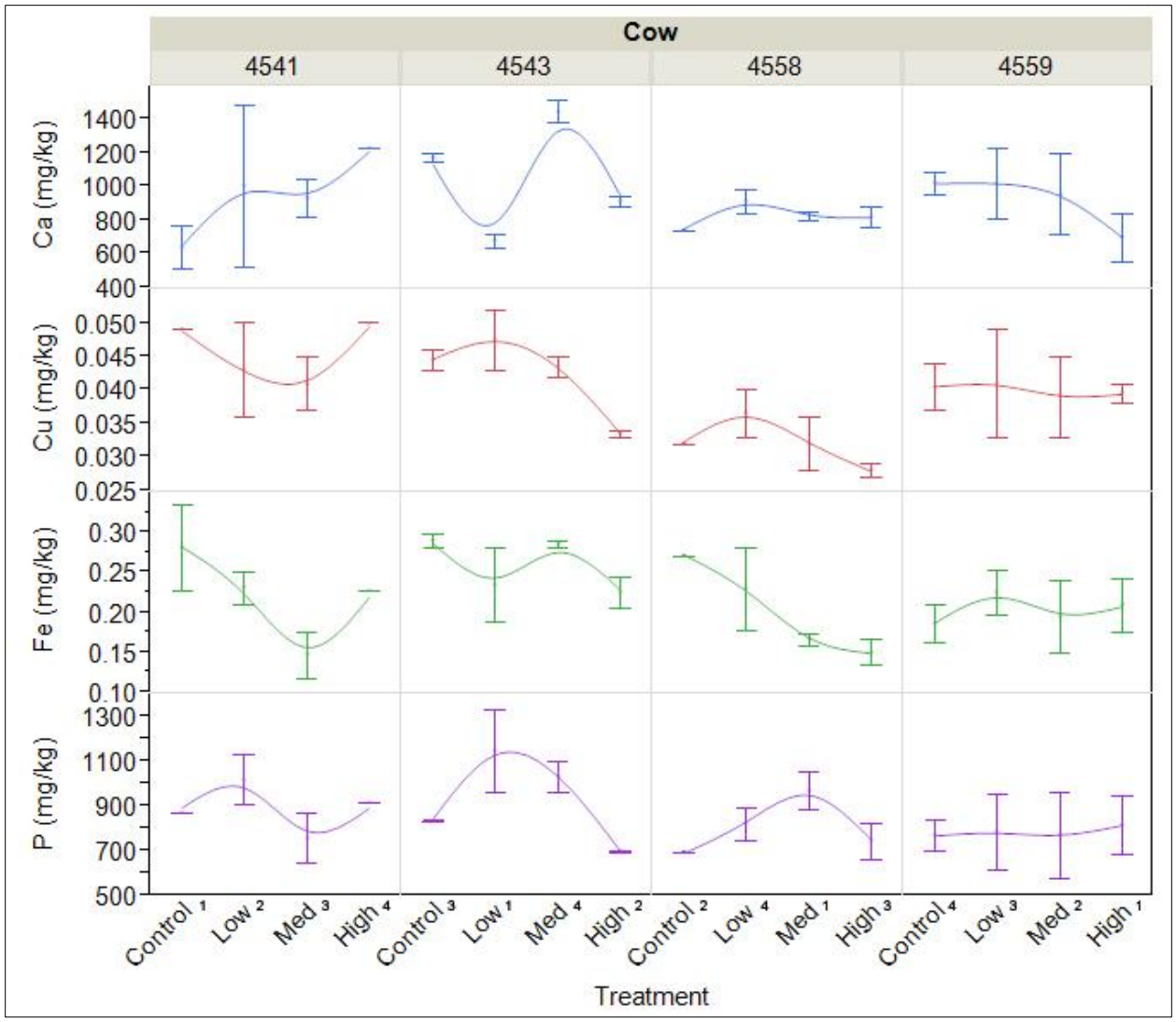

Figure 3.2: Experiment 3, Treatment vs. Mineral Content by Individual Cow. Mineral content (mg/kg; mean; n=4, (d1, d8) values as duplicates) by individual cow as determined by inductively coupled plasma mass spectrometry on whole processed (pasteurized, homogenized) milk. Dietary iron water concentrations for the cattle were control (0 mg/kg), low (200 mg/kg), medium (500 mg/kg) and high (1250 mg/kg). Numbers indicate period in which treatment was received. Four two week periods of milk collection are represented. Milk was stored for 11 d (4°C; no light exposure). There were no significant differences found among means ($P > 0.05$) for each mineral using ANOVA.

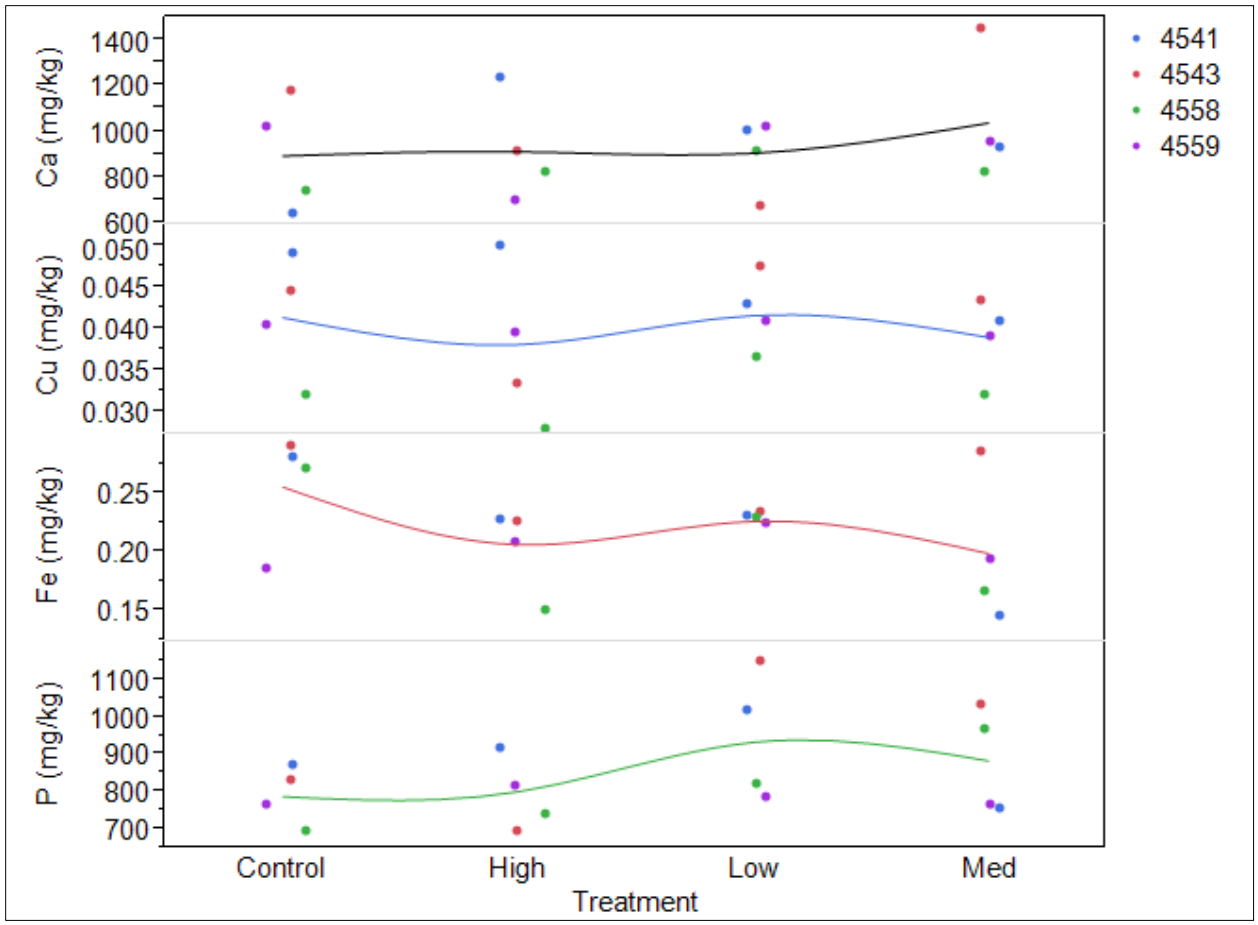

Figure 3.3: Experiment 3, Treatment vs. Mineral Content by Cow. Mineral content (mg/kg; mean; n=4, (d1, d8) values as duplicates) as determined by inductively coupled plasma mass spectrometry on whole processed (pasteurized, homogenized) milk. Dietary iron water concentrations for the cattle were control (0 mg/kg), low (200 mg/kg), medium (500 mg/kg) and high (1250 mg/kg). Four two week periods of milk collection are represented. Each color represents each of four cows. Milk was stored for 11 d (4°C; no light exposure). There were no significant differences found among means (P > 0.05) for each mineral using ANOVA.

Oxidative Stability. TBARS values of processed milk for the first and second week of analyses were averaged as there were no significant differences with time (P > 0.05) (Table J1); mean values ranged from 1.0 to 1.9 mg MDA/kg milk. Processed milk in experiment 1 had lower starting MDA levels than experiment 3 processed milk, likely due to different handling methods. There was no overall treatment effect (P>0.05), although milk from three of the four cows showed a general increase with increasing iron infusion concentration (Figure 3.4 and 3.5; Figure J1). The linear regression of MDA levels in response to treatment concentration was not

significant (P = 0.1854; R^2=0.02811) (Figure J1). However, when cow 4558 was omitted, the effects of treatment on MDA were significant (P = 0.0040; R^2=0.166112) (Figure J2), suggesting that cow variation may have influence on the MDA levels in the processed milk. A possible explanation is spontaneous oxidation which can occur seemingly without reason and often in specific cows (Frankel, 1991; Nicholson and Charmley, 1993; Timmons et al., 2001). Iron contamination of water may lead to spontaneous oxidation in the final milk product (Hegenauer et al., 1979b). However, with spontaneous oxidation we would expect cow 4558 to consistently show more oxidized milk than other cows in the study. This inconsistency could be associated with order of iron treatment or an effect of iron treatment.

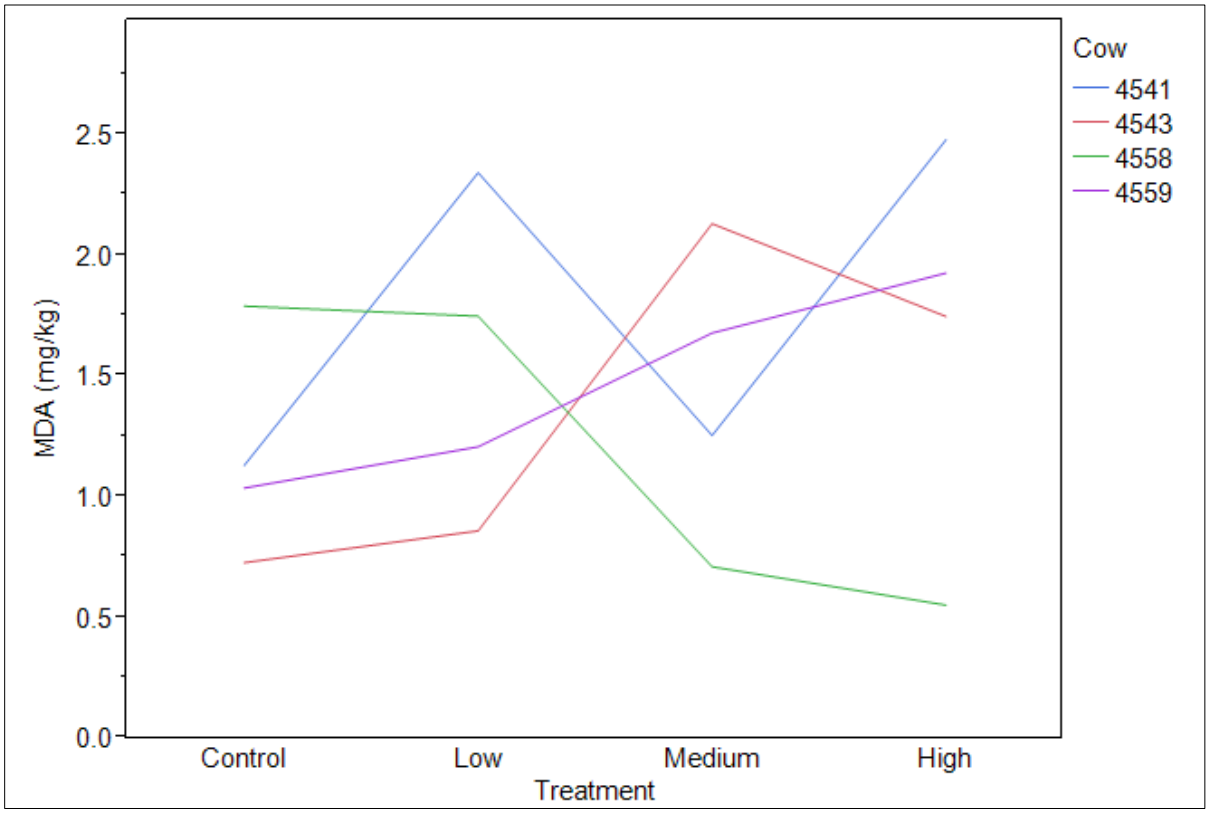

Figure 3.4: Experiment 3, Treatment vs. MDA by Cow. MDA (mg/kg; mean; n=4, (d1, d8) values as duplicates) by individual cow as determined by TBARS assay on whole processed (pasteurized, homogenized) milk. Dietary iron water concentrations for the cattle were control (0 mg/kg), low (200 mg/kg), medium (500 mg/kg) and high (1250 mg/kg). Tests were run in duplicate. Four two week periods of milk collection are represented. Milk was stored for 11 d (4°C; no light exposure).

Mean MDA concentrations of 1.61±0.238 mg/kg have been reported within 34 h of milk collection (Suriyasathaporn et al., 2006). No significant differences in MDA in milk were observed from the infusion of iron-containing water into the abomasum, although variation in samples from individual cows was high (Table J2). When iron-contaminated water was directly added to milk in the direct iron addition experiment, there was an increase in oxidation and the standard deviations were not as high as observed in this study (Figure 3.1, Table G1). While the TBARS analysis is limited to the detection of malondialdehyde, it can be an indication that similar secondary oxidative products are also in the milk such as other aldehydes (Moore and Roberts, 1998).

The test power is weak (0.14) for the analysis of treatment effect on MDA. There is no significant difference between cow and the corresponding MDA values in the milk using a Tukey-Kramer analysis ($P > 0.05$). Each cow is represented in each of the four treatments. Processing conditions can easily affect oxidation rates as temperature and light exposure affect oxidative stability and consequently may be reflected in the MDA concentration of samples (Suriyasathaporn et al., 2006; Drake et al., 2007; Clark et al., 2009). Laboratory conditions were controlled as completely as possible. Guzun-Cojocaru et al. (2011) used the TBARS procedure to find values for MDA over a week-long period to show the time effect of iron added to emulsions and found MDA levels to be higher in emulsions with iron compared to the control with no iron (Guzun-Cojocaru et al., 2011). TBARS assay has been used to compare relative oxidation rates in comparison studies between different iron fortification levels (Hegenauer et al., 1979a; Hegenauer et al., 1979b).

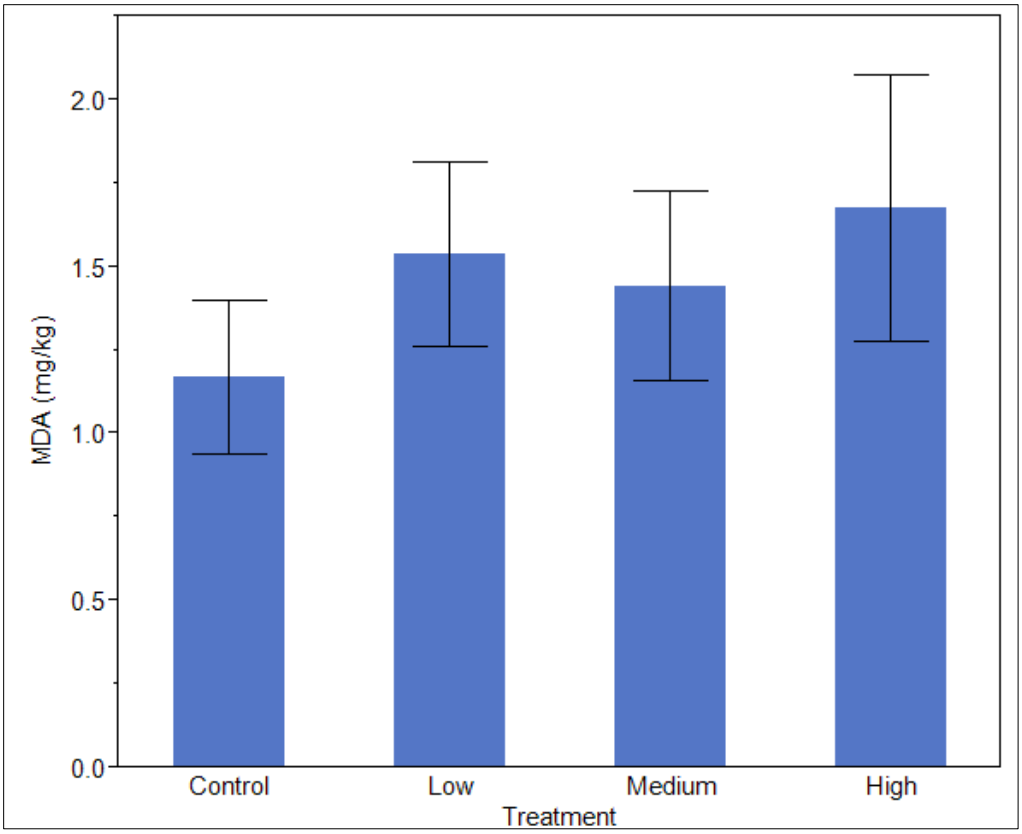

Figure 3.5: Experiment 3, Treatment vs. MDA. Malondialdehyde concentration (mg/kg; mean; n=4, (d1, d8) values as duplicates), as indication of oxidation on whole processed (pasteurized, homogenized) milk. Dietary iron water concentrations for the cattle were control (0 mg/kg), low (200 mg/kg), medium (500 mg/kg) and high (1250 mg/kg). All of the four two week periods of milk collection are represented. Milk was stored for 12 total days (4°C; no light exposure). There were no significant differences found between the two weeks for each mineral ($P > 0.05$) using a Tukey-Kramer analysis. Four two week periods of milk collection are represented. Bars indicate mean values; error bars display standard error from the mean. Mean MDA values are 1.17, 1.53, 1.44, and 1.68, respectively. There were no significant differences found among means ($P > 0.05$) using ANOVA.

Concentration of hexanal and pentanal, volatile aldehydes that are often measured as an indication of oxidation of milk, did not change in peak area during refrigerated storage for one week or in response to the treatments (Tables J4 and J5).

While analytical tests did not indicate any effects of abomasal infusion of ferrous lactate solutions on oxidative stability of the processed milk, sensory tests, using triangle testing discrimination methods, showed that the panelists could discern differences between each treatment sample when compared to the control ($P < 0.01$) (Table 3.2). Differences were

observed on both day 1 and day 8 between the control and each treatment, but more panelists were able to detect differences on day 8. Triangle tests, commingled over the four test periods with two sessions per period (n=288, p_d=30%, alpha=0.05, beta=0.001), had a high degree of power (0.999) compared to each individual session (n=36, p_d=30%, alpha=0.05, beta=0.3), where the power was fair (0.7). The preset proportion of discriminators was 30% for the triangle test for difference but the actual proportion of discriminators, based on the commingled pool of panelists, was 27%, 36% and 39% for low, medium and high treatments.

Table 3.2: Experiment 3, Sensory Test. Sensory triangle test for difference on whole processed (pasteurized, homogenized) milk[1].

Sensory triangle test for difference				
	Number correct[2]		Critical values	
Treatment	Day 0	Day 7	α=0.05	α=0.01
Low (200 mg/kg)	66*	82*	58	62
Medium (500 mg/kg)	79*	87*	58	62
High (1250 mg/kg)	80*	91*	58	62

* Detectable differences milk samples in comparison to the control are statistically significant ($P < 0.05$) at α=0.05 (β=0.001; p_d=30; n=144); based on 8 sessions with 36 panelists each.
[1]Milk obtained from cows (n=4) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milk was stored for a total of 11 days (4°C; no light exposure).

It is possible that the differences detected in the milk by the panelists are related to very low concentrations of aldehydes and other flavor compounds associated with oxidation that are below the detection limits of the GC-MS. It is possible that sensory differences might be attributed to compounds other than hexanal or pentanal (Kristensen et al., 2004; Webster, 2009). Sensory perception is often used as it is more sensitive than analytical methods (Ogden, 1993). Often a gas chromatography olfactometry is used to compensate for the inability of GC-MS to detect important aroma compounds. The human nose is more sensitive to aroma compounds than GC-MS as only select compounds can be identified at a time (Drake et al., 2007).

However, since this study used one cow per treatment per period, it is possible that differences in milk flavors could be associated with cow-to-cow variation. Though the Latin square design allowed for each cow to be her own control in evaluating effects of the iron treatments, within each period the control milk used for the sensory testing was from one cow and compared to the treatment milks from three different cows. The study did not include the assessment of milk in all cows when there was no abomasal infusion, which could have provided a control to verify if any cow-to-cow variation in milk flavor was detectable. While processing conditions were controlled as much as possible, each batch of milk (cow/treatment) was processed independently and slight variations in temperature and time in the pasteurization vat may have occurred. This could cause slight differences in protein denaturation, leading to flavor variations, though this impact was likely minimal in this study (Powell, 2001).

Sensory testing cannot be exclusively replaced by other analytical tests (Barrefors et al., 1995). The human senses capture more than just one stimulus at a time, unlike many instrumental methods. However, it is possible for relationships to be established between instrumental and sensory tests (Drake, 2007). When comparing the treatments (low, medium, high) to the control milk, sufficient numbers of panelists were able to discern a detectable difference between the samples. This is primarily an indication that the added iron in the dietary water may have a sensory effect on the milk that is otherwise undetectable by chemical analysis (Jenness, 1974).

Experiment 4 (Case Study): Effect of Low and High Iron Sources in Bovine Drinking Water on Mineral Composition and Oxidative Stability of Raw Milk

Milk samples for the case study were pooled sets from two treatment levels. Cattle received water *ad libitum* from a water hydrant (0.99 mg/kg) noted as high iron and from a free stall water source (0.018 mg/kg) noted as low iron. Cattle in both herds were separated. In

comparison to the treatment levels made for the experimental study (0, 2, 5, 12.5 mg/kg drinking water) these levels were low, but this unique variation in iron level treatment in water on one farm with portion of the herd receiving each water source provided an opportunity to look at the effects of chronic exposure to water with an iron source above the EPA SMCL. Iron levels vary in water on dairy farms in the southwestern region of Virginia (Martel et al., 2013). This case study provided an opportunity to evaluate effects of long term exposure to higher iron levels in drinking water in comparison to low levels.

DHIA laboratory analyses reported milk fat (3.85%±0.04%) and protein (3.06%±0.02%), each within normal ranges (Goff and Hill, 1993; Jensen, 1995). MDA concentrations were not different ($P>0.05$) between the two iron treatments (low 0.76±0.04 and high 0.78±0.05 mg/kg MDA) with standard errors much lower than observed in the abomasal infusion study. The MDA values in this study were slightly lower than the MDA values in the experimental study and were in the normal range. The milk in the case study was raw, compared to processed (homogenized, pasteurized) milk in the experimental study but had received a mild heat treatment during the DHIA composition testing (Suriyasathaporn et al., 2006). However, the heat treatment was not comparable to the heat treatment used in a processing scenario, a factor that can affect oxidation (Eric A. Decker, 2010). Despite the low TBARS values, the coefficient of variation (4.2%) was lower than the experimental study which may be due to the pooled design of the case study compared to the testing of individual cows in the experimental design.

No statistically significant differences ($P > 0.05$) were detected between the iron treatments and mineral content (834±20 mg/kg Ca, 0.043±0.009 mg/kg Cu, 0.232±0.017 mg/kg Fe, 671±15.4 mg/kg P) of the milk (Table K1). Other published data have reported mineral levels to be ca. 1100-1300 mg/kg calcium, 0.1-0.6 mg/kg Cu, 0.3-0.6 mg/kg Fe, and 900-1000 mg/kg P

in raw milk (Goff and Hill, 1993). Fe and Cu, measured using ICP-MS technology, were 1.18-1.35 mg/kg and 0.31-0.32 mg/kg respectively in raw milk (Brescia et al., 2003). Phosphorus concentration in raw milk, based on ICP, ranged from 768-856 mg/kg, or were determined using other methods such as photometry after a sulfuric acid digestion (Brescia et al., 2003). This could perhaps explain the lower levels of phosphorous consistently found by this study. Similar to the validation study, Ca and P levels were lower than expected. Another study on raw milk reported higher copper values than those found in the case study, but copper in the same study was extreme (Moreno-Rojas et al., 1993). The mineral content can vary depending on lactation stage, climate, feed type, and time of year (Murthy et al., 1972; Moreno-Rojas et al., 1993; Havemose et al., 2006). These factors were not controlled for in this case study.

The analyses run by DHIA are also included in Appendix K. These include kilograms of milk given per cow on the collection day, fat percent of milk and protein percent of milk. An interesting difference to note is that the milk yield (between the cows receiving the low iron water (average = 31±0.58 kg) and the cows receiving the high iron-containing water (27±0.54 kg) differed significantly ($P < 0.05$). This could be a difference in separation of herds (fresh and sick cattle on low iron; medium milk producers on high iron) but it is also possible that cows drinking the higher iron water may not be as willing to drink the water with metallic flavor as the cows drinking the low iron water (less potential of metallic taste in the water). No significant difference ($P > 0.05$) was noted in milk yield when cattle were infused with iron treatments (Feng et al., 2013). It is known that the amount of iron in water can affect the palatability of the water (National Primary Drinking Water Regulations, 2011) and cows that are on restricted water intake, whether from lack of availability (drought) or lack of willingness to consume (metallic flavor) may produce less milk (Genther and Beede, 2013).

CONCLUSIONS

Iron added directly to milk has been shown to increase the oxidation rates of milk. In dairy processing this contamination may be through use of wash water in the processing plant. Dairy production on the farm also should consider mineral content of water. Until now the effect of dietary iron of bovine drinking water on milk quality was largely unstudied. It is likely that high levels (> 0.3 mg/kg, EPA SMCL) of iron given to cattle in their drinking water does not accelerate oxidation rates of milk nor alter mineral composition in a significant manner; however sensory characteristics of milk from cows given iron treatments of more than 0.3 mg/kg may differ. High iron contamination of bovine drinking water may affect some cows more than others and perhaps may play a role in spontaneous oxidation. It is possible that cows receiving higher levels of dietary iron produce less milk. Awareness of mineral concentration in water is important in protecting the quality of the final milk product. Both farmers and processors should be wary of mineral content of the water used for operations. It is possible that long-term effects of dietary iron on cow health may be negative; further research on the topic is needed.

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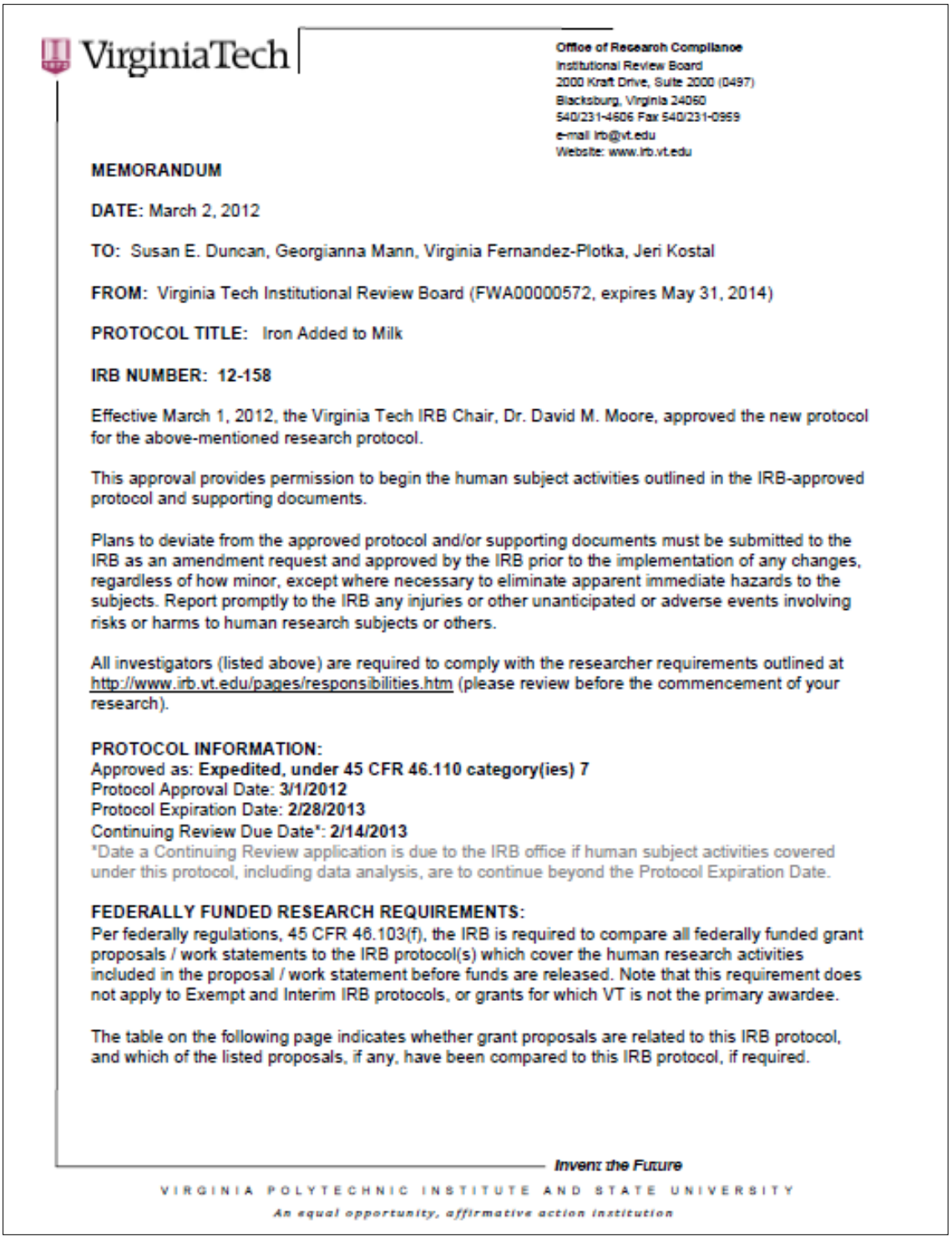
CHAPTER IV

APPENDICES

Appendix A: Experiment 1. IRB Approval Letter



VirginiaTech

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MEMORANDUM

DATE: March 2, 2012

TO: Susan E. Duncan, Georgianna Mann, Virginia Fernandez-Plotka, Jeri Kostal

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires May 31, 2014)

PROTOCOL TITLE: Iron Added to Milk

IRB NUMBER: 12-158

Effective March 1, 2012, the Virginia Tech IRB Chair, Dr. David M. Moore, approved the new protocol for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at http://www.irb.vt.edu/pages/responsibilities.htm (please review before the commencement of your research).

PROTOCOL INFORMATION:
Approved as: **Expedited, under 45 CFR 46.110 category(ies) 7**
Protocol Approval Date: **3/1/2012**
Protocol Expiration Date: **2/28/2013**
Continuing Review Due Date*: **2/14/2013**
*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:
Per federally regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals / work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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Appendix B: Experiment 1. Consent Form

Virginia Polytechnic Institute and State University

Informed Consent for Participants in Research Projects Involving Human Subjects (Sensory Evaluation)

Title Project: Effects of iron content in water used for milk processing: Does iron content affect milk stability?

Investigators: Georgianna Mann, Jeri Kostal, Tina Plotka, Susan E. Duncan, PhD, RD

I. Purpose of this Research/Project

You are invited to participate in a study about effects of iron in milk. Water containing high levels of iron may be used to clean equipment that is necessary for milk processing. Excess minerals may affect milk quality. This research will help describe effects of water quality on milk quality.

II. Procedures

After you sign the consent form, there will be a sensory test, which will last approximately 20 minutes. You will be presented with three sets of milk samples; each set contains three samples. For each set, please taste samples from left to right, and identify the different sample among the three milk samples.

Please follow these two steps when you taste the milk:

1) Take a generous sip, roll the milk around in the mouth, and then swallow or expectorate in the cup provided.

Between each set, eat a few crackers to clean the palate, rinse your mouth with water, and wait one minute before evaluating the next set of samples.

III. Risks

There are no more than minimal risks for participating in this study. If you are aware of any allergies to dairy proteins or lactose intolerance, please do not participate.
Your participation in this study will provide valuable information about milk flavor as affected by iron, which will be useful to the food and dairy processing industries. If you would like a summary of the research results, please contact the researcher at a later time.

V. Extent of Anonymity and Confidentiality

The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results.

VI. Compensation
You will be compensated with a small edible treat for participating in this study.

VII. Freedom to Withdraw
If you agree to participate in this study, you are free to withdraw from the study at any time without penalty. There may be reasons under which the investigator may determine you should not participate in this study. If you have allergies or lactose intolerance to dairy products, or are under the age of 18, you are asked to refrain from participating.

VII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

> Smell and taste the milk products and identify the odd sample based on aroma and taste.

IX. Subject's Permission

I have read the Consent Form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

_____ Date

_____
Subject Signature

_____
Subject Printed Name

----------For human subject to keep----------

Should I have any pertinent questions about this research or its conduct, and research subjects' rights, and whom to contact in the event of a research-related injury to the subject. I may contact:

Georgianna Mann, Graduate Research Assistant, Investigator	(770) 316-0784; gmann89@vt.edu
Jeri Kostal, Graduate Research Assistant, Co-Investigator	(434) 738-8918; jkost07@vt.edu
Tina Plotka, Research Associate, Co-Investigator	(540) 231-9843; tplotka@vt.edu
Susan Duncan, Faculty, Investigator	(540) 231-8675; duncans@vt.edu
David Moore Chair, Virginia Tech Institutional Review Board for the Protection of Human Subjects Office of Research Compliance 1880 Pratt Drive, Suite 2006 (0497) Blacksburg, VA 24061	(540) 231-4991; moored@vt.edu

Appendix C: Experiment 1 and 3. Instruction Ballot

test administration
purposes only
Sample Set: _____

Instructions: Enter as **"Anonymous"**.

Test number: _____ Ballot number: _____

Verify the numbers on the samples match the code order on the screen. Taste the samples on the tray from left to right. Two samples are identical; one is different. Select the odd/different sample and indicate selecting the odd sample's code on the screen.

Please slide your finished samples under the hatch and wait for your next set.

Appendix D: Experiment 3. Study Design

Table D1: Experiment 3, Cow Feed. Ingredients and nutrient content of diets.

Item	% of DM
Ingredient	
Corn silage	40.35
SS grain mix[1]	20.55
Grass hay, mid bloom	14.01
Corn grain dry, ground	12.44
Grass/legume mix silage	6.06
Brewer's grain, wet	3.31
Soybean meal, solvent 48%	2.80
Bentonite	0.48
Chemical composition	
DM	54.3
CP	15.0
NDF	34.7
ADF	17.5
Ca	0.66
P	0.39
Fe, mg/kg	651

[1] Ingredient components (As fed): hominy feed 20.65%; soybean meal (48%) 20.65%; cottonseed meal (36 CP) 20.65%; wheat midds 8.7%, Pro-Lak 10.35%; animal fat 2.05%; molasses dehydrated 4.35%; urea (45%) 1.05%; limestone 4.78%; sodium bicarbonate 2.61%; magnesium oxide 0.87%; Dynamate 1.30%; Salt-white 1.30%; Availa-4 0.26%; Selenium (0.06%) 0.13%; Vit. E-60000 0.07%; Rumensin 90 0.03%; Vit ADE 0.20%

Table D2: Experiment 3, Sample Period Layout. The study consisted of four periods where each cow received one of four treatments[1] for one period. Milk was picked up to be processed on day 13.

Day of the week	Period day	Treatment
Tuesday	1	Washout[2]
Wednesday	2	Washout
Thursday	3	Washout
Friday	4	Washout
Saturday	5	Washout
Sunday	6	Washout
Monday	7	Washout
Tuesday	8	Infusion[3]
Wednesday	9	Infusion
Thursday	10	Infusion
Friday	11	Infusion
Saturday	12	Infusion
Sunday	13	Infusion
Monday	14	Infusion

[1] Control (0 mg/kg), low (200 mg/kg), medium (500 mg/kg) and high (1250 mg/kg) ferrous lactate. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection.

[2] The washout period consisted of no abomasal infusion.

[3]The infusion period was when the abomasal infusion of ferrous lactate solution occurred.

Appendix E: Experiment 3. IRB Approval Letter

VirginiaTech

Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24060
540/231-4606 Fax 540/231-0959
e-mail irb@vt.edu
Website: www.irb.vt.edu

MEMORANDUM

DATE: March 5, 2012

TO: Susan E. Duncan, Georgianna Mann, Virginia Fernandez-Plotka

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires May 31, 2014)

PROTOCOL TITLE: Effects of Iron on Milk Stability

IRB NUMBER: 12-227

Effective March 2, 2012, the Virginia Tech IRB Administrator, Carmen T. Green, approved the new protocol for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at http://www.irb.vt.edu/pages/responsibilities.htm (please review before the commencement of your research).

PROTOCOL INFORMATION:
Approved as: **Expedited, under 45 CFR 46.110 category(ies) 7**
Protocol Approval Date: **3/2/2012**
Protocol Expiration Date: **3/1/2013**
Continuing Review Due Date*: **2/15/2013**
*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:
Per federally regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals / work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

Appendix F: Experiment 3. Consent Form

Virginia Polytechnic Institute and State University

Informed Consent for Participants in Research Projects Involving Human Subjects (Sensory Evaluation)

Title Project: Effects of mineral content in bovine drinking water: Does iron content affect milk quality?

Investigators: Georgianna Mann, Tina Plotka, Susan E. Duncan, PhD, RD

I. Purpose of this Research/Project

You are invited to participate in a study about effects of dietary iron on cattle and their resulting milk. Water containing high levels of iron may be used nourish cattle in times of water shortage. Excess minerals may affect milk quality. This research will help describe effects of water quality on milk quality.

II. Procedures

After you sign the consent form, there will be a sensory test, which will last approximately 20 minutes. You will be presented with three sets of milk samples; each set contains three samples. For each set, please taste samples from left to right, and identify the different sample among the three milk samples.

Please follow these two steps when you taste the milk:

1) Take a generous sip, roll the milk around in the mouth, and then swallow or expectorate in the cup provided.

Between each set, eat a few crackers to clean the palate, rinse your mouth with water, and wait one minute before evaluating the next set of samples.

III. Risks

There are no more than minimal risks for participating in this study. If you are aware of any allergies to dairy proteins or lactose intolerance, please do not participate. Your participation in this study will provide valuable information about milk flavor as affected by dietary iron in dairy cattle, which will be useful to the food and dairy industries. If you would like a summary of the research results, please contact the researcher at a later time.

V. Extent of Anonymity and Confidentiality

The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results.

VI. Compensation
You will be compensated with a small edible treat for participating in this study.

VII. Freedom to Withdraw
If you agree to participate in this study, you are free to withdraw from the study at any time without penalty. There may be reasons under which the investigator may determine you should not participate in this study. If you have allergies or lactose intolerance to dairy products, or are under the age of 18, you are asked to refrain from participating.

VII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

Smell and taste the milk products and identify the odd sample based on aroma and taste.

IX. Subject's Permission

I have read the Consent Form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

_____ Date _____
Subject Signature

_____
Subject Printed Name

----------For human subject to keep----------

Should I have any pertinent questions about this research or its conduct, and research subjects' rights, and whom to contact in the event of a research-related injury to the subject. I may contact:

Georgianna Mann, Graduate Research Assistant, Investigator	(770) 316-0784; gmann89@vt.edu
Tina Plotka, Research Associate, Co-Investigator	(540) 231-9843; tplotka@vt.edu
Susan Duncan, Faculty, Investigator	(540) 231-8675; duncans@vt.edu
David Moore Chair, Virginia Tech Institutional Review Board for the Protection of Human Subjects Office of Research Compliance 2000 Kraft Drive, Suite 2000 Blacksburg, VA 24060	(540) 231-4991; moored@vt.edu

Appendix G: Experiment 1. TBARS and Sensory Test

Table G1: Experiment 1, TBARS. Malondialdehyde concentration (mg/kg; mean ± SE; n=3 duplicate values), as indication of oxidation in commercially processed milk[1] treated with iron (ferrous sulfate) solutions at four levels.

Treatment	Mean (mg/kg)
Control	0.282 ± 0.00
Low	0.421 ± 0.03
Medium	1.09* ± 0.04
High	1.68* ± 0.07

* Treatment mean is significantly different ($P < 0.05$) from control mean based on ANOVA.
[1] Milk treated with ferrous sulfate solutions (n=3; duplicate values) were made with distilled water in the following levels: control (0 mg/kg), low (0.3 mg/kg), medium (3 mg/kg) and high (30 mg/kg). 30 mL was added to 300 mL of commercially processed milk. Milk iron concentrations were control (0 mg/kg), low (0.0028 mg/kg), medium (0.028 mg/kg) and high (0.28 mg/kg). Milk was stored for 3 days (4°C; no light exposure).

Table G2: Experiment 1, Sensory Test. Sensory triangle test for difference in commercially processed milk[1] treated with iron (ferrous sulfate) solutions at four levels.

Sensory Test	Number correct	Result
Low (0.3 mg/kg)	22*	P<0.01
Medium (3 mg/kg)	25*	P<0.001
High (30 mg/kg)	32*	P<0.001

* Detectable differences milk samples in comparison to the control are statistically significant ($P < 0.05$) at $\alpha=0.05$ ($\beta=0.2$; $p_d=30$; n=43); critical value = 21 correct responses
Detected sensory differences are significant, above the critical number of 20, at ($P < 0.01$). Above 22 are statistically significant at ($P < 0.001$). All additions of iron revealed significant sensory differences when compared to the control milk.
[1] Milk treated with ferrous sulfate solutions (n=3; duplicate values) were made with distilled water in the following levels: control (0 mg/kg), low (0.3 mg/kg), medium (3 mg/kg) and high (30 mg/kg). 30 mL was added to 300 mL of commercially processed milk. Milk iron concentrations were control (0 mg/kg), low (0.0028 mg/kg), medium (0.028 mg/kg) and high (0.28 mg/kg). Milk was stored for 3 days (4°C; no light exposure).

Appendix H: Experiment 2. ICP-MS and TBARS

Table H1: Experiment 2, Mineral Content. Mineral content (mg/kg; mean ± SE), as indication of oxidation in commercially processed milk[1].

Mineral content				
Milk type	Calcium (mg/kg) $\bar{x} \pm SE$	Copper (mg/kg) $\bar{x} \pm SE$	Iron (mg/kg) $\bar{x} \pm SE$	Phosphorous (mg/kg) $\bar{x} \pm SE$
Nonorganic	897 ± 17.1	0.029 ± 0.001	0.237 ± 0.018	639 ± 14.9
Organic	903 ± 20.7	0.030 ± 0.004	0.192 ± 0.004	661 ± 14.3
Average	900 ± 13.3	0.029 ± 0.002	0.215 ± 0.008	650 ± 10.3
USDA reported values[2]	113	0.110	0.300	910

[1] Milk, commercially processed organic and nonorganic, (n=5) was prepared using a nitric acid digestion prior to inductively-coupled plasma spectroscopy analysis. Milk was stored for 1 day (4°C; no light exposure).
[2] www.milkfacts.info/NutritionFacts

Table H2: Experiment 2, TBARS. Malondialdehyde concentration (mg/kg; mean ± SE; n=4), as indication of oxidation in commercially processed milk[1].

Malondialdehyde (mg/kg)		
Milk type*	Day	Mean[2] $\bar{x} \pm SE$
Nonorganic[A]	1	0.76 ± 0.04
Organic[A]	1	0.74 ± 0.08
Nonorganic[B]	7	1.56 ± 0.07
Organic[C]	7	1.41 ± 0.04

*Statistically significant ANOVA differences among means represented by superscript letters ($P < 0.05$).
[1] Milk, commercially processed organic and nonorganic, (n=4; duplicate values) was stored for 14 days (4°C; no light exposure).
[2] Mean MDA is 0.75 for day 1.

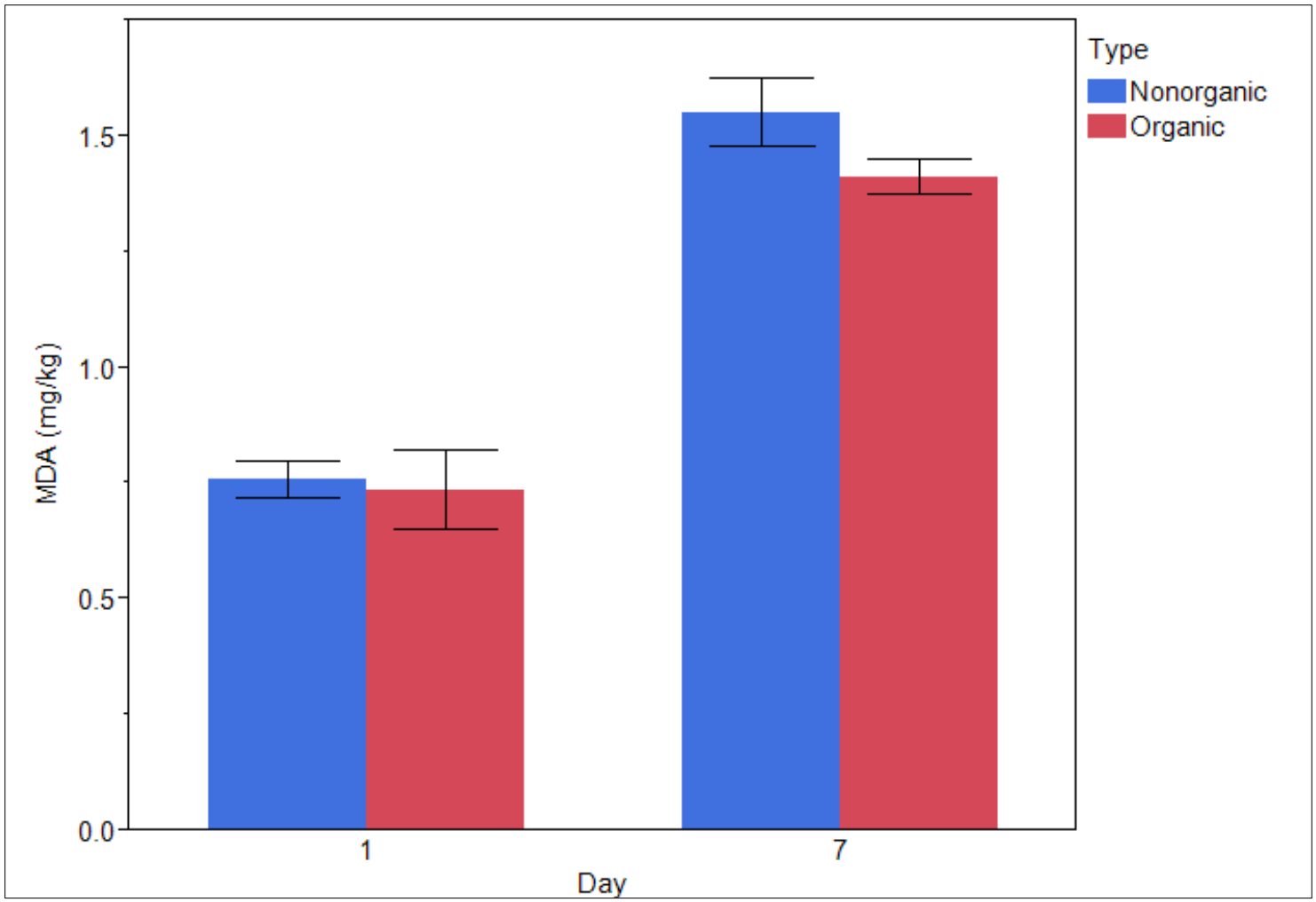

Figure H1: Experiment 2, Day vs. MDA. Malondialdehyde concentration (mg/kg; mean ± SE; n=4), as indication of oxidation in commercially processed milk (organic and nonorganic). Milk was stored for 14 days (4°C; no light exposure). Bars indicate mean values; error bars display one standard error from the mean. Mean MDA values per milk type (nonorganic, organic) were 0.76^{A} and 0.74^{A} for day 1, and 1.56^{B} and 1.41^{C} for day 7. Differences among means, found by ANOVA, represented by superscript letters ($P < 0.05$).

Appendix I: Experiment 3. Gross Composition

Table I1: Experiment 3, Mineral Content. Mineral content (mg/kg; mean ± SE), as determined by inductively coupled plasma mass spectrometry, on whole processed (pasteurized, homogenized) milk[1].

	Mineral content, averages (n=4)[2]			
Treatment	Calcium (mg/kg) $\bar{x} \pm SE$	Copper(mg/kg) $\bar{x} \pm SE$	Iron (mg/kg) $\bar{x} \pm SE$	Phosphorous (mg/kg) $\bar{x} \pm SE$
Control (0 mg/kg iron)	925 ± 128	0.042 ± 0.003	0.255 ± 0.029	794 ± 43
Low (200 mg/kg iron)	901 ± 119	0.042 ± 0.003	0.230 ± 0.021	943 ± 103
Medium (500 mg/kg iron)	1041 ± 160	0.039 ± 0.003	0.198 ± 0.034	881 ± 97
High (1250 mg/kg iron)	871 ± 78	0.036 ± 0.004	0.196 ± 0.018	771 ± 56
Average[3]	935 ± 26	0.040 ± 0.001	0.220 ± 0.010	847 ± 27
USDA reported values[4]	113	0.110	0.300	910

[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milk was stored for a total of 11 days (4°C; no light exposure).

[2] There were no statistically significant differences ($P > 0.05$) found among the different treatments using ANOVA.

[3] Day 1 and day 8 differences in mineral composition were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

[4] www.milkfacts.info/NutritionFacts

Table I2: Experiment 3, Gross Composition. Fat, protein and ash (%; mean ± SE), of whole processed (pasteurized, homogenized) milk[1].

	Treatment				
Analyses	Control (0 mg/kg iron) $\bar{x} \pm SE$	Low (200 mg/kg iron) $\bar{x} \pm SE$	Medium (500 mg/kg iron) $\bar{x} \pm SE$	High (1250 mg/kg iron) $\bar{x} \pm SE$	Average[2] $\bar{x} \pm SE$
Fat (%)	3.15%±0.00%	3.21%±0.04%	3.16%±0.07%	3.21%±0.06%	3.18%±0.04%
Protein (%)[3]	3.99%±0.54%	3.66%±0.54%	3.91%±0.55%	3.53%±0.77%	3.77%±1.33%
Ash (%)[3]	0.64%±0.03%	0.53%±0.03%	0.58%±0.03%	0.58%±0.02%	0.58%±0.21%

[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milkfat analyses were in duplicate, d1 only. Protein and ash were in duplicate on d1 and d8. Outliers, determined by an outlier box plot, were excluded. Milk was stored for a total of 11 days (4°C; no light exposure).

[2] There were no statistically significant differences ($P > 0.05$) found among the different treatments using ANOVA.

[3] Day 1 and day 8 differences in gross composition were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

Table I3: Experiment 3, Gross Composition F Table. F statistic and P values for specific contrasts with gross composition (fat, protein, ash) as response on whole processed (pasteurized, homogenized) milk[1].

Contrast	F Statistic	P value
	Milkfat (%)	
Treatment*Period	0.8146	0.54
Treatment*Cow	0.3037	0.82
Cow*Period	0.0631	0.98
Treatment*Cow*Period	0.6989	0.70
	Protein (%)	
Treatment*Period	2.1563	0.21
Treatment*Cow	2.1264	0.22
Cow*Period	0.3331	0.80
Treatment*Cow*Period	3.523	0.01†
	Ash (%)	
Treatment*Period	0.3221	0.81
Treatment*Cow	0.3059	0.82
Cow*Period	0.5082	0.69
Treatment*Cow*Period	0.8787	0.56

†Notes a statistically significant effect among effects ($P < 0.05$)

[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milkfat analyses were in duplicate, d1 only. Protein and ash were in duplicate on d1 and d8. Outliers, determined by an outlier box plot, were excluded. Milk was stored for a total of 11 days (4°C; no light exposure).

Table I4: Experiment 3, Mineral Composition F Table. F statistic and P values for specific contrasts with mineral composition (Ca, Cu, Fe, P) as response on whole processed (pasteurized, homogenized) milk[1].

Contrast	F Statistic	P value
	Ca (mg/kg)	
Treatment*Period	1.2683	0.33
Treatment*Cow	2.584	0.05†
Cow*Period	1.0801	0.43
Treatment*Cow*Period	1.9527	0.10
	Cu (mg/kg)	
Treatment*Period	1.2683	0.33
Treatment*Cow	2.584	0.05†
Cow*Period	1.0801	0.43
Treatment*Cow*Period	4.227	0.00†
	Fe (mg/kg)	
Treatment*Period	2.1882	0.09
Treatment*Cow	3.1045	0.03†
Cow*Period	1.8159	0.13
Treatment*Cow*Period	2.6138	0.04†
	P (mg/kg)	
Treatment*Period	1.8654	0.14
Treatment*Cow	1.8953	0.13
Cow*Period	0.8206	0.61
Treatment*Cow*Period	1.7606	0.14

†Notes a statistically significant effect among effects ($P < 0.05$).

[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milk was stored for a total of 11 days (4°C; no light exposure).

Appendix J: Experiment 3. Oxidative stability

Table J1: Experiment 3, TBARS. Malondialdehyde concentration (mg/kg; mean ± SE), as indication of oxidation on whole processed (pasteurized, homogenized) milk[1].

Treatment[1]	Malondialdehyde (mg/kg)[2]		
	Day 1 $\bar{x} \pm SE$	Day 8 $\bar{x} \pm SE$	Overall[3] $\bar{x} \pm SE$
Control (0 mg/kg iron)	0.975±0.285	1.37±0.141	1.17±0.22
Low (200 mg/kg iron)	1.76±0.421	1.32±0.138	1.53±0.25
Medium (500 mg/kg iron)	1.71±0.375	1.18±0.137	1.44±0.25
High (1250 mg/kg iron)	1.91±0.480	1.45±0.336	1.68±0.41
Average	1.59±0.074	1.33±0.020	1.46±0.04

[1] Milk obtained from cows (n=4) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Each test was run in duplicate. Milk was stored for a total of 11 days (4°C; no light exposure).
[2] There were no statistically significant differences ($P > 0.05$) found among the different treatments using ANOVA.
[3] Day 1 and day 8 differences in MDA (mg/kg) were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

Table J2: Experiment 3, TBARS by Cow. Malondialdehyde concentration (mg/kg; mean ± SE), as indication of oxidation on whole processed (pasteurized, homogenized) milk[1].

Malondialdehyde (mg/kg)				
	Cow[3]			
Treatment[1,2]	4541 $\bar{x} \pm SE$	4543 $\bar{x} \pm SE$	4558 $\bar{x} \pm SE$	4559 $\bar{x} \pm SE$
Control (0 mg/kg iron)	1.13±0.370	0.73±0.076	1.79±0.333	1.04±0.443
Low (200 mg/kg iron)	2.34±0.428	0.86±0.214	1.75±0.462	1.21±0.040
Medium (500 mg/kg iron)	1.25±0.036	2.13±0.741	0.71±0.055	1.68±0.092
High (1250 mg/kg iron)	2.48±0.624	1.75±0.702	0.55±0.089	1.93±0.591
Average[2]	1.80±0.258	1.37±0.306	1.20±0.166	1.46±0.206

[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Each test was run in duplicate. Milk was stored for a total of 11 days (4°C; no light exposure).

[2] There were no statistically significant differences ($P > 0.05$) found among the different treatments or cows using ANOVA.

[3] Day 1 and day 8 differences in mineral composition were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

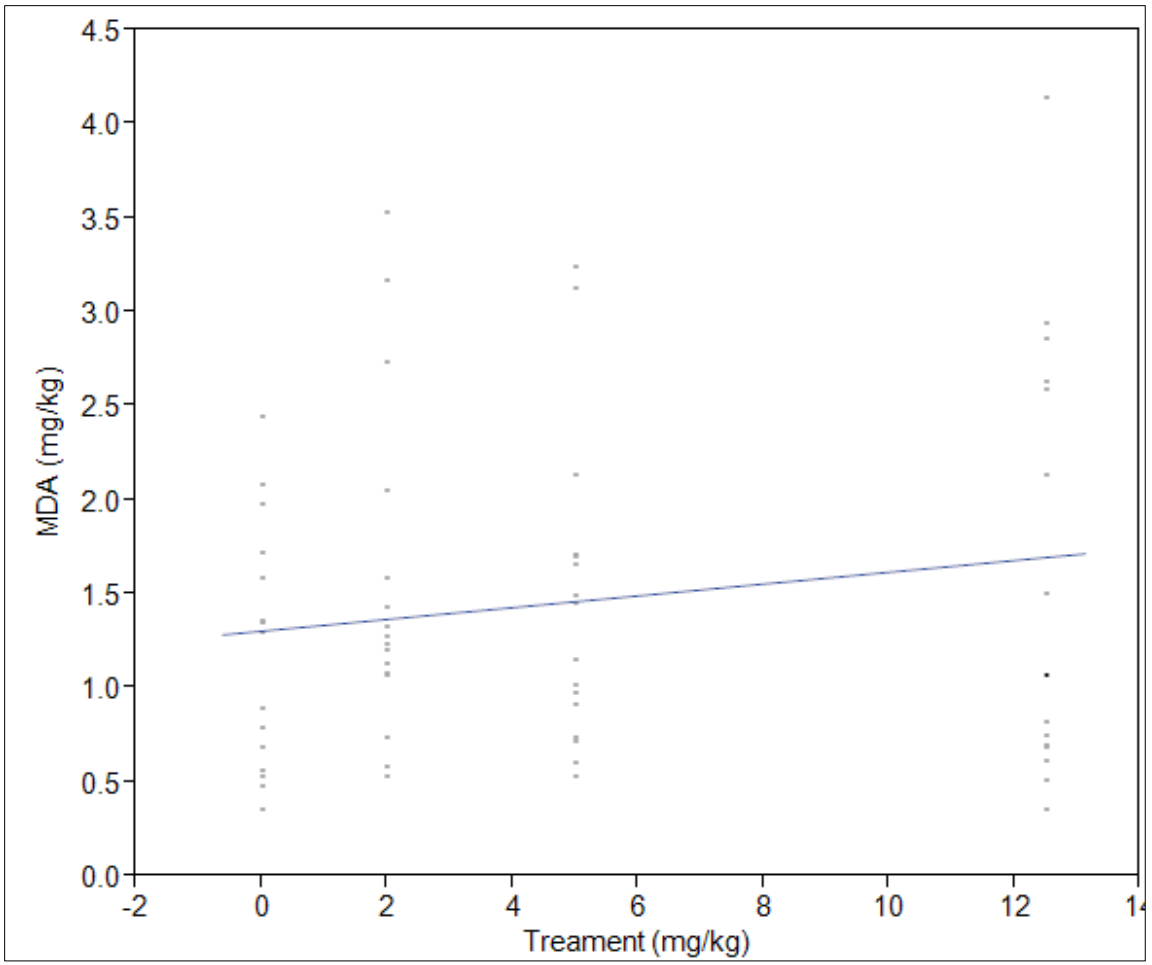

Figure J1: Experiment 3, Treatment vs. MDA. Malondialdehyde concentration (mg/kg), as indication of oxidation on whole processed (pasteurized, homogenized) milk in response to iron treatment (mg/kg). Milk obtained from cows (n=4; duplicate values (d1, d8) per cow) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Each test was run in duplicate. Milk was stored for a total of 11 days (4°C; no light exposure). Treatment concentration did not have a significant effect on MDA using ANOVA ($P = 0.1854$; $R^2=0.02811$). Day 1 and day 8 differences in mineral composition were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

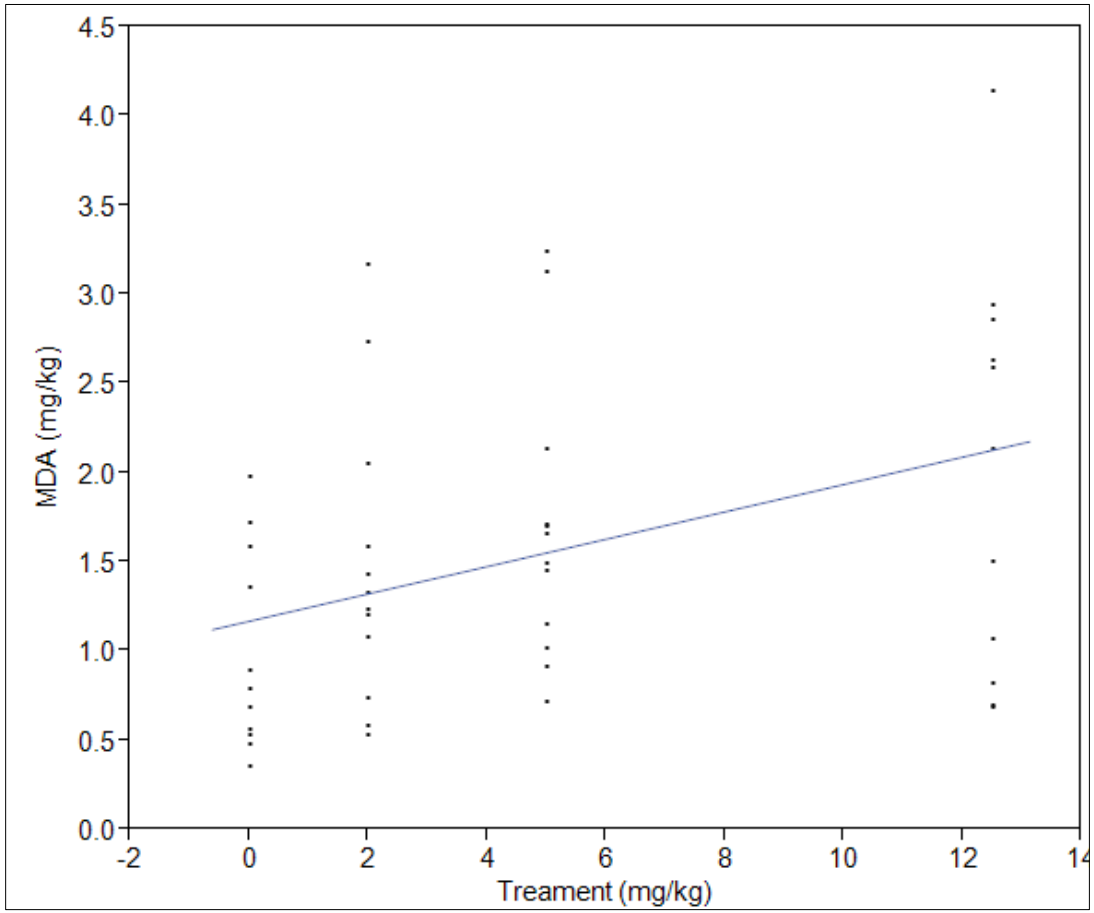

Figure J2: Experiment 3, Treatment vs. MDA (Cow 4558 Excluded). Malondialdehyde concentration (mg/kg), as indication of oxidation on whole processed (pasteurized, homogenized) milk in response to iron treatment (mg/kg). Milk obtained from cows (n=4; duplicate values (d1, d8) per cow) infused in the abomasum with ferrous lactate solution at four concentrations. Cow 4558 excluded (Holstein, 41 months old). Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Each test was run in duplicate. Milk was stored for a total of 11 days (4°C; no light exposure). Treatment concentration did have a significant effect on MDA using ANOVA ($P = 0.0040$; $R^2=0.166112$). Day 1 and day 8 differences in mineral composition were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

Table J3: Experiment 3, MDA F Table. F statistic and P values for specific contrasts with oxidative stability (MDA) as response on whole processed (pasteurized, homogenized) milk[1].

Contrast	F Statistic	P value
	TBARS (mg/kg)	
Treatment*Period	0.7233	0.68
Treatment*Cow	1.3451	0.29
Cow*Period	0.6742	0.72
Week[Treatment][2]	0.5957	0.45
Treatment*Cow*Period	1.0844	0.43

[†]Notes a statistically significant effect among effects ($P < 0.05$).
[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milk was stored for a total of 11 days (4°C; no light exposure).
[2] Week nested within treatment

Table J4: Experiment 3, Hexanal. Hexanal (mean ± SE; n=8), determined by gas chromatography mass spectrometry as indication of oxidation on whole processed (pasteurized, homogenized) milk[1].

	Hexanal area[2]	
Treatment[3,4]	Day 1 $\bar{x} \pm SE$	Day 8 $\bar{x} \pm SE$
Control (0 mg/kg iron)	$2.2 \times 10^6 \pm 0.24 \times 10^6$	$1.8 \times 10^6 \pm 0.35 \times 10^6$
Low (200 mg/kg iron)	$2.2 \times 10^6 \pm 0.27 \times 10^6$	$1.7 \times 10^6 \pm 0.17 \times 10^6$
Medium (500 mg/kg iron)	$1.9 \times 10^6 \pm 0.35 \times 10^6$	$2.1 \times 10^6 \pm 0.21 \times 10^6$
High (1250 mg/kg iron)	$2.1 \times 10^6 \pm 0.42 \times 10^6$	$2.4 \times 10^6 \pm 0.46 \times 10^6$

[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milk was stored for a total of 11 days (4°C; no light exposure).
[2] Units are shown in area under the curve. Area directly correlates to amount of compound present.
[3] There were no statistically significant differences ($P > 0.05$) found among the different treatments or cows using ANOVA.
[4] Day 1 and day 8 differences in mineral composition were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

Table J5: Experiment 3, Pentanal. Pentanal (mean ± SE; n=8), as indication of oxidation on whole processed (pasteurized, homogenized) milk. All of the four two week periods of milk collection are represented. Milk was stored for 12 total days (4°C; no light exposure).

	Pentanal area[1]	
Treatment[2,3]	Day 1 $\bar{x} \pm SE$	Day 8 $\bar{x} \pm SE$
Control (0 mg/kg iron)	$2.5 \times 10^6 \pm 0.64 \times 10^6$	$2.7 \times 10^6 \pm 0.42 \times 10^6$
Low (200 mg/kg iron)	$2.4 \times 10^6 \pm 0.57 \times 10^6$	$2.1 \times 10^6 \pm 0.28 \times 10^6$
Medium (500 mg/kg iron)	$2.1 \times 10^6 \pm 0.57 \times 10^6$	$3.0 \times 10^6 \pm 0.60 \times 10^6$
High (1250 mg/kg iron)	$1.6 \times 10^6 \pm 0.35 \times 10^6$	$2.7 \times 10^6 \pm 0.71 \times 10^6$

[1] Milk obtained from cows (n=4; (d1, d8) values as duplicates) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milk was stored for a total of 11 days (4°C; no light exposure).
[2] Units are shown in area under the curve. Area directly correlates to amount of compound present.
[3] There were no statistically significant differences ($P > 0.05$) found among the different treatments or cows using ANOVA.
[4] Day 1 and day 8 differences in mineral composition were not statistically significant ($P > 0.05$) using a Tukey-Kramer analysis.

Table J6: Experiment 3, Sensory Test. Sensory triangle test for difference on whole processed (pasteurized, homogenized) milk[1].

	Sensory triangle test for difference							
	Number correct[2]							
Treatment[1]	1.1[3]	1.2	2.1	2.2	3.1	3.2	4.1	4.2
Low (200 mg/kg)	13	18*	17	21*	15	19*	21*	24*
Medium (500 mg/kg)	22*	15	29*	28*	16	21*	12	23*
High (1250 mg/kg)	18*	19*	25*	30*	21*	25*	16	17

* Detectable differences milk samples in comparison to the control are statistically significant ($P < 0.05$) at α=0.05 (β=0.3; p_d=30; n=36); critical value = 18 correct responses
[1] Milk obtained from cows (n=4) infused in the abomasum with ferrous lactate solution at four concentrations. Ferrous lactate solutions were made using ultrapure water and were provided for four days prior to milk collection. All four two week period of milk collection are represented. Milk was stored for a total of 11 days (4°C; no light exposure).
[2] Represents period 1, week 1. Other notations follow the same format.

Appendix K: Experiment 4. Gross Composition and TBARS

Table K1: Experiment 4, Gross Composition. Fat and protein (%; mean ± SE), MDA (mg/kg; mean ± SE) as determined by TBARS, and mineral content (mg/kg; mean ± SE) as determined by inductively coupled plasma mass spectrometry on raw milk from cows receiving water with low and high levels of iron[1].

Analyses	Low (0.014 mg/kg) $\bar{x} \pm SE$	High (0.99 mg/kg) $\bar{x} \pm SE$	Average[2] $\bar{x} \pm SE$
		Gross Composition[3]	
Fat (%)	3.91±0.0617	3.78±0.0437	3.85±0.0408
Protein (%)	3.06±0.0235	3.06±0.0231	3.06±0.0164
		TBARS[4]	
MDA (mg/kg)	0.76±0.044	0.78±0.049	0.77±0.033
		Mineral Composition[4]	
Calcium (mg/kg)	842±25.8	825±31.6	834±20.0
Copper (mg/kg)	0.022±0.002	0.065±0.026	0.043±0.009
Iron (mg/kg)	0.228±0.024	0.235±0.025	0.232±0.017
Phosphorus (mg/kg)	672±17.3	670±27.4	671±15.4

[1] Iron levels refer to water given to cattle *ad libitum* containing low iron (0.014 mg/kg) and high iron levels (0.99 mg/kg). Gross composition analyses completed by DHIA labs. Milk was treated with bronopol and natamycin to prevent bacterial and yeast growth.
[2] Differences among means were statistically not significant using ANOVA ($P > 0.05$).
[3] Gross composition was tested by individual cow (n=136).
[4] Milk was pooled 12 h (10 mL each from 17 individual cow samples) after receipt from DHIA labs. Pooled samples were transferred into plastic low density polyethylene bottles, labeled appropriately, and stored in a dark cooler (4^{o}C) for 4 h before TBARS and 1 d before ICP-MS preparation by nitric acid digestion. TBARS (n=8; duplicate samples) and mineral composition (n=8) completed on pooled samples.

Table K2: Experiment 4, Mineral Composition in Literature. Literature referencing mineral content of raw milk.

	Mineral content			
Reference	Calcium (mg/kg)	Copper (mg/kg)	Iron (mg/kg)	Phosphorous (mg/kg)
Fransson and Lonnderdal, 1983	830-1389	0.019-0.204	0.107-0.573	
Goff and Hill, 1993	1068-1262	0.097-0.583	0.291-0.583	874-971
Birghila et al., 2008		0.17	0.72	1608
Sikiric et al., 2003	1125.76-2019.04	0.2-0.69	0.10-0.16	
Rodríguez et al., 1999	1888		0.500	

Table K3: Experiment 4, Milk Yield. Results of DHIA collection of milk (kg; mean ±SE), from cows receiving water with low and high levels of iron[1].

Iron Level	Milk (kg)[3] $\bar{x} \pm SE$		
Low (0.014 mg/kg)	31.91^{A}	±	0.5772
High (0.99 mg/kg)	26.69^{B}	±	0.5356

* Statistically significant differences among means represented by superscript letters determined by ANOVA ($P < 0.05$).

[1] Iron levels refer to water given to cattle ad libitum containing low iron (0.014 mg/kg) and high iron levels (0.99 mg/kg). Milk yield measured on farm from cows (n=126) prior to DHIA lab analysis.

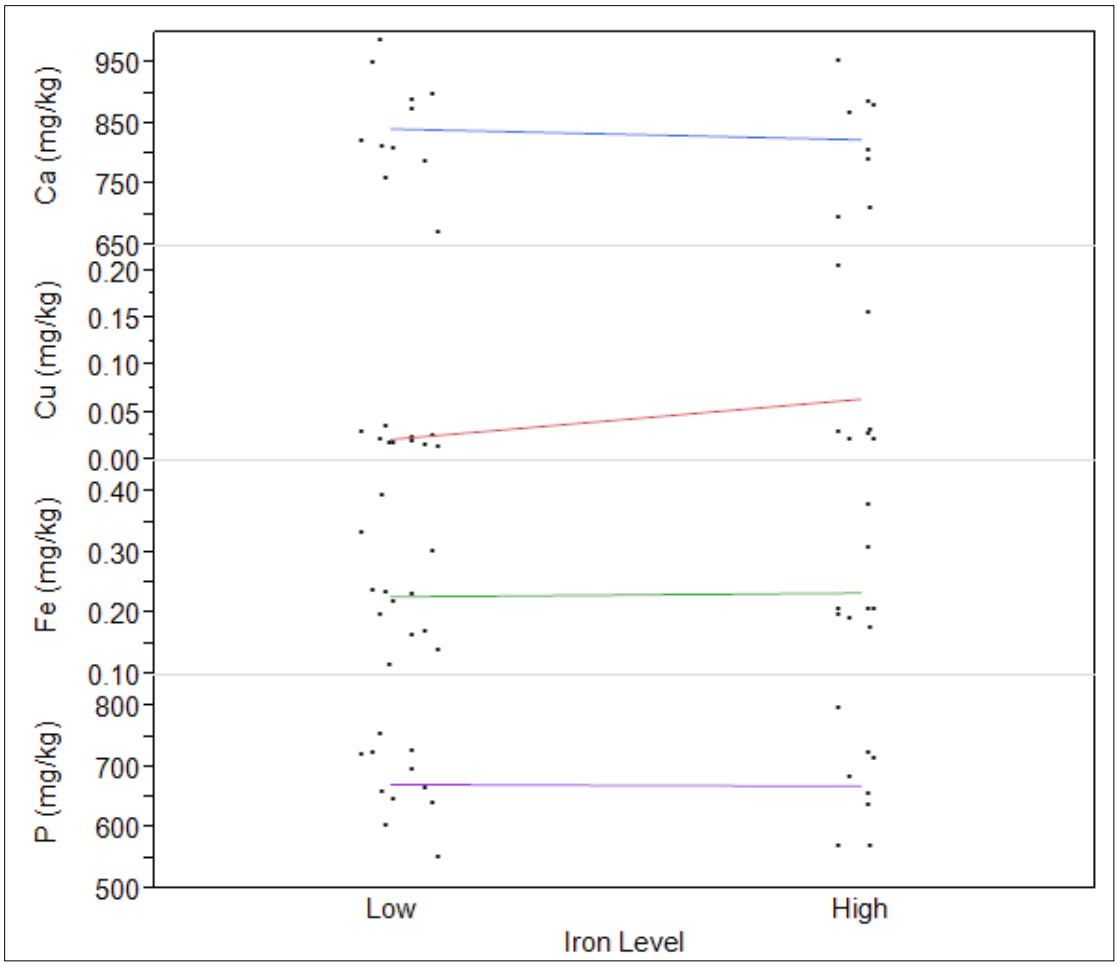

Figure K1: Experiment 4, Treatment vs. Mineral Content. Mineral content (mg/kg), as determined by inductively coupled plasma mass spectrometry on raw milk. Iron levels refer to water given to cattle ad libitum containing low iron (0.014 mg/kg) and high iron levels (0.99 mg/kg). Milk (n=8) was analyzed by a DHIA laboratory and treated with bronopol and natamycin to prevent bacterial and yeast growth prior to the ICP-MS analysis. Milk was pooled (10 mL each from 17 individual cow samples) after receipt from DHIA labs. Pooled samples were transferred into plastic low density polyethylene bottles, labeled appropriately, and stored in a dark cooler (4^{o}C) for 1 d before ICP-MS preparation by nitric acid digestion. Differences among means were not significant using ANOVA ($P > 0.05$).

Appendix L: Virginia State Dairyman Article, January 2013

Water Use in the Dairy Industry: Learning from New Zealand

By: Georgianna Mann, Dr. Susan Duncan
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Drought is a term that seems to be on the minds of many Americans after the dry summer of 2012. The impacts of drought on dairy production causes difficulty with production, and subsequently, higher milk and dairy product costs. Drought affects water availability and quality, which are important for cow health and milk quality. Georgianna Mann, a graduate student in Food Science and Technology at Virginia Tech, travelled to New Zealand to investigate the issues of water availability and quality on the New Zealand dairy industry. She talked with farmers, a dairy cooperative, and dairy representatives at the New Zealand Field Days, the largest agribusiness event in the Southern Hemisphere. Georgianna was specifically interested in minerals in water sources for dairies.

Regardless of whether the dairy production is in the Southern or Northern hemisphere, water is used on the dairy farm for animal cooling, wash-down, and hydration. In addition water is needed for cleaning and sanitation of the operations, parlor and equipment, and cooling of milk. In the U.S. dairy industry over the past 70 years, water usage requirements for producing a gallon of milk have decreased by 65% but there are some aspects that cannot be reduced. About 30 gallons of drinking water are needed per cow per day for maintaining health and producing milk. This means that over 3,000 gallons of good quality water are required daily on a 100-cow farm just to sustain the animals.

While the USA has far more renewable water resources than New Zealand, the 2012 drought suggests that we might learn from the industry in New Zealand. Most of the concerns about water quality and availability in New Zealand are largely tied to the new government

implementation of "water take" regulations, a policy designed to curtail wasteful water usage on the farms. These "water take" regulations are quite similar to water restrictions that may be implemented in the United States in the face of a drought, but deep wells where water is taken from, called bores, will be equipped with meters on them to closely monitor the amount of water used. New Zealand has "controlled activity consents" which require farmers to obtain consent for any water needed above the stock water supply (standard allocations per herd) if there are over 215 cows in the herd, according to a regional council member. Herds smaller than 215 are not considered to be of significant impact on the full allocation of water resources but in the future it is likely all farms will need to meet the strict regulations. An astonishing 3,500 farms will be requiring these consents in the Waikato region on the North Island of New Zealand over the next three years. This is reflective of New Zealand's growing concern for their water supply.

In one particular New Zealand water "catchment", akin to a watershed, the farms are using "using all the water resources we've got", according to a regional council member. Most water for dairy cattle is derived from bores and tested for quality before use. In some areas the water cannot be used due to overloads of heavy metals, especially iron. One farmer noted that "the water was orange. We had to cap the bore; it was too full of iron." He elaborated on methods used to improve the metal-laden water, using "huge filters on the deep bores… but it's quite expensive and you have to keep changing the medium since the lifespan is 4-5 months. It back flushes every hour. It's horrible water coming out."

New Zealand, a country boasting the "100% Pure" tourism advertisements, seeks to entice visitors by selling its closeness to raw nature. While New Zealand is actively assessing water needs from a conservation standpoint, they are taking no risk of wasting this valuable resource. One of the ways farmers reuse water is to irrigate land with the effluents. This,

however, is strictly monitored to ensure too much nitrogen and phosphorus is not being returned to the soil. The New Zealand government and its citizens are devoted to keeping water resources pristine, particularly lakes. The regional council member noted that Lake Taupo has "very low losses but what we're seeing with a lake that is so pristine, any farming in the catchment is creating an effect so we're starting to see deep algal blooms accumulating in the lake". The New Zealand drive for water conservation stems from the desire to preserve this valuable resource.

Virginia did not suffer directly from the 2012 drought like other regions of the U.S. However, the dairy industry in Virginia can recognize that the water resources available today may not be as abundant in the future. The United States, in contrast to New Zealand, has a water withdrawal per capita that is more than three times that of New Zealand. Virginia appears "rich" in water resources. However, when water availability and quality become stressed and costs for available water increase, the effects will be felt in the dairy industry. Restrictions, whether regulated or imposed because of lack of water resources, could indirectly limit dairy herd size and quality milk production. Dairy producers and processors can be thinking proactively about options for water conservation, including reuse and recycling of water, and consideration of the implications these options have on dairy production and processing.