

**The Interaction of Iron with Proteins and Sugars in Biological Fluids and
Beverages**

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Abstract-Academic

Iron is one of the most common trace elements in natural water sources and an important component to living systems. The existence of iron may not only cause metallic flavor, it may also deeply impact health of human and animals by interacting with constituents in the related biological fluids such as saliva and milk. The overall goal of this study is to investigate the taste interaction between iron and sweeteners, and the interaction between iron and proteins in bovine milk and human saliva. Based on pairwise-ranking sensory test, we have found that sweetness of sweeteners was varied with different concentrations of minerals in water and with different types of sweeteners. Sweet-metallic taste interaction between sucrose and ferrous ions significantly ($p < 0.05$) increased the acceptance of very hard water (3 mg Fe/L). The sweet-metallic interaction created a unique selection of the emotional term “mild”. High iron concentration in bovine drinking water (2 mg Fe/L or higher), causing oxidative stress in dairy cattle, affected expression of both casein and whey proteins in the milk. Direct addition of iron above 5 mg Fe/L in processed whole commercial milk led to lipid oxidation during storage at 4°C. Oxidation level was positively associated with increasing concentration of added iron. Minerals (Mg, P, Na, K, Ca, Zn) in milk were not affected with the added iron in milk. Dietary supplementation with metal-binding protein significantly decreased ($p < 0.05$) taste and smell abnormality score in cancer patients receiving chemotherapy, and this effect lasted at least 30 days after the treatment was ended. Although supplementation did not effectively reduce ($p > 0.05$) the metallic taste intensity stimulated by ferrous sulfate solution (1 mg Fe/L), it significantly ($p < 0.05$) decreased

salivary Fe for both healthy subjects and cancer patients. The production of metallic taste perception both induced by chemotherapy and ferrous sulfate solution, might be associated with the decreased expression of low-abundance proteins (pH 5.5-8.5, MW 25-75kDa), which were mainly immune proteins in saliva. Supplementation may improve taste disorder by recovering low-abundance salivary proteins in cancer patients.

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Abstract-Public

Iron is one of the most common trace elements in natural water source and important components to living systems. The existence of iron may not only cause metallic flavor, it may also deeply impact health of human and animals. The overall goal of this study was to investigate how iron in drinking water influences taste perception in sweetened beverage and quality of cow milk, and the effect of iron-binding protein in improving human health. Results showed that sweet-metallic taste interactions varied with different concentrations of iron and with different types of sweeteners. Higher water hardness may increase perception of sweetness of sucrose, honey and ace-K, but may decrease perceived sweetness of sucralose. Addition of sucrose in very hard water largely improved taste perception of the water and elicited a “mild” feeling. Our results provide valuable information to the beverage industry relating to water quality used for beverage production and implications relating to type of sweeteners used to generate the most optimal taste in beverage. Excess iron in bovine drinking water (2 mg Fe/L and higher) decreased the quality of milk including protein degradation, mineral concentrations changes, and development of off-flavor. Contamination of iron in processed whole commercial milk also decreased the nutrition value and taste perception of milk. Changes in milk proteins as affected by iron may affect yield and quality of dairy products, such as cheese. Results of this study may assist dairy farmers, by guiding them about implications of bovine drinking water quality, and remind the dairy processing industry to control metal contamination in milk/ milk sources during transport, processing, packaging and storage. Protein supplementation effectively improved

metallic taste disturbance symptom in cancer patients receiving chemotherapy, and benefited their immunity system by increasing the production of salivary immune proteins. This study suggests that protein supplementation over long-term (60 days) may be an effective treatment for reducing taste disturbance symptoms caused by chemotherapy, which may benefit the recovery of cancer patients by increasing appetite, food intake, nutrition level and immunity.

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Chapter 1

Introduction

Iron is one of the most important trace elements for living systems. It is the fourth most common element found in the Earth's crust and forms much of Earth's outer and inner core (Folkl, 2011). In living cells, it is involved in a variety of biological processes including oxygen transport, oxidative phosphorylation, DNA biosynthesis and xenobiotic metabolism (Carpenter and Mahoney, 1992; Hentze et al., 1996).

Iron is commonly found as binary compounds in nature; the most common forms are ferrous oxide (Fe_2O_3) and ferric oxide (Fe_3O_4) (Stumm et al., 1992). As an essential component for oxygen utilization, iron is readily oxidized both in natural environment and living cells. Iron is stable in dry air as solid metal or in pure water (without oxygen) as soluble ferrous iron. However, when iron is exposed to an environment both with water and oxygen, it will react with oxygen to form iron (II) hydroxide, which will be rapidly oxidized to iron (III) oxide (Fe_3O_4) (Lenntech, 2013). In living systems, iron oxidation causes oxidative stress, which leads to the accumulation of reactive oxygen species (ROS) (Halliwell, 2007). Failure to detoxify ROS will release toxic compounds that severely destroy the membranes, proteins and DNA (Halliwell et al., 1984). Such damage alters the structure of proteins, such as proteolysis or post-translational modification, which results in a decrease of protein bioactivity and a change of protein composition. As a consequence, biological functions of living cells are damaged, which may lead to observable effects such as change or loss in taste perception in human beings or decrease of milk quality of dairy cattle.

Extra iron or other minerals in drinking water will not only induce oxidative stress, it may also result in taste change because of interaction with compounds. In the food industry, water

used for beverage production has to meet drinking water standards established by the US Environmental Protection Agency (USEPA, 2012). However, mineral thresholds can be detected even under the maximum amount of drinking water standard. For example, iron can impart metallic flavor; the overall (estimated population) sensory threshold for ferrous (Fe^{2+}) iron in drinking water was as low as 0.17 mg/L (Mirlohi et al., 2011). This threshold is lower than the drinking water secondary standard for iron (0.3 mg/L; USEPA 2012), indicating that iron may cause metallic flavor in beverage if the concentration of iron in the water source approaches the maximum limit. On the other hand, although artificial sweetening agents are commonly used in commercialized beverage industry, little is known about the interaction between sweeteners and minerals. Previous studies have proven that low concentration of sodium (salt) can enhance sweetness, while moderate intensity of sweetener (sweet) can inhibit salty taste (Kreast et al., 2003). However, the taste interaction between sweeteners and other minerals and their effective concentration of interaction still lacks study.

Excess iron in drinking water will not only influence the taste, it may also induce oxidative stress in living cells and affect health of human and animals. Water supplied to dairy cattle on the farm is commonly from a reservoir or well, in which iron concentration often exceeds the secondary maximum contaminant levels (SMCC) of 0.3 mg Fe/L (USEPA, 2012). The excess amount of iron found in bovine drinking water may be due to high iron-containing water from local ground water or contamination from metal container/pipelines (Lenntech, 2013). Lactating dairy cattle consume a large quantity of water every day (~ 100 L/day) (Feng et al., 2013). Since water contributes more than 80 % of the total weight of milk, water chemistry may deeply impact milk quality.

Cow's milk is a nutritious food containing numerous essential nutrients, especially milk proteins that serve as an excellent source for essential amino acids. Milk proteins are comprised of caseins (80%) and whey proteins (20%). Caseins are well known to carry calcium and phosphate with many bioactive functions as well as contributing to efficient digestion (Haug et al., 2007). Whey proteins possess a variety of nutritional and biological properties, thus are widely used in reducing the risks of diseases such as cancer (Gill and Cross, 2000; de Wit, 1998), inflammation (Clare et al., 2003), chronic stress-induced disease (Ganjam et al., 1997), and HIV (Oona et al., 1997; Micke et al., 2002). Bovine whey proteins have many biological functions including β -lactoglobulin (mediate and transport immunoglobulins during colostrum formation), α -lactalbumin (lactose synthase component and possible antimicrobial/anticancer activity), immunoglobulins (serving as antibodies to protect the mammary gland from infection), serum albumin (anti-mutagenic, anticancer, and immunomodulation activity), lactoferrin (iron-binding, iron transport, antimicrobial/anti-inflammatory/anticancer activities, immune system modulation), and lactoperoxidase (antimicrobial and antioxidant properties) (Alonso-Fauste et al., 2012; Haug et al., 2007; Swaisgood, 1995; Levieux and Ollier, 1999; Loimaranta et al., 1999; Adlerova et al., 2008). However, the effect of high levels of iron in bovine drinking water on milk quality, especially milk protein synthesis has not been well studied yet.

Proteins in human saliva play an important role in taste perception. However, a majority of cancer patients experience changes in taste perception after receiving chemotherapy and/or radiation treatments (Bernhardson et al., 2007), which is associated with the alteration of saliva proteome caused by these treatments. It has been reported that the occurrence of taste alteration in chemotherapy patients can be as high as 69.9 % (Wickham et al., 1999; Ravasco et al., 2005). A significant proportion of taste change has been found in head and neck cancer patients, where

88.8 % of patients reported changes in one taste and 66.7 % for more than one taste (Redda et al., 2006). The prevailing taste alteration in cancer patients is perception of metallic flavor, which is described as “a peculiar mouthfeel, which is similar to that observed when an iron nail or metal foil is placed in the mouth” (Clark et al., 2009). This taste alteration is commonly produced during the chemotherapy/radiotherapy, which can last several hours, several weeks, or even several months after the completion of the treatments (Bernhardon et al., 2007; Rhodes et al., 1994; Steinbach et al., 2009). As a frequent side effect of chemotherapy, this taste alteration largely decreases cancer patients’ appetite, food intake, nutrition level, immunity, and subsequently impairs their life quality (Bernhardson et al., 2007; Zabernigg et al., 2010). However, the biological understanding for the production of metallic flavor and an effective therapy for cancer patients undergoing chemotherapy still lacks study.

Although metallic taste disturbance is widespread and is frequently a grievance of cancer patients, there are no established therapies that are reliable to prevent or treat this problem. Treatment strategies currently suggested for cancer patients with taste disturbance are using plastic utensils instead of metallic silverware (Hong et al., 2009), eating cold food (Rehwaldt et al., 2009), adding strong herbs and spices (Boltong et al., 2012), and using sweeteners or other taste enhancers, which are only able to temporarily reduce metallic taste perception. A long-term effective treatment method is still urgently needed to improve the metallic taste disturbance of cancer patients.

Recently, lactoferrin, which is found in milk and saliva, has received attention due to its diversity of bioactive functions. As an iron-binding protein, lactoferrin works as a metal chelator to remove excess iron from living cells by binding and transporting iron ions. Based on its metal chelation property, lactoferrin is able to restrict lipid peroxidation and potential radical-

generating reactions through scavenger of lipid byproducts in vitro (Sies, 1997). Due to its anti-inflammatory and antioxidant functions, lactoferrin is involved in a wide range of biological activities such as prevention of cell injury and tissue damage, antimicrobial defense, immune modulation, cellular growth and differentiation, and cancer prevention (Bellamy et al., 1992; Wright and Gallin, 1979; Varadhachary et al., 2004; Spadaro et al., 2007). Recently, lactoferrin has been found to effectively decrease metallic flavor stimulated by ferrous sulfate solution in healthy subjects. In our previous study (Ömur-Özbek et al., 2012), a post-rinse of the oral cavity with a lactoferrin solution (10.4 mg/L) after ferrous iron (1 mg Fe/L), completely removed the metallic flavor for all 19 participants (10 females, age 19-53 years). Talactoferrin (3g/day for 12 weeks) in other studies was well tolerated without any significant toxicity (Jonasch et al., 2008). In this study, we will test whether daily intake of lactoferrin supplement can reduce taste disturbance in cancer patients.

Objectives

The overall goals of this study are to investigate (1) the interaction of iron and sweeteners and the effect on metallic and sweet perception, (2) the interaction between iron and iron-binding proteins and their effect on biological fluids including bovine milk and human saliva. This is accomplished through several designed studies.

In the first study, the influence of different concentrations of iron and water hardness on taste perception and emotional response of sweetened water beverages is studied. Sweet-metallic taste interaction is investigated by detecting sweet and metallic intensity generated from combinations of ferrous ions (0, 0.3, 1 and 3 mg Fe/L) and five commercial sweeteners including sucrose, honey, sucralose, saccharin, and acesulfame potassium (ace-K). In addition, emotional responses using check-all-that-apply (CATA) term ballot in combination with hedonic response is applied

to study binary taste interactions. Results of this study are useful in understanding taste interaction between sweet (commercial sweeteners) and metallic (ferrous ions) flavors, and influence on consumer acceptability. Our results provide valuable guidance information to beverage industry for water quality used for beverage production and type and amount of sweetener used to generate the most optimal taste in beverage.

In the second study, how iron in water sources affects milk protein composition, oxidative stability and minerals concentration is investigated. We evaluate the interaction of water-sourced iron (low, medium and high level), as determined through in vivo (water provided to dairy cattle) and in vitro (direct addition to whole processed milk) delivery, on milk proteome and implications on other milk quality parameters including oxidative state and mineral content. Our hypothesis was that both methods of iron infusion would affect milk proteome, affect milk mineral content, and would contribute to changes in oxidative stability. Results of this study deliver knowledge to dairy farm and processing industry about how different levels of iron in bovine drinking water impact the quality of raw milk and the related dairy products. This study may assist dairy farms in establishing good agricultural practices for bovine drinking water standard for milk quality control. Results of this study also may assist the dairy industry in regulating iron content to prevent iron-induced oxidation in milk products during transport, processing, packaging and storage.

In the third study, we evaluated the effects of protein supplements on reducing long-term metallic taste disturbance of cancer patients receiving chemotherapy and temporary metallic flavor perception stimulated by ferrous sulfate solution. As comparison, effect of supplement intake was studied also in healthy subjects. Salivary proteome analysis conducted by two-dimensional electrophoresis (2-DE) was used to detect the relationship between salivary

proteome and metallic taste perception. In addition, concentration of salivary minerals, metallic taste intensity, and self-reported taste abnormality along with supplement treatment were also recorded and compared. We hypothesized that protein supplement could be clinically used to alleviate taste disturbances in cancer patients. This study suggests a long-term effective treatment in reducing taste disturbance symptom in cancer patients receiving chemotherapy and/or radiotherapy, which may benefit the recovery of cancer patients by increasing appetite, food intake, nutrition level and immunity.

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Chapter 2

Literature Review

2.1 Background

2.1.1 Iron Source

Iron is one of the richest elements on earth, forming five percent of the earth's crust (Folkl, 2011). Iron naturally exists in soil and sediments and can be dissolved by rain and further brought to rivers and seas. In addition, natural deposits, industrial wastes and corrosion of iron-containing metals, especially iron pipes, introduces iron into water systems (Lenntech, 2013). Generally, the iron concentration in wells and aquifers is between 0.5-1 ppm (Folkl, 2011) and in groundwater is about 100 ppm but the iron level in sea water varies largely among different districts (Lenntech, 2013). In drinking water, the iron level is required to below 0.3 ppm according to National Secondary Drinking Water Regulations (USEPA, 2012).

Iron can be present in water as soluble ferrous iron (typically Fe^{2+} compounds such as iron carbonate, iron sulphide and iron vitriol) or insoluble ferric iron (generally Fe^{3+} compounds like iron oxide, iron hydroxide, iron carbide and iron penta carbonyl) (Lenntech, 2013). Dissolved iron appears as $\text{Fe}(\text{OH})^{2+}$ under acidic and neutral oxygen-rich conditions while presented as binary iron when lacking oxygen. Iron is relatively stable in pure water (without oxygen) or dry air. However, when iron-containing water is exposed to air, the colorless water will turn to reddish brown and a precipitate, which mainly consists of iron (II) hydroxide, may form. This process is called iron corrosion (Lenntech, 2013).

Iron is an essential trace element for living cells. In plants, iron is crucial to carry oxygen through their systems. In animals, iron consists of the central part of hemoglobin, which transports oxygen to each organ of body. Plants obtain iron through soil, which is mainly as a

form of ferric oxide (Hewitt, 1963). For human and animals, the principle source of iron comes from food intake. Specifically, animals such as dairy cattle absorb iron from their dry diet (National Research Council, 1956) while humans ingest iron through meat, eggs, fortified cereal products, and vegetables (Block et al., 1985). Although the iron concentration in ground water can be as high as 100 ppm (Lenntech, 2016), it is not considered as a normal iron source for animals. The low amount of iron in drinking water for human (≤ 0.3 mg Fe/L) (USEPA, 2012) is also not included in the dietary iron source.

2.1.2 Iron and Health

Iron is not only responsible to transport oxygen in living system, it is also involved in various cellular processes including oxidative phosphorylation, DNA biosynthesis and xenobiotic metabolism (Carpenter and Mahoney, 1992; Hentze et al., 1996). Besides producing energy for the body, iron is also a significant component of various proteins including hemoglobin, cytochromes, oxygenases, flavoproteins, and redoxins (Mackenzie et al., 2008).

Dietary iron serves as the primary source of bioavailable iron for human and animals, including inorganic forms as ferrous and ferric compounds (e.g. vegetables and cereal) and organic forms as heme iron (e.g. meat and eggs) (Carpenter and Mahoney, 1992). Heme iron (15%-35%) has much higher bioavailability than nonheme iron (2%-20%), and it can directly combine with hemoglobin to transport oxygen (Hurrell and Egli, 2010). Nonheme iron, including inorganic iron and other forms of iron with very low absorption, is utilized by body for hemoglobin synthesis (Yip, 1996) and formation of an iron storage pool through binding with other compounds to form complexes such as polypeptides or form chelates with ligands such as ascorbic acid, sucrose and amino acids (Carpenter and Mahoney, 1992). Nonheme iron in the pool may be absorbed and utilized by body through iron-binding proteins such as transferrin and

ferritin (Carpenter and Mahoney, 1992). Although nonheme iron is low in bioavailability, its quantity in the diet is manifold higher than heme iron (Monsen et al., 1978). Therefore, control of iron absorption is regulated by levels of iron-binding proteins such as transferrin, lactoferrin and ferritin.

However, excess iron also can be dangerous to living cells. When iron exceeds the demand of living cells, there will be an increase in the concentration of reactive oxygen and nitrogen species (such as OH , O^{2-} , and NO), which is called oxidative stress (Puntarulo, 2005). Oxidative stress is “a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage” (Sies, et al., 1985). The resistance of a biological system to oxidative stress depends on its ability to readily detoxify the reactive oxygen species (ROS) or to repair the resulting damage (Halliwell, 2007). Failure to detoxify ROS will lead to production of peroxides and free radicals that damage the cell, which further induces the disruption of cellular signaling (Sies, 1997; Halliwell, 2007). During the iron-dependent conversion, superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) transfer to the extremely reactive hydroxyl radical (OH) (Haber-Weiss reaction) and release toxic compounds that severely destroy the membranes, proteins and DNA (Halliwell and Gutteridge, 1984). Such damage may lead to the peroxidation of nearby lipids, oxidative damage of DNA and other macromolecules (Papanikolaou and Pantopoulos, 2005), and cell membrane damage and, thus, interrupt several biological reactions (Lobo et al., 2010). As a consequence, humans or animals may suffer damage of blood vessels, reduced activity of natural killer cells and lymphatic system (Weinberg, 1990), increased risk of microbial or virus infection (Metwally et al., 2004), formation of tumor sites because of iron deposition (Weinberg, 1990), heart failure caused by the great affinity of myocardial cells with iron (Weinberg, 1990), and other chronic diseases such as

chronic liver diseases (Sikorska et al., 2003), type II diabetes (McCarty, 2003) and hypertension (Piperno et al., 2002).

One direct consequence of oxidative stress is the accumulation of oxidized protein, which is derived from the oxidation of amino acid residue side chains and protein backbone, formation of protein cross-linkages and aggregation, and generation of protein carbonyl derivatives. Protein oxidation was reported to associate with aging and a number of diseases in humans such as Alzheimer's disease, respiratory distress syndrome, muscular dystrophy, amyotrophic lateral sclerosis and rheumatoid arthritis (Schuessler and Schilling, 1984). When free iron is available, which is determined by the concentrations of iron-binding proteins (such as lactoferrin, transferrin) and iron-responsive factors (control the binding and release of iron from iron-binding proteins), derivatives of protein oxidation such as H_2O_2 can generate the even more toxic OH by iron-catalyzed cleavage through the Fenton reaction (Berlett and Stadtman, 1997). On the other hand, free iron also catalyzes the formation of free radicals, which will hasten oxidative stress and further oxidize oxygen-derived radicals (Hentze and Kühn, 1996). It has been demonstrated that the primary oxidative damage to proteins was metal-catalyzed oxidation (Stadtman and Berlett, 1998; Berlett and Stadtman, 1997).

Excess iron ingestion not only influences human health; it also impacts the health of animals. Previous studies reported that dairy cattle can tolerate no more than 1000 ppm dietary iron under most conditions, especially if other mineral constituents are adequately supplied (National Research Council, 1956). Excess ingestion of iron was found to impact bovine health through increasing reactive oxygen species (oxidative stress), which has been related to an increase in mastitis and retained fetal membranes as well as a decrease in immune function (Linn, 2008). Higher iron concentration also may result in metallic flavor in water, which reduces water

consumption and milk production of cows (Linn, 2008). In addition, higher iron levels in water affect cow's performance (Feng et al., 2013), including the decrease of digestibility of DM, NDF, and nitrogen.

To minimize the damage (protein aggregation and cross-linking) induced by protein oxidation, a series of antioxidant defense mechanisms are activated within dairy cattle. Mammalian cells serve as the first protective defense to rescue moderately damaged polypeptides. Both prokaryotic and eukaryotic cells have enzymes that can directly repair some covalent modifications to the primary structure of proteins, and restore the functions of defective proteins through reduction of oxidized disulfide bonds, repair function of disulfide bonds and reduction of amino acid side chains (Grune et al., 1997). However, mammalian cells have only limited ability to directly repair oxidized protein. Most oxidized proteins are degraded by proteolytic pathway to minimize protein aggregation and remove potentially toxic protein fragments, which is considered as the secondary antioxidant defense (Berlet and Stadtman, 1997).

2. 2 Minerals and Sensory Perception in Water

2.2.1 Hard Water and Soft Water

Hard water has high amounts of dissolved calcium, magnesium and other minor minerals such as sodium, iron, strontium and manganese. Soft water contains similar minerals but with much lower levels of calcium, magnesium and sometimes iron. The hardness of water is commonly expressed as an equivalent concentration of calcium carbonate (CaCO_3) (USEPA, 1986). The more minerals present, the harder the water is. The typical classification of the hardness of water is given in the Table 1 (DES, 2008).

Table 1. Classification of Water by Hardness Content

Description	Conc. mg/L CaCO ₃
Soft	0-75
Moderately hard	75-150
Hard	150-300
Very hard	300 and up

Minerals in natural water are dissolved from soil and rock during the water movement; small amounts of minerals are dissolved and retained in water (Lenntech, 2013). During the transportation, water may further dissolve metal ions when passing through the metal pipes. However, metals such as iron, copper and lead, which are soluble in water, are potentially toxic when reaching certain concentrations.

Hard water is not a health hazard but is a nuisance because of mineral buildup on plumbing fixtures and poor soap or detergent performance (Cowens, 2004). Excessive minerals in drinking water may result in potential health risk and metallic flavor perception (Mirlohi et al., 2011). Thus, some municipalities and industries try to soften the hard water before supplying to consumers. There are various ways of softening hard water including ion exchange, chelating agents or distillation. One of the commonly used methods is ion exchange, in which the calcium and magnesium ions are replaced with sodium or potassium ions. The harder the water, the more sodium that has to be added in the exchange process to soften the water (Greenleaf et al., 2006).

2.2.2 The Influence of Minerals on Taste of Drinking Water

Minerals in water may deeply influence the taste and much of a “mouth feel” of water. Minerals such as magnesium, iron, copper, zinc, sodium can add salty, sweet, or bitter tastes to water (Dietrich and Burlingame, 2015).

Calcium is common in water and it is dissolved in tap water as forms of Ca(HCO₃)₂, CaSO₄ and CaCl₂ (Whelton, 2007). The taste threshold concentration for calcium is 100–300 mg/L,

which varies according to its associated anion (WHO, 2011). Calcium concentration between 10 and 100 mg/L is considered as good-tasting water (Burlingame et al., 2007). However, this is probably due to its lower hardness rather than the effect of calcium ions. It is still unclear whether calcium directly influences the drinking water taste; soft water (0-70 mg/L, calcium) has no deteriorated effect on the taste (Burlingame et al., 2007). Most drinking water has calcium concentration less than 50 mg/L (van der Leeden et al., 1990); mineral water may have calcium levels above 150 mg/L (van der Aa, 2003).

Magnesium is commonly present in water as MgCO_3 , $\text{Mg}(\text{HCO}_3)_2$, MgSO_4 , and MgCl_2 (Whelton, 2007). Magnesium can impart an astringent or bitter taste and can be detected by consumers at 100–500 mg/L (Zoeteman et al., 1978). In tap water, magnesium is normally below 20 mg/L although in some areas the magnesium can be as high as 120 mg/L (van der Leeden et al. 1990). However, water containing magnesium salts at 1,000 mg/L has been considered acceptable (Bruvold and Pangborn, 1966). Water containing magnesium concentrations above 10 mg/L in water causes an offensive and bitter taste (Zoeteman et al., 1978).

Iron is normally found in water as soluble, suspended, or hydroxide forms (Whelton et al., 2007). Iron in water can cause metallic taste, rusty color, sediment, and reddish/orange staining of plumbing and laundry (USEPA, 1979), if its concentration is above SMCL standard (0.3 mg Fe/L; USEPA 2012). However, the overall population threshold of ferrous can be as low as 0.17 mg Fe^{2+} /L; younger group (19-50 years) is 0.045 mg Fe^{2+} /L and older group (50-84 years) is 0.498 mg Fe^{2+} /L (Mirlohi et al., 2011). This indicated that ferrous ions in drinking water can be perceived even under the water standards. Ferric ion normally has no taste or odor since it cannot be oxidized and will not produce off-flavor volatiles through lipid oxidation (Ömür-Özbek and Dietrich, 2011).

Copper presented in natural water is at low ug/L concentrations, and the SMCL standard for copper in the drinking water is 1 mg Cu/L to prevent aesthetic effects and unpleasant taste (USEPA, 1979). Threshold of copper is 0.4-0.8 mg soluble Cu/L in water (Cuppett et al., 2006). When above the threshold, copper can be tasted as metallic, bitter and astringent in mouth, with lingering aftertastes that develop 1-2 min after ingestion (Hong et al., 2010). Copper can enter into drinking water system by dissolving from the corrosive copper pipes (Dietrich et al., 2004). Copper ions in water also generate a blue or blue-green color when its concentration approaching 0.5 mg Cu/L in water (USEPA, 1979).

Sodium is commonly found in water and is typically present in water as NaHCO_3 and Na_2SO_4 (Whelton et al., 2007). Sodium is responsible for salty taste and its taste threshold concentration is 30–460 mg/L according to various factors such as serving temperature, diet and age of person (USEPA, 2003). The US Environmental Protection Agency (USEPA) recommends that the concentration of sodium in water should not exceed 60 mg/L to avoid any salty taste (USEPA, 2003), even though most drinking water contains less than 50 mg/L sodium. Water taste is disliked by consumers when NaHCO_3 exceeds 630 mg/L and Na_2CO_3 exceeds 75 mg/L (Whelton et al., 2007).

Anions such as chloride can also impact the taste in water. The taste threshold concentration for chloride varies from 200 to 300 mg/L (Lockhart et al., 1955; WHO, 2004). Potassium chloride and magnesium chloride are less noticeable in taste than sodium chloride and calcium chloride (Burlingame et al., 2007). Most tap water contains less than 50 mg/L chloride ions (van der Leeden et al., 1990). In the presence of sodium, calcium, potassium, and magnesium in water, increasing chloride levels can cause objectionable water taste (Burlingame et al., 2007). Consumer acceptance testing has indicated that water containing NaCl less than 290 mg/L is

acceptable and NaCl above 465 mg/L is disliked. Testing also revealed that CaCl₂ less than 120 mg/L in water is neutral in taste, while CaCl₂ above 350 mg/L is disliked (Whelton, 2007).

2.2.3 Mineral Interactions Influencing Taste and Flavor

Beverage and food products contain multiple components. Water is a principal ingredient in many products, potentially contributing minerals to the product composition. The interaction of minerals from the water ingredient with other molecules can influence the odor, taste and flavor of beverages and foods. The flavor is the result of comprehensive effects of various tastants. Metallic, bitter or astringent off-flavors can create unpleasant sensations that influence quality of the food experience.

2.3 Taste Interactions

2.3.1 Taste-Taste Interactions

Taste stimuli are perceived by taste buds/receptors on the tongue or mouth surfaces (Noble, 1996). When single taste compounds are mixed together, they may enhance or suppress other tastes to present a totally new flavor rather than simply adding up the tastes. Interactions between taste compounds can take place at three different levels including chemical interactions, oral physiological interactions and central cognitive interactions (Keast and Breslin, 2002a).

Chemical interactions occur in the food matrix, resulting in changes in taste intensity or even taste qualities. For example, sodium can bind with gluten in bread and lose its taste intensity (Liem et al., 2011). Precipitation caused by several compounds in aqueous solution will result in weaker or no taste perception (Keast and Breslin, 2002a). In oral physiological interactions, two compounds are mixed. There is a potential that one compound may interfere with taste receptor cells or taste transduction mechanisms associated with a second compound (Keast and Breslin, 2002a). In a study demonstrating whether sodium can suppress bitter (Kroeze

and Bartoshuk, 1985), researchers applied a bitter stimulus on one side of tongue and a sodium salt on the other side. Both stimuli were applied independently and simultaneously. Then these two stimuli were mixed together and applied on the same tongue locations again. The perception of bitterness was not reduced until the stimuli were applied as a mixture. This kind of reaction is classified as peripheral interaction and can take place at a number of sites or taste receptor cells (Keast et al., 2001). Cognitive interactions refer to central processing of taste stimuli signals in the brain. In Kroeze and Bartoshuk's (1985) study, salty taste was decreased after mixing bitter and sodium salt stimulus; this is an example of cognitive interaction (Kroeze and Bartoshuk, 1985). When the stimuli interacted with taste transduction mechanisms in the tongue, the afferent signals were sent to the nucleus of the solitary tract and to upstream taste processing regions of the brain where the signal was decoded and reduced salty taste was then perceived.

There are a number of examples to show taste interactions caused by minerals in foods or beverages. Sodium chloride (NaCl) presents salty taste and improves the sensory properties of foods by increasing saltiness, decreasing bitterness, or increasing sweetness (Keast and Breslin, 2002a). Sodium enhances sweetness at low intensities/concentrations but inhibits or has no effect on sweetness at high intensity/concentration. On the other hand, sweetness can also inhibit salty taste at moderate intensity (Keast and Breslin, 2002a). In a study detecting the taste interaction between sodium (salt), sucrose (sweet) and urea (bitter) in a mixture (Breslin and Beauchamp, 1997), the authors found that perceived bitterness was suppressed when sodium was added to a bitter-sweet mixture. The perception of sweetness was also increased due to the decrease of bitterness. The effect of reducing the use of salt in a taste mixture has been conducted by a series of experiments designed to investigate the taste interactions between sucrose, citric acid and NaCl (Pangborn, 1960; Pangborn, 1962). Several different kinds of food matrix were used

including pear nectar, tomato juice and lima bean puree. The results proved that removing sodium caused bitterness to be released from suppression, thereby decreasing sweetness (Bresli and Beauchamp, 1997).

2.3.2 Taste-Aroma Interaction

Although metallic flavor is always described as bitter, salty taste and astringent mouthfeel (Ömur-Özbek & Dietrich, 2011), metallic sensation is a result of combination of taste and odor, which is classified as “metallic flavor”. Flavor is defined as “the psychological interpretation of a physiological response to a physical stimulus” (Heymann et al., 1993), which is a comprehensive result of nasal and oral stimulation (Noble, 1996). When food is broken down in the mouth, it will interact with saliva and release food volatiles. The volatiles in the oral cavity headspace are transferred through the nasal passages from the back of the mouth, through the cribiform plate, and perceived through olfactory receptors. Food volatiles can be perceived by inhaling the volatile headspace through the mouth (Noble, 1996). Taste-aroma interactions belong to central cognitive effect, which take place at the central processing level (Noble, 1996).

Many studies have shown that odors can enhance or suppress in food products (Caporale et al., 2004; Labbe et al., 2007). For example, ethyl butyrate (fruit aroma) can increase the sweetness in sucrose-sweetened solution (Hornung and Enns, 1994). Furthermore, taste-aroma interactions are not necessarily associated with the concentration of odours or taste compounds. Although aroma effects may be inconsistent in some cases (S áenz-Navajas et al., 2010), it still presented the ability in inhibiting off-flavor and potentially used to suppress metallic smell in mineral water. In addition, previous studies have proven that metallic sensation can be largely decreased if olfaction of panelists was occluded by nose-clips (Mirlohi et al. 2011). Thus, it is

reasonable to speculate that the addition of aroma compounds such as ethyl butyrate may decrease the perception of metallic flavor.

Aroma used for modifying taste perception has to be the congruent aroma-taste pair. For example, strawberry aroma can increase sweetness perception because people associate strawberry odor with sweetness, whereas peanut butter does not affect sweetness rating (Frank and Byram, 1988). Some taste-aroma interactions can increase both the smell and taste, but some interactions only take effect on one. When ethyl butyrate was added to a sucrose-sweetened solution, both sweetness of sucrose and ethyl butyrate aroma were increased due to the existence of each other (Hormung and Enns, 1994). In addition, the intensity of sweetness of sucrose was increased along with the increasing concentration of ethyl butyrate, and vice versa (Hormung and Enns, 1994). In contrast, the increase of peach essence in glucose-sweetened beverage enhances both the intensity and duration of sweetness of glucose. However, increasing concentration of glucose did not increase the intensity of fruitiness even though the persistence of peach flavor was extended (Cliff and Noble, 1990).

2.3.3 Masking Metallic and Bitter Off-Flavors

Metallic and bitter flavors are common problems in pharmaceuticals. They also occur in beverages, foods and supplements that include some phytochemicals such as polyphenols. Water, as an additional source of metals that may contribute to these off-flavors, can enhance the problem. One approach is to mask the effects. Enhancing sweetness is often used for masking these off-flavors.

Three types of taste interactions are generally used to mask the bitterness of a compound: 1) physico-chemical interactions in a food or beverage matrix, 2) oral peripheral physiological interactions with receptor cells, 3) central cognitive mixture suppression such as taste-taste and

taste-aroma interactions (Keast, 2008; Keast and Breslin, 2002b). Physio-chemical interactions reduce intensity of bitterness by changing the chemical structure or composition of food compounds. Weak attractive forces (e.g. hydrogen, hydrophobic bonding) between different taste compounds will result in altered structures, and precipitation of the compounds may lead to them taste weaker or even tasteless (Keast and Breslin, 2002b). Alteration of chemical composition, changing from an aqueous to emulsion system (oil-in-water), reduced the bitterness of original food matrix (Metcalf and Vickers, 2002). The “masking” compounds, whether or not elicit a taste, can have effects by occupying common taste receptor sites on the tongue (e.g. sweet compounds occupy bitter taste receptors to reduce bitterness in a sweet-bitter mixture (Lawless, 1979; Keast and Breslin, 2002a and 2002b), or via interactions in the oral periphery by using fats and fatty acids that have physio-chemical influence on bitterness (Koriyama et al., 2002).

Central cognitive effects take place in a mixture of taste stimuli, in which the perceived intensity of one or more taste is diminished by the perception of the others (Keast, 2008). Suppression of bitterness by sweeteners is one of the examples of central cognitive effects. For example, when adding sugar to coffee, both the sweetness of the sugar and the bitterness of the coffee are reduced (Keast, 2008). Taste-aroma interaction is another example to influence the taste and intensity through central cognitive effects. However, the aroma used for modifying taste perception has to meet the congruency of the aroma-taste pair. Astringency has been reported to be decreased by the increase of sweetness perception (Lyman and Green, 1990; Ares et al., 2009). This effect was due to an increase in salivary volume and the lubrication characteristics of sucrose solutions (Lyman and Green, 1990).

To mask bitterness or other off-tastes, several techniques have been evolved (Ley, 2008):

- 1) Physical barriers such as micro/nano encapsulation, coatings, emulsions, suspensions;

- 2) Scavengers, complexing agents;
- 3) Strong flavors or tastants such as salt, sweeteners, acid, and strong fruit flavors;
- 4) Congruent flavors such as chocolate, grapefruit, and coffee;
- 5) Masking flavors (e.g., against rancid or fishy flavor of poly unsaturated fats); and
- 6) Bitter taste reducing compounds on a molecular level.

Sweeteners and other compounds can offer some masking properties. Polydextrose is a polysaccharide often used as fat-mimetic in low-fat dairy product and also used as an alternative sweetener in various desserts (Mitchell, 1996; Roland et al., 1999). It can mask unpleasant flavors through interacting with polar, hydrogen bonds or dipole-dipole interactions (Plug and Haring, 1993). But the effectiveness of polydextrose in reducing bitterness depends on the type and concentration of the antioxidant components in the food product. Fat emulsions are reported to inhibit the bitterness of quinine (Metcalf and Vickers, 2002). Selected cyclodextrins were able to reduce or even fully eliminate bitterness of the bitter taste compounds dissolved in an aqueous solution when formed in inclusion complex (Szejtli and Szente, 2005). Zn-lactate and Na-gluconate were reported to effectively reduce bitterness of caffeine (71% and 31% bitterness inhibition respectively) through oral peripheral strategy (Keast, 2008). Since Zn ions have a high affinity for both thiol and hydroxyl group, it can readily bind with the amino acids/proteins in extracellular portions of the bitter taste receptor (TAS2Rs) form a complex. Once combined, the native configuration of TAS2Rs can be altered and are unavailable for bitterness reception (Meyerhof et al., 2010).

Recently, an interesting result was shown that milk was an effective way to mask bitterness of caffeine (Keast, 2008). In addition, Ares et al. (2009) found that milk was able to significantly ($p < 0.01$) decrease the bitterness and astringency of antioxidant extracts from *A.satureioides*. One

of the possible mechanisms for the reduction of bitterness by milk is the reaction between milk proteins and polyphenolic compounds that yields insoluble or at least incapable compounds for interacting with the taste receptors (Keast, 2008). However, how to mask metallic flavor has not been specifically studied yet.

Over the past decades, only a few studies focused on the causes of metallic taste and how to control it. Hettinger et al. (1990) found that FeSO_4 produced a retronasal smell rather than any other salts. In addition, the metallic taste from ferrous sulfate was decreased when the nose was occluded. It was reported that the taste of placing copper penny in the mouth differs from that of rinsing with FeSO_4 solutions, which produces a retronasal smell (Lawless et al., 2004). In a study of metallic taste and retronasal smell, Lawless et al. (2004) compared a series of metal solutions and found the metallic perception of FeSO_4 was reduced to baseline with nose occlusion, but no such reduction was observed for CuSO_4 or ZnSO_4 . The metallic taste stemming from FeSO_4 is likely due to development of a retronasal smell which is probably followed by a lipid oxidation reaction in the mouth (Lawless et al., 2004). Ömür-Özbek et al. (2012) further demonstrated that ferrous ion solution caused the greatest flavor perception and lipid oxidation compared with cupric and cuprous ions in the study of metallic flavor in the oral cavity. Sensory evaluation showed that lactoferrin effectively removed the metallic flavor of all the samples as well as artificial chelating agents EDTA, while antioxidants (vitamins E and C) minimally reduced the metallic flavor (Ömür-Özbek et al., 2012).

2.4 Interaction between Iron and Biological Fluids

Biological fluids in mammals, including saliva, blood, milk, and intra- and extracellular fluids, exhibit a critical role in body by involvement in a variety of bioactivities. As one of the most common minerals in living cells and natural environment, iron may directly or indirectly

interact with biological fluids in mammals and deeply influence the components and functions of these fluids. This study focuses on human saliva and milk, with an emphasis on interactions between iron from water source and components within these fluids.

Human saliva is composed of 99.5 % water and 0.5 % other components include a variety of electrolytes, proteins, enzymes and nitrogenous products (Table 2). Saliva is a mixture of secreted fluid present in the oral cavity and is produced by three main salivary glands: the major and minor salivary glands (for ca 90 %) and the gingival crevicular fluid (for ca 10 %) (Hirtz et al., 2005). With a role in digestion, lubrication and formation of a pellicle that coats and protects teeth and other oral surfaces, saliva is crucial for the maintenance of oral health by forming the first line of oral cavity defense against bacterial and viral insult (Amerongen and Veerman, 2002). On the other hand, inadequate volumes of saliva will lead to a painful tongue and mucosa, decreasing taste ability, swallowing and chewing problem, and an increased risk for infections (Vissink, 1985; Mandel, 1989; Axell, 1992).

Table 2. Major components of biological fluids (bovine milk and human whole saliva) and the major composition of proteins and minerals in each type of biological fluid

Components	Biological fluids	
	Milk ¹	Human whole saliva ²
Water	88%	99.5%
Proteins	3.3%	< 0.5%
Minerals	0.7%	<0.5%
Fat	3.4%	<0.5%
Lactose	4.9%	—
Major protein composition		
Lactoferrin	√	√
Transferrin	√	√
Immunoglobulins	√	√
Casein	√	—
β-lactoglobulin	√	—
α-lactalbumin	√	—
Amylases	—	√
Albumins	—	√
Major minerals composition³(mg/L)		
Mg	60.5	5.49 ± 1.35
P	739.8	166.69 ± 47.45
Na	779.7	169.23 ± 41.11
K	1140.4	776.28 ± 130.81
Ca	330.0	67.04 ± 13.08
Fe	3.9	1.28 ± 0.21
Zn	11.8	0.54 ± 0.12

¹Milk composition percentage data is from Jenness, 1988.

²Human whole saliva composition percentage data is from Hirtz et al., 2005.

³Data of milk minerals composition is from USDA reported value:

<http://ndb.nal.usda.gov/ndb/foods/show/69>; data of salivary minerals composition in human is from Mirlohi et al., 2015.

Milk is considered as a nearly complete food since it contains water, protein, fat, minerals, vitamins and more than twenty different trace elements (Table 2) that play a significant role in nutrition and health (Enb et al., 2009). The largest portion of milk is water, which is approximately 88 % of total content. As an aqueous medium, water enables the suspension of organic components of milk and helps the movement of milk from mammary gland to the

neonate (Bionaz et al., 2012). The rest of constituents in bovine milk include 4.9 % lactose (carbohydrate), 3.4% fat, 3.3% protein, and 0.7% minerals (ash) (Jenness, 1988).

Proteins in saliva and milk are important for sequestering and transporting iron. In cows, iron is most absorbed and utilized as the form of heme iron, which has high bioavailability (15%-35%) and can directly combine with hemoglobin to transport oxygen (Hurrell and Egli, 2010). The transportation of heme iron to hemoglobin is conducted by iron-binding proteins including transferrin and lactoferrin. The storage of iron in the bovine body is performed by ferritin. Control of iron absorption is regulated by levels of iron-binding proteins such as transferrin, lactoferrin and ferritin. In human saliva, transferrin and lactoferrin appear as immune proteins as the first defense of human body. However, when excess of iron is ingested through mouth, salivary iron-binding proteins will perform its chelating ability to bind with the free ferrous ions.

2.4.1 Metal-Binding Proteins and Metalloproteins

The binding between protein and metallic ions is crucial in biological activity. Metallic ions are essential to life since a wide variety of enzymes require metals for their catalytic activity and other fundamental biological activity including photosynthesis, respiration and nitrogen fixation (Andreini et al., 2008). Other metallic ions such as iron play an important role in mammal by producing blood. Copper is crucial in forming haemoglobin and promoting iron absorption, and calcium is used for building bones, enzyme regulation, acid-base equilibrium and cardiovascular permeability degradation. Most metal ions are combined with specific proteins or enzymes, either directly attached to the polypeptide backbone or via a prosthetic group, where they exert their effects as active or structural centers of proteins (Dong et al., 2003). Thus, proteins (peptides) with multiple binding sites for metallic ions can regulate the metal ion homeostasis,

detoxification, participation in unspecific immuno-reactions, transportation and storage, and regulation of the gene expression by governing signaling process (Kennedy and Ginbey, 2001).

About 40 % of proteins and enzymes contain metal ions in their structures. These can be further defined as metalloproteins and metal-binding proteins. As a metalloprotein, the protein conformation needs to have the appropriate type and number of ligands. Once these ligands are in correct geometry, they are able to encapsulate and activate the metal ion (Kennedy and Ginbey, 2001).

Metal-binding proteins have similar binding mechanisms as metalloproteins. However, the affinity of interactions between metal ion and protein differentiates these two groups.

Metalloproteins have high-affinity interactions with metal ion, which is not lost during the protein purification (isolation and dilution), while the interaction affinity of metal-binding proteins is much lower and easily broken (Herald et al., 2003). The high interacting affinity always happens on binding with transition metal ions such as Cu^{2+} , Fe^{2+} , Mn^{2+} and Mo^{2+} . Their characteristics, including their density, small atomic radius, and interaction through electromagnetic and electrostatic forces, form strongest coordinating interactions and they are found in most metalloproteins (Herald et al., 2003). Ions such as Ca^{2+} and Mg^{2+} combine moderately with proteins while monovalent ions like K^{+} and Na^{+} interact weakly with proteins (Herald et al., 2003). Thus, the ability of binding with specific metal ions decides the metal-binding property of a protein.

2.4.2 Iron-Binding Proteins

Iron is essential to the living system and acts as cofactor in multiple biological processes including blood production, respiration and DNA replication (Aisen et al., 2001). However, free iron is toxic since it is able to damage membranes and other essential biological constituents

through catalyzing the formation of free radicals, which will further oxidize to damage oxygen-derived radicals (Hentze et al., 1996). Iron-binding proteins are important in sequestering overabundant iron to quickly decrease the oxidative stress. Besides, iron-binding proteins can transport and deliver iron to cells in all organisms for utilization or storage. Thus, iron-binding proteins are crucial in regulating iron ion in living organisms.

2.4.2.1 Transferrin

Transferrin is a family of metalloproteins that have a high ability of binding iron and high capacity for iron storage (Dunkov and Georgieva, 2006). The transferrin family includes proteins such as serum transferrin, ovotransferrin, lactoferrin, melanotransferrin (Lambert et al., 2005). The molecular weight of transferrin is around 79 kD (Parkkinen et al., 2002). A unique character of transferrin is its pink color with visual absorbance (max 465nm) resulting from the binding of iron. The ratio of the absorbance at 280 nm to the visible absorbance can be an indicator of purity and whether iron is bound by each lobe (Lambert et al., 2005).

Transferrin is a polypeptide chain whose N⁺ and C⁻ terminal sequences consist of globular lobes. Each lobe contains two domains, which interact with each other to form hydrophilic iron-binding sites between two lobes. Amino acids that are identical located on both lobes (two tyrosines, one histidine and one aspartic acid) are responsible for binding the iron to the transferrin (Lambert et al., 2005). In addition, an anion (two oxygen molecules donated by a carbonate molecule) is also required to ensure the binding process by stabilizing the iron atom (Hirose, 2000). Besides, amino acid residues such as Gly-65, Glu-83, Tyr-85, Arg-124, Lys-206, Ser-248 and Lys-296, which surround the N-terminal lobe are also considered to further stabilize the iron-binding site. What's more, they are important in iron release also (MacGillivray et al., 1998). The affinity of transferrin for iron ion is highly dependent on the pH. Fe (III) is

progressively combined (10^{23} M^{-1}) at pH 7.4 but is decreased largely at pH below 7 (Aisen et al., 1978). The progressive affinity at physiological pH protects Fe (III) from hydrolysis at biological system and sequester Fe (III) to be the catalytic agent of superoxide radical formation via Fenton reactions (Gutteridge, 1994) or growth of pathogens (Bulle and Griffith, 1988). The binding and release of iron by transferrin also depends on temperature, chelator and ionic concentrations (He et al., 2000).

Transferrin is mainly synthesized by liver and distributed in various body fluids including blood plasma and secretory fluids such as bile, amniotic, lymph and breast milk (Qian et al., 2002; Evans et al., 1999). The concentration of transferrin is important for healthy growth and its level in plasma is maintained from 2 -3 g/L since birth (van Campenhout et al., 2003). When transferrin concentration is below 0.1 g/L, infection or growth retardation and anemia always appears (Hayashi et al., 1993).

The primary role of transferrin is to bind iron and transport it in redox-inactive form to tissues all over the body (Gomme, 2005). Transferrin is crucial during erythropoies and active cell division (Macedo, 2008). Serum transferrin (STF), which is synthesized by liver and secreted into the blood, is responsible for iron transport. The STF gene is also expressed in tissues such like “central nervous system, testes, ovary, spleen, mammary gland and kidney” (Lambert et al., 2005). Another important function of transferrins is that they can inhibit the growth of pathogens (Teehan et al., 2004). Free iron can promote the growth of pathogens and lead to the increase of bacterial infection. Therefore the binding of transferrin and iron ion will reduce infection by decreasing the free iron level (Von Bonsdorff et al., 2003). What’s more, apo-transferrin (without binding with iron) was also proven to have the ability to decrease adhesion of gram-positive and gram-negative bacteria to cell surfaces (Ardehali et al., 2003). In

addition, transferrins were also reported to have the effects in growth and differentiation activities such as myotrophic (Shimo-Oka et al., 1986), mitogenic (Sirbasku et al., 1991) and neurotrophic (Bruinink et al., 1996). Since different cell types will respond to different stimulatory and inhibitory signals and such response changes during the cell life cycle, transferrins will work on specific cells at certain stages (Gomme, 2005). Besides, transferrins also present the capability of protecting cells by binding and restoring iron in the cell for preventing cell death (Lesnikov et al., 2001).

As described above, transferrins were developed for multiple clinical applications, such as treating atransferrinemia resulting from iron overload (Van Campenhout et al., 2003), repairing ischemia reperfusion injury caused by oxidative stress with iron canalization (De Vries et al., 2004), sequestering free iron in cardiovascular disease and radiotherapy impaired by lipid peroxidation (Van Campenhout et al., 2003), therapy for cancer by promoting cytotoxicity, growth of lymphokine-activated killer (LAK) cells and natural killer cells through combining with factors like insulin-like growth factor 1 (IGF-1) (Okamoto et al., 1996). Recently, transferrin and its receptor have been found to have the ability to decrease tumour cells by attracting antibodies through receptors (Macedo, 2008).

2.4.2.2 Lactoferrin

Lactoferrin is widely known as one of the most important proteins in milk and saliva, and originally isolated from milk. Although the amount of lactoferrin varies widely between different species and at different stages of lactation, it can occur in high concentration in milk (Masson et al., 1971). Lactoferrin is a single –chain glycoprotein with a molecular weight of about 80 kDa (Kuwata et al., 1998). It functions to bind and transfer iron ions, thus belonging to a transferrin family where all the members have the same polypeptide folding pattern (Baker and Lindely,

1993). Lactoferrin, however, differs from transferrin in its helical inter-lobe connecting peptide while in transferrin it is irregular (Baker et al., 2002). The diversity in functions of lactoferrin may be attributed to its various isoforms. Among the three isolated isoforms, lactoferrin- α could bind with iron but without ribonuclease activity while lactoferrin- β and lactoferrin- γ demonstrate ribonuclease activity but they do not exhibit iron-binding function (Furmanski et al., 1989).

Lactoferrin has a higher PI (8-9) than transferrin, which probably stems from a unique basic region in the N-terminal region of the molecule that is not found in transferrin. This provides lactoferrin the ability to bind in a “pseudospecific” way to many acidic molecules such as heparin and various cell surface molecules (Lampreave and Pineiro, 1990; Gasymov et al. 1999).

Lactoferrin could be eliminated from the organism by two methods. One is through receptor-mediated endocytosis of phagocytic cells including macrophages, monocytes and other cells belonged to the reticuloendothelial system and then transfer iron to ferritin. The other way is directly through the uptake by the liver (Levay et al., 1995; Hutchens et al., 1991).

The model of iron binding of lactoferrin is illustrated in the study of the relationship between the structure and function of lactoferrin and transferrin (Baker et al., 2002). In this model, there exist two kinds of configurations: one is open and the other is closed. In the iron-free state as shown in Fig.1, both of them are accessible for the iron ions although the protein is predominantly in the open state. The initial binding is expected on domain 2 since it provides anion such as Tyr ligands and the CO_3^{2-} ion, which is believed to bind with metal ion first (Baker, 1994). Then, conformational dynamics will allow the lactoferrin occasionally turn into the closed state. Under this condition, if iron is present, the lactoferrin is locked into the closed state by coordination to the Asp (D) and His (H) ligands on domain 1. If no iron is present, the lactoferrin will immediately open again.

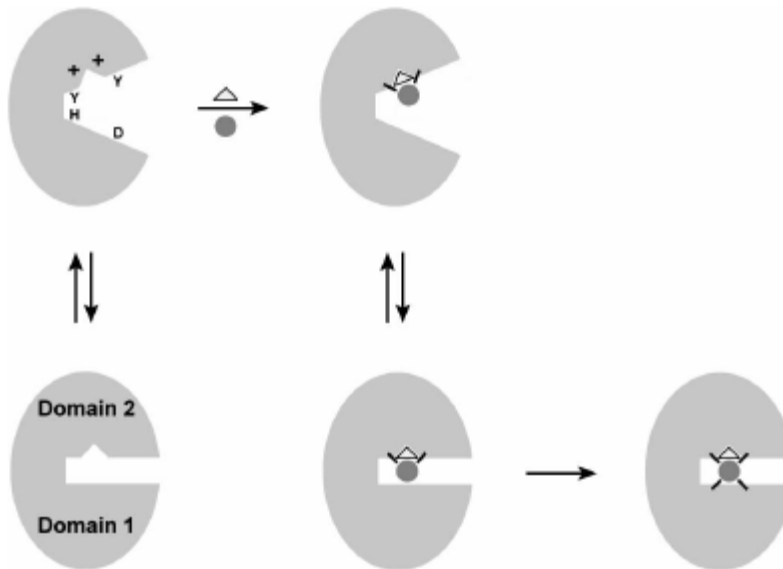


Figure 1. The mechanism of iron binding with lactoferrin protein (Baker et al., 2002).

There are three possible factors influencing iron release by opening the domain. The first is the involvement of specific receptors that may trigger the domains open. However, there is no evidence to prove this for lactoferrin. The second one is the reduction of the bound Fe^{3+} . Lowering the pH is taken as the third factor, which appears to be the main differences between lactoferrins and transferrins. Transferrins release iron at pH 6 whereas lactoferrins do not begin releasing until pH 3.5 (Baker et al., 2002).

The ability for binding iron by lactoferrin is two times higher than that of transferrin (Adlerova et al., 2008) and one lactoferrin molecule binds with two ferric ions. In addition, lactoferrin is able to retain bound iron to a much lower pH of ~3.5 than ~5.5 of transferrin (Mazurier and Spik, 1980). According to the iron saturation, lactoferrin exists in three forms: apolactoferrin (iron free), monoferric form (one ferric ion), and hololactoferrin (binds two ferric ions) (Jameson et al., 1998). Apart from iron, lactoferrin is also capable of binding many other compounds such as lipopolysaccharides, heparin, glycosaminoglycans, DNA and metal ions like

Al^{3+} , Ga^{3+} , Mn^{3+} , Co^{3+} , Cu^{2+} , Zn^{2+} , although its affinity for these ions is much lower (Baker, 1994).

Besides binding iron, lactoferrin has a wide variety of biological functions. Lactoferrin was determined as acute-phase protein due to its increased concentration during most inflammatory reactions and some viral infections (Kanyshkova et al., 2001). Besides, lactoferrin could also inhibit bacterial (and fungi) growth in vitro by sequestering the iron in the medium that is required for microbial metabolism (Kontoghiorghes and Weinberg, 1995), or counteract the inhibitory effect through synthesis of low molecular weight high affinity chelators or by producing specific lactoferrin receptors that could help iron removal from the protein. Lactoferrin can also reduce infectivity of a number of different viruses and shows an antitumour role in vitro (Brock, 2002).

Based on its functional properties, lactoferrin has been used in some clinical trials to evaluate its effect of oral administration in animal models and in humans. Lactoferrin-enriched infant formula was reported to contribute to the formation of a bifidobacteria-rich flora in low birth weight infants (Kawaguchi et al. 1988). In a study of the effect of gamaglobulin and lactoferrin on eliminating endotoxins, the author found that the most effective lactoferrin-enriched bovine colostrum was able to reduce the maximum plasma endotoxin value by 67 % as compared with the albumin group (Döhler and Nebermann, 2002). Malet et al. (2011) found that bovine lactoferrin supplementation can have a beneficial effect on postmenopausal bone loss by modulating bone formation, resorption and immune function. In a study of dietary effect of lactoferrin-enriched fermented milk on acne vulgaris, it was found that lactoferrin-enriched fermented milk ameliorated acne vulgaris with a selective decrease of triacylglycerols in skin surface lipids (Kim et al., 2010). Oral administration of lactoferrin suppressed formation of

precancerous lesions in the large intestine in a colon cancer rat model induced by subcutaneous injection of a carcinogen (Sekine et al., 1997).

Industrial lactoferrin is produced from whey. As existed as a cationic protein, lactoferrin could be readily adsorbed to a cation exchange resin and then eluted using salt solutions. Then the crude lactoferrin is desalted and concentrated. After freeze-drying or spray-drying, purified lactoferrin powder with a purity of 95 % or higher is obtained (bovine lactoferrin). Lactoferrin shows various applications in food industry along with increasing recognition of its biological function. Lactoferrin- supplemented infant formula, follow-up milk and nutritional supplements are being marketed (Tomita et al., 2002).

2.4.2.3 Caseins

In recent decades, casein, which accounts for more than 80% of milk proteins, was also found to have high affinity in binding with Fe ions through the clusters of their phosphoserine residues. Caseins with multiple clusters of phosphoserine residues, especially α_{s1} -, α_{s2} -, and β -casein, present high iron-binding capacity (Horne, 1998). In the study of iron-binding properties of calcium-depleted milk (Mittal et al., 2015), it was found that among milk proteins (casein and whey proteins), iron was bound mostly with casein irrespective of their state of aggregation.

Besides caseins themselves, caseinophosphopeptides (CPP) and casein hydrolysates, which are hydrolytic products of casein, are also able to bind with metal ions such as iron through chelation by their phosphoserine residues. Enriched CPP and casein hydrolysates were reported as antioxidants in corn oil-in-water emulsions at pH 7.0 and 3.0 (D áz et al., 2003) and cooked ground beef (D áz and Decker, 2004). However, CPP only effectively inhibited lipid oxidation promoted by ferric/ascorbate when it was lower than 1.0 mg/mL. Higher amount of CPP (>1.0 mg/mL) were prooxidant in the same reaction. In this study, large hydrolytic products of casein

were found in low, medium and high iron treatment. Their concentration may be much higher than 1.0 mg/mL and turn out to be prooxidants along with iron and other free radicals.

2.4.3 Analysis of Proteins

Identification and quantification of proteins in complex systems are the basis of analyzing complete complements of proteins. Tools for the separation of proteins have developed significantly in recent years and the most common techniques are various types of chromatography for the identification of structure and electrophoresis for the separation of complex protein mixtures. Among them, 2-DE combined with mass spectrometry is widely used and taken as core technology for proteome analysis (Ong and Pandey, 2001).

The principle of 2-DE is to separate proteins according to their isoelectric point along a pH gradient by isoelectric focusing (IEF) in the first dimension, and based on their molecular weight by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Normally, each sample will form an average of 1,000- 2,500 spots corresponding to ~ 300- 1,000 proteins. In some conditions, a protein gel can produce as many as 10,000 spots along with 3,000 proteins at each run (Carrette et al., 2006). Since 2-DE can present hundreds of proteins at the same time on a single gel, it provides a direct and overall profile of a sample proteome. By comparing with the published reference 2-DE maps, scientists can easily detect protein isoforms, variants, posttranslational modifications (Carrette et al., 2006) and identify and quantify the changes of proteins among different treatments of a sample. What's more, the proteins are well protected in the gel media during the sample running and exhibit high sensitivity in 2-DE technology.

Two-dimensional electrophoresis can be applied to almost all type of protein-containing samples, such as biological fluids (saliva and milk), eukaryotic tissues and its extracts, cells and

organelles, seeds and plants (Carrette et al., 2006). For the analysis of protein composition in milk, several studies chose 2-DE technology to analyze its proteome. Lindmark-Månsson et al. (2005) constructed analysis methods for separating proteins at the pH ranges 4-7 and 6-11 in the first dimension, and in the second dimension by SDS-PAGE gel on 12.5 % homogeneous gels. Based on this method, the variations of milk proteins under different heating treatments were clearly shown (Lindmark-Månsson et al., 2005). Pisanu et al. (2012) used the 2-DE/mass spectrometry to characterize the sheep milk fat globule proteins and establish a reference map. Among them, 29 milk fat globule proteins were successfully identified by tandem MS from 61 spots and characterized for gene ontology (Pisanu et al., 2012). Chevalier et al. (2009) applied reducing and non-reducing or combined SDS-PAGE gel when using 2-DE technology with mass spectrometry to separate the raw milk proteins. The study had detected majority of proteins spots of raw milk and this analysis strategy can be proposed to analyze reduction/oxidation of milk and dairy product proteins under different processing treatments (Chevalier et al., 2009). Alonso-Fauste et al. (2012) has applied 2-DE technology to characterize and compare the difference of milk proteome between healthy and mastitis cows. Different mass spectrometry has also been chosen to work with 2-DE technology. For example, matrix-assisted laser desorption/ionization mass spectrometry coupled with 2-DE was used to detect the protein composition in commercial bovine milk. This method helped to resolved the difficulty of identifying basic proteins with low molecular masses by limiting the time-of-flight mass spectrometry analysis to the linear mode when examining the ability of reliable relative molecular masses of the intact proteins in their characterization (Galvani et al., 2001).

In bovine mature milk, 80 % of proteins are caseins (α -S₁, α -S₂, β -, and κ -). The abundant caseins will cover the low abundance proteins when running the gel with whole milk. However,

many low-abundance proteins including serum albumin, β -lactoglobulin and α -lactalbumin need to be further isolated and characterized since parts of them showed biological functions such as enzymatic activities or immunoassays (Swaisgood, 1995). Yamada et al. (2002) developed the method to characterize the low-abundance proteins in bovine colostrums and mature milk using 2-DE. After two major milk proteins were removed from milk effectively, several low-abundance proteins including bovine serum albumin, serotransferrin and lactoferrin were observed. Based on this method, significant differences in low-abundance protein patterns were detected between colostrum and mature milk (Yamada et al., 2002).

Two-dimensional electrophoresis is also applied to characterize proteome of human saliva (Ghafouri et al., 2003; Vitorino et al., 2004; Hu et al., 2005). Since most proteins show quite different expression in saliva compared to serum or tears, salivary proteome has demonstrated unique clinical diagnostic value for initial disease identification (Lee and Wong, 2009). Therefore, many studies compare the differences of salivary proteome between healthy subjects and patients in order to identify the biomarker for certain diseases, which might provide a fast and effective way for clinical diagnosis. Salivary proteome has been studied for dental caries (Mungia et al., 2008; Rudney et al., 2009), periodontal disease (Aurer et al., 2005; Wu et al., 2009), oral cancer (Jou et al., 2010), lung cancer (Xiao et al., 2012), diabetes mellitus (Rao et al., 2009), cystic fibrosis (Minarowski et al., 2008), auto-immune deficiency syndrome (AIDS) (Landrum et al., 2005), and breast cancer (Zhang et al., 2010).

In human normal whole saliva, the most abundant proteins are salivary amylases (58 kDa, pI 5.2-7.8), albumins, transferrin, and immunoglobulins (Adkins et al., 2002). Other abundant proteins include zinc-alpha-glycoprotein, cystatins, and carbonic anhydrase (Hu et al., 2005). Salivary proteins are often reported as biomarkers or associated with certain diseases. For

example, transferrin has been reported to increase in oral squamous cell carcinoma (Jou et al., 2010), head and neck squamous cell carcinoma (Dowling et al., 2008), and oral cancer (Jou et al., 2010). Lactoferrin increased in patients with dental caries (Vitorino et al., 2006), Sjögren's syndrome (Hu et al., 2007), acute and chronic episodes of recurrent parotitis (Tabak et al., 1978), periodontal disease (Groenink et al., 1999), and diabetes (Harrison and Bowen, 1987). Salivary α -amylase increased in patients with gingivitis (He et al., 2011), aggressive periodontitis (Goncalves et al., 2010), and dental caries (Vitorino et al., 2006). IgA increased in patients with dental caries, periodontal disease (Bachrach et al., 2008), oral cancer patient (Jou et al., 2010), and diabetes (Harrison et al., 1987). Annexin A1 increased in patients with lung cancer (Xiao et al., 2012). Zinc- α -2-glycoprotein increased in patients with aggressive periodontitis (Wu et al., 2009), lung cancer (Xiao et al., 2012), and female breast and male prostatic tumors (Diez-Itza et al., 1993). Carbonic anhydrase VI increased in patients with non-invasive breast cancer (Zhang et al., 2010).

2.4.4 Milk

2.4.4.1 Milk Composition

Milk contains all the vitamins required by mammals such as vitamin A, D, E, K, B1, B2, B3, B5, B6, B12 and minerals like calcium and phosphorous. Milk also has numerous other components such as urea, ammonia and cellular metabolites from glycolysis, citric acid cycle, RNA and DNA synthesis and fatty acid synthesis (Jensen, 1995). Milk is not a rich source of iron; most iron in milk is associated with proteins. Proteins binding with iron in bovine milk mainly include lactoferrin, transferrin, casein, and membrane proteins surrounding the fat globule (Fransson and Lönnerdal, 1983). Both lactoferrin and transferrin binds with two ferric ions but lactoferrin presents a much higher binding affinity. Lactoferrin releases its bound iron

below pH 2 and transferrin releases its bound iron at or below pH 4 (Lönnerdal et al., 1981). Casein was found mostly with iron irrespective of their state of aggregation (Mittal et al., 2015). Besides caseins themselves, caseinophosphopeptides (CPP) and casein hydrolysates, which are hydrolytic products of casein, are also able to bind with metal ions such as iron through chelation by their phosphoserine residues (D'Áz et al., 2003). Very limited literatures discuss how iron is synthesized into bovine milk, but it is speculated that iron in milk is from diet/water of cows or contamination from the environment. It has been reported that contamination from metal receptacles can increase the iron content in bovine milk (Feuillen and Plumier, 1952). However, other studies also stated that iron supplementation for cows did not appreciably affect iron concentration in the milk (Archibald, 1958; Pond et al., 1965).

The proteins in the milk can be separated to two groups: caseins and whey proteins. The primary component of milk protein is the caseins, which takes about 82 % of total protein content. Caseins are highly digestible in the intestine while most whey proteins cannot be fully digested and even stimulate a localized intestinal or systemic immune response such as allergy. Caseins are source of nutrients for the neonate by supplying amino acids, calcium and phosphate. The major whey proteins in the milk are β -lactoglobulin (~65 %) and α -lactalbumin (~25 %). α -Lactalbumin is required for synthesis of lactose and thus is necessary for the process of milk synthesis (Haug et al., 2007). Although the specific function of β -lactoglobulin is still not clear, it is generally considered as mediate that transports high levels of immunoglobulins during colostrum formation (Levieux et al., 1999). Other whey proteins include enzymes, which are involved in milk protein processing; hormones, which affect the development of mammary gland such as growth hormone; immunoglobulins, which serve as antibodies that protect the mammary gland from infection; serum albumin, whose concentration will increase during mastitis of cow

and during mammary involution (Swaisgood, 1995); lactoferrin and transferrin, which have the ability to bind with iron ion and decrease the levels of lipid oxidation in human body (Gomme et al., 2005).

2.4.4.2 Influencing Factors on Quality of Milk

The quality of milk is affected by various factors including genetic (breed of cow), physiological (animal health, stage of lactation, milking frequency), and environmental variables (temperature, humidity, water quality, seasonal changes, milking system) (Lindmark- Månsson et al., 2005). In a study of proteomic characterization of bovine serum and whey from healthy and mastitis affected farm animals, the researchers have found that the composition of normal and mastitis whey was highly different (Alonso-Fauste et al., 2012). Although the most abundant proteins in milk such as caseins were detected in both normal and mastitis samples, minor whey proteins like lactoferrin, transferrin, and many cellular proteins were only found in the inflamed animal samples. The absence of cellular proteins from normal milk is the main reason for the great differences between healthy and mastitis whey samples, because cellular proteins were responsible for the biological function related to immune defense.

In a study of the effect of minerals in water on dairy cows (Linn, 2008), it was summarized that higher amount of mineral components including total dissolved solids (TDS), sodium chloride, sulfur and nitrate affected animals' performances, which may reduce bovine intake of water and further decrease milk production. However, calcium, magnesium and water hardness are not considered to affect water intake or performance of animals. In addition, high quality water cannot enhance bovine consumption of water or milk production (Linn, 2008). Iron concentration in drinking water was always reported to exceed the safety requirement due to iron

pipe corrosion or environmental contamination, but few researchers have studied the relationship between iron containing water and the quality of milk.

2.4.5 Human Saliva

2.4.5.1 Inorganic Constituents of Human Saliva

The inorganic components in human saliva are a variety of electrolytes, containing sodium, potassium, calcium, chloride, magnesium, bicarbonate, phosphate and so on (Berkovitz et al., 2002). These constituents have their own function, such as bicarbonate allows buffering while calcium and phosphate maintain the tooth mineral integrity (Dodds et al., 2005). In addition, inorganic components like ionic composition also influence the activity of organic components. For example, lysozyme activity is influenced by electrolytes and salivary anions of low-charge density (Mandel, 1989).

Iron is well known as an essential element for the human body, which is involved in a variety of bioactivities including oxygen transportation through formation of hemoglobin, production of enzyme, and immune surveillance (Beard, 2001). However, function of salivary Fe has not been fully studied yet. Recently, level of salivary iron has been found to relate with several diseases and may potentially serve as an efficient tool for the early diagnosis of some disease. In Shetty et al. (2014), decreased levels of salivary Fe was associated with potentially malignant disorders and oral cancer. Mirlohi et al. (2015) has found that salivary Fe was significantly lower ($p < 0.05$) in cancer patients with primary malignant gliomas compared with healthy subjects.

2.4.5.2 Organic Constituents of Human Saliva

Saliva contains a wide variety of proteins that have particular biologic functions for oral health. The main composition of human saliva is proteins and peptides (salivary proteome), which maintain homeostasis in the oral cavity. Saliva proteins with high levels (35–40 %) of

proline are designated as proline-rich proteins (PRPs). PRPs comprise about 70% of the total protein content of human saliva and can be further divided into three groups based on charge and degree of glycosylation: acidic (MW 16 kDa), basic (MW 6-9 kDa) and glycosylated basic PRPs (MW 36 kDa) (Bennick, 1982). PRPS are crucial in maintaining oral health and intact dentition. The acidic PRPs are multifunctional proteins with separating bacterial and hydroxyapatite-binding domains. When adsorbed to the tooth surface, acidic PRPs may provide highly specific sites to combine with certain oral bacteria. It has been suggested that the attachment of PRPs to hydroxyapatite/enamel lead to a conformational change in the protein, thus exposing bacterial binding sites (termed cryptipopes) hidden in the tertiary structure (Gibbons et al., 1988; 1989). Acidic PRPs also allow saliva to maintain its state of supersaturation by binding with calcium and phosphate ions (Hay et al., 1984). Thus, acidic PRPs may prevent the formation of salivary stones through inhibiting spontaneous calcium phosphate precipitation (Saitoh et al., 1985). In addition, when attached on the tooth surface, the acidic PRPs inhibit apatitic crystal growth by blocking specific mineral growth sites (Aoba et al., 1984). In addition, PRPs can bind with dietary tannins, which protect from the potentially deleterious effects of tannins commonly found in the human diet (Mehansho et al., 1987). It has been suggested that PRPs are also involved in the bitter taste sensation (Azen et al., 1990; Hong et al., 2009a).

The other 30 % of the human saliva proteins are histatins, mucins, amylase, lysozyme, lactoferrin, peroxidase and secretory IgA. These minor components are much more important in destroying or inhibiting the growth of microorganisms in the oral cavity (Xu et al., 1991). Histatins are small (3–5 KDa) and histidine-rich proteins. They can destroy the structure of gram-negative bacteria by neutralizing the lipopolysaccharides of their external membranes (Xu et al., 1991). Mucins are large glycoproteins with viscoelastic properties, which provide

lubrication and prevent desiccation in high degree. Mucins may also inhibit bacteria through “binding to toxins, agglutinating bacteria, interacting with host cells, and are important components of the acquired pellicle and plaque matrix” (Dodds et al., 2005). Among immunologic components (e.g. IgA, IgG and IgM), secretory immunoglobulin A (IgA) is the most abundant one in human saliva. It can neutralize viruses, enzyme toxins and aggregate bacteria through inhibiting their adherence to oral surface (Williams et al., 1972). Similarly, lysozyme can also aggregate and inhibit the attachment of bacteria onto the oral surface. Besides, its strong cationic property can hydrolyze the cellular wall of some bacteria. Peroxidase can protect cells from the attack of hydrogen peroxide (Tenovuo, 1991). Lactoferrin presents antimicrobial activity by fighting for free iron, which is required by microorganisms for their survival, such as the *Streptococcus mutans* group. Lactoferrin also provides anti-inflammatory, antiparasitic activity, host defense and tumor inhibition (Adlerova et al., 2008).

2.4.5.3 Factors Affecting Saliva Composition

Aging Although numerous studies have investigated the relationship between age and saliva, the effect of aging on salivary gland is still controversial. Yeh et al. (1998) indicated that salivary secretion declines with increasing age in both healthy individuals and type 2 diabetes patients. Specifically, flow rates of unstimulated whole saliva, stimulated parotid saliva, as well as unstimulated and stimulated submandibular/ sublingual saliva all decreased with increasing age (Yeh et al., 1998; Dodds et al., 2000; Johnson et al., 2000). However, functional studies among healthy people demonstrated that aging itself does not necessarily cause diminished glandular ability to produce saliva (Nagler et al., 2004).

Disease such as diabetes may be associated with alteration of saliva according to different age categories. When studying the effects of diabetes on human saliva, researchers had found

that flow rates for subjects with diabetes tended to be lower than non-medicated controls of the younger age groups while this effect was not apparent in the oldest age group (Dodds et al., 2000). This study implies that diseases and their treatments may cause a greater harmful effect on saliva function in younger than in older individuals.

Systemic Diseases and Nutrition Chronic diseases, psycho-emotional state and nutritional deficiencies may impact the composition and function of human saliva (Dodds et al., 2000).

Diabetes has been reported to significantly decrease the output of both stimulated and unstimulated submandibular/sublingual saliva. A number of proteins were observed to have an obviously increase in diabetic subjects, including “stimulated parotid salivary lactoferrin, myeloperoxidase and peroxidase, as well as stimulated submandibular/sublingual salivary total protein, albumin, lactoferrin and secretory IgA” (Dodds et al., 2000). The increase of lactoferrin level in diabetes may be due to “specific induction of lactoferrin secretion by acinar and/or ductal cells, or there is a glandular lymphocytic infiltrate associated with chronic sialadenitis” (Tabak et al., 1978).

Drugs Dry mouth is considered as one of the most common side effects of many drugs, although it is still debatable (Sreebny et al., 1997; Wynn et al., 2001). However, it has been proven that increasing intake of medication is associated with the reduction of saliva output. These side effects will notably impact the daily life of patients by decreasing appetite for food; especially those who are suffer chronic diseases and need a long period of medication. However, few research studies focus on the measurement of salivary flow and look for the treatment of this problem.

2. 4.5.4 Taste Alteration of Cancer Patients

Taste alteration is widely reported by cancer patients undergoing chemotherapy and radiation treatments (Hong et al., 2009b; Bernhardson et al. 2008) and is among the most common side effects such as hair loss, nausea, vomiting and constantly being tired (Lindley et al., 1999; Bernhardson et al., 2008). It was found that the prevalence of taste alteration in chemotherapy patients can be as high as 69.9 % (Wickham et al., 1999; Ravasco et al., 2005). Also, the changes in taste can happen in various cancers. In breast cancer, 21 % of the patients reported the abnormal taste acuity in breast cancer (McDaniel and Rhodes, 1998) while 23 %-25 % in lung cancer (Johnson, 2000). Significant proportion of taste changes was found in head and neck cancer patients, where 88.8 % patients reported changes in at least one taste and 66.7 % for more than one taste (Redda and Allis, 2006).

Significantly decreased olfactory and gustatory functions were observed during chemotherapy and older patients suffered more than that of younger patients. However, the taste alteration did not show obvious differences to age or diagnosis during chemotherapy. Instead, it differed with the chemotherapeutic agent and taxane-based chemotherapy caused the most severe disorders (Steinbach et al., 2009).

As a frequent side effect of chemotherapy, taste alteration largely decreases the food intake of cancer patients and subsequently impaired their quality of life. The loss of appetite caused by taste alteration induces malnutrition and weight loss (Sarhill et al., 2003; Hong et al., 2009b). A significant correlation was found between the time from diagnosis to death and the severity of weight loss of cancer patients (Sarhill et al., 2003). Similarly, treatment-related malnutrition is the primary cause of death for as many as 20% of cancer patients (Otty, 1994). In addition, the changes in taste also lead to fatigue and psychological anxiety, which may further associate with

the decrease in social life (Zabernigg et al., 2010). The symptoms start very early under the processing of chemotherapy while the recovery of the taste function took completely three months after chemotherapy (Steinbach et al., 2009).

The prevailing taste alteration is the perception of metallic flavor and bitter taste. Metallic taste was reported in 16 % of lung cancer patients and 32.2 % of patients with colorectal, breast, head and neck, stomach, lung and other cancers (Newell et al., 1998). Possible sources of metallic and/or bitter taste have been associated with low levels of irradiation and chemotherapy with cyclophosphamide, doxorubicin, 5-fluorouracil, methotrexate and cisplatin (Comeau et al., 2001; Capra et al., 2001; McDaniel and Rhodes, 1998). Although it is widely known that cancer status, type and duration of antineoplastic therapy and the use of antioxidants is directly related with taste alteration, the mechanism of alteration that is influenced by numerous exogenous and endogenous factors is still unknown. However, researchers clearly agree that there are two independent causes out of this multifactor system: damage to sensory receptor cells and abnormal neuronal activities (Hong et al., 2009b). Damages in sensory receptor cells by chemotherapy and radiotherapy include the decrease in the number of normal receptor cells, the changes of cell structure or receptor surface like taste-bud structure and interruption of neural coding. Abnormal neuronal activities may lead to the failure of stimulating taste receptors or the presence of corresponding flavor molecules, which resulted in taste alteration (Davidson et al., 1998). Metallic flavor may lead to decrease in appetite, weight loss and deterioration of cancer due to the lost interest in food.

Several other causes of taste alteration are discussed in different studies. In a study of the effect of high-dose chemotherapy on oral condition and quality of life, researchers found that many bitter-tasting compounds from chemotherapeutic drugs can enter the mouth and lead to

bitter taste (Epstein et al., 2002). Drugs working by a free radical-mediated mechanism may also impair the taste perception by increasing oxidative stress in patients (Look and Musch, 1994; Faber et al., 1995). Other influencing factors for taste alteration are poor oral hygiene and mouth care, and oral infections, use of alcohol and tobacco products (Peregrin, 2006). Metallic or medicinal tastes may be associated to zinc deficiency (Ripamonti et al., 1998). Oral mucositis, which is produced from head and neck radiotherapy or certain chemotherapeutic agents, is another cause for the taste and smell abnormalities (Nowak and Janczak, 2006). Oral mucositis can affect up to 100% of patients undergoing high-dose chemotherapy and hematopoietic stem cell transplantation (Trotti et al., 2003). Mucositis damages the oral epithelial cell membranes and may increase oxidative stress at the site of the mucositis, which further produce off-flavor in the mouth (Rubenstein et al., 2004; Rosen et al., 2006).

Lipid oxidation has been suggested to be a responsible reason for the metallic flavor on food materials such as beef (Campo et al., 2006), raw milk (Barrefors et al., 1995) or oil (Kochhar, 1996) and on the mouth of human (Lawless et al., 2004). This is similar to the metallic odor left on the hand after holding keys or doorknob for a while, which produce a series of aldehydes and ketones that are caused by lipid oxidation (Glindemann et al., 2006). In the mouth, lipid peroxidation of oral epithelial cells results in the formation of carbonyl compounds that may cause metallic flavor perceptions (National Cancer Institute, 2013). Antioxidants are assumed to be effective in decreasing the metallic perception since they can prevent or reduce lipid peroxidation in food and body systems. However, an imbalance between oxidants and antioxidants may enhance lipid peroxidation and strengthen the metallic taste perception, especially in the presence of iron and copper (Hong et al., 2009a; 2009b).

Since taste alteration has been neglected for a long time as a common side effect and the mechanism of generating metallic flavor by chemotherapy is still under study, the prevention or reduction of such alteration is also poorly understood.

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Chapter 3

Effect of Iron on Taste Perception and Emotional Response of Sweetened Beverage under Different Water Conditions

Abstract: Sweeteners are widely used additives in beverages, however, taste interaction between sweeteners and minerals in water is rarely reported. The study objective was to investigate the influence of different concentrations of iron and water hardness on taste perception of sweetened beverages and characterize the corresponding emotional profiles. Sweet-metallic interaction was developed by dissolving five natural and artificial sweeteners [sucrose, honey, sucralose, saccharin, and acesulfame potassium (ace-K)] into four synthetic waters [soft water (0mg Fe/L), moderate hard water (0.3mg Fe/L), hard water (1mg Fe/L) and very hard water (3mg Fe/L)], respectively. Sweet and metallic intensity of different combinations were compared by pairwise ranking tests. Acceptability and emotional response on sucrose sweetened beverage with and without the addition of iron was evaluated by 9-point hedonic score test and check-all-that-apply emotional term ballot. Iron (Fe^{2+}) created metallic flavor in drinking water and *bored* and *disgusted* feelings for consumers. Other minerals such as Ca^{2+} , Mg^{2+} and Na^+ at subthresholds impacted taste perception of water. Sweet-metallic taste interactions were variable: sweetness of sucrose-sweetened very hard water was significantly higher ($p < 0.05$) than in hard water; sweetness of ace-K- and honey-sweetened hard water was significantly higher ($p < 0.05$) than in other types of water; metallic flavor of honey-sweetened very hard water was significantly higher ($p < 0.05$) than other water conditions. Sweet-metallic interaction significantly ($p < 0.05$) increased the acceptance score of sucrose-sweetened very hard water (3 mg Fe/L), and *mild* was determined as the unique term for the water. Distribution of emotional profiles could be different between samples with the same hedonic scores.

3.1 Introduction

Water is critical to beverage production and its quality directly influences the taste and acceptability of the corresponding beverage products. Water quality is classified by the composition/concentration of the containing minerals, which can add salty, bitter, sweet or metallic flavor to water (Burlingame et al., 2007; Dietrich and Burlingame, 2015). When minerals reach certain concentrations, they may deeply impact taste perception of final products by interacting with each other or other food constituents. In the food industry, water used for beverage production has to meet drinking water standards established by US Environmental Protection Agency. However, the National Primary Drinking Water Regulations (USEPA, 2016a) are mandatory health-based standards for about 100 organic, inorganic, and microbial contaminants in drinking water. USEPA also provides Secondary Drinking Water Regulations (USEPA, 2016b) as guidance for public water systems. These suggested guidelines are related to aesthetic quality targeted toward providing desirable drinking water with acceptable taste and appearance. These aesthetic guidelines are usually followed on a voluntary basis, although some states enforce some or all of them on a mandatory basis.

Many companies provide additional water filtration and treatment steps to standardize their water supply. However, such processes are expensive and may not be feasible for small companies. Consumers may also have filters at the tap to reduce minerals in their drinking water. However, their home made beverages (such as coffee, tea, beer, wine) may be affected by residual minerals. Therefore, high concentration of mineral ions found in the supplied water may greatly impact the taste perception if used in beverage production.

Humans are sensitive to many minerals; some thresholds are below the maximum amount set by Secondary Drinking Water Regulations. Copper can be detected by humans at very low

concentration ranging from 0.5-13 mg/L (Jesse et al. 1960; Cuppett et al. 2006) and the Secondary Drinking Water Standards for copper is 1.0 mg/L (USEPA, 2012). Iron is one of the most noticeable minerals since it can impart metallic flavor in drinking water as low as 0.17 mg/L (Mirlohi et al., 2011) which is below the secondary drinking water standard for iron set at 0.3 mg/L. Furthermore, iron is found most frequently in water supplies since it is the fourth most common element found in the Earth's crust and widely spread in the natural environment. Effects of iron and other minerals on metallic flavor of drinking water have been reported. In a study about the influence of water quality on recognition thresholds of each basic tastes (sweet, salty, sour, bitter, umami) as well as metallic flavor, iron (II) sulfate was best represent metallic sensation in deionized water. In addition, taste sensitivity of sweet (sucrose) and metallic (ferrous sulfate) could be weakened when exposed to higher concentrations of minerals (Hoehl et al., 2010). However, a commercial food product is a comprehensive flavor system with many interactions; a single taste never exists. Thus, how minerals influence taste perception of beverages through interaction with other taste stimuli still needs further study.

Taste interactions are important to the development and modification of food products and have been widely studied for decades. Taste interaction between same taste stimuli including sweet (Ayya and Lawless, 1992; Schiffman, 1995), umami (Yamaguchi, 1967), salt (Breslin et al., 1995), sour (Bartoschuk and Cleveland, 1977) and bitter (Ke et al., 2002), and between multiple taste stimuli such as umami/salt (Woskow, 1969; Keast and Breslin, 2002b), salt/sour (Breslin, 1996), bitter/salt (Breslin et al., 1995; Breslin and Beauchamp, 1997), bitter/sour (Pangborn, 1960; Keast and Breslin, 2002a), sweet/salt (Beebe-Center et al., 1959; Breslin, 1996), sweet/sour (Curtis et al., 1984), and bitter/sweet (Schiffman et al., 1994; Calvino et al., 1990) were reported in previous studies. However, binary taste interaction between sweet and

metallic have not been previously reported; metallic flavor is most often associated with bitter perception. Different from bitterness, metallic sensation is a combination of taste and odor. Metallic taste is characterized as bitter and salty taste as well as astringent mouthfeel (Ömur-Özbek & Dietrich, 2011) and often carries a lingering aftertaste (Hong, Duncan, & Dietrich, 2009). However, metallic sensation may be 2 to 30 times lower if olfaction of panelists was occluded by nose-clips (Epke and Lawless, 2007). Therefore, metallic flavor is comprised of metallic taste and retronasally perceived odor (Mirlohi et al. 2011). Metallic flavor is widely observed as an undesirable sensory side effect. It has been associated with polyphenols in cloudy raw fruit juices, some meat products, and metal ions found in water or metal pipes (Sarin *et al.*, 2004), and attributed to metal from equipment or containers during production, storage and transportation. Some alternative sweeteners such as acesulfame potassium and saccharin may also contribute low level bitter, astringent or metallic characteristics at certain levels (Horne et al., 2002). It is recognized that metallic flavor is not a positive attribute and can reduce product acceptability.

Consumer acceptability of a food product is the traditional approach for estimating potential purchase/consumption behavior; however, product success does not always follow this measure. Correlation between consumer emotional response and sensory experience during food consumption has been explored in recent years to provide additional information on which to predict product success. Metallic flavor, however, may influence both acceptability and emotional response to beverage. Emotional response provides an additional layer of information that can help describe the impact of sensory quality on consumer hedonic response. Few studies have related emotional response to taste perception of binary taste interactions.

In this study, influence of different concentrations of iron and water hardness on taste perception and emotional response of sweetened water beverages was studied. Sweet-metallic taste interaction was investigated by detecting sweet and metallic intensity generated from combinations of ferrous ions (0, 0.3, 1 and 3 mg Fe/L) and five sweeteners including sucrose, honey, sucralose, saccharin, and acesulfame potassium (ace-K). In addition, emotional response using check-all-that-apply (CATA) term ballot in combination with hedonic response was applied to study binary taste interactions. Results of this study will be useful in understanding how iron and natural and alternative sweeteners interact to influence beverage sensory quality and influence acceptability. This will provide guidance for water standards in beverage production, and provide information for usage of sweeteners to generate the optimum taste in food/beverage products, functional foods, and medicines.

3.2 Materials and Methods

3.2.1 Materials

Food-grade chemicals including $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, NaHCO_3 , $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and KCl were purchased from Spectrum Chemical (New Brunswick, NJ). Acesulfame potassium (ace-K; Wego Chemical & Mineral Co.; Great Neck, NY) and sucralose (Sucral; Tate & Lyle; London) were received by donation. Saccharin, honey (clover) and sucrose (Kroger; Cincinnati, OH) were purchased at the local supermarket. Distilled water, drinking water and unsalted soda crackers were purchased from Kroger (Kroger brand, Cincinnati, OH).

3.2.2 Preparation of Iron-Containing Sweetened Beverage

Preparation of Synthetic Water

The preparation of synthetic soft water followed the formulation of minerals for hard and soft natural waters in 100 large cities in the United States as described by Burlingame et al. (2007)

and Smith et al. (2002) with slight modification. To prepare synthetic water with appropriate hardness (DES, 2008), mineral concentration in moderate hard, hard and very hard water was determined through multiplying mineral ions concentration in soft water by 2.5, 5, and 7.5, respectively (Table 5). Synthetic very hard water (4L) was prepared daily with food grade chemicals following the compositions in Table 5. Distilled water (3.8 L) (The Kroger Co., Cincinnati, OH, 45202) was purchased in a clean glass bottle, to which was added 461.9 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 635.5 mg NaHCO_3 and 114.7 mg KCl . The solution was aerated with magnetic stirrer overnight. The next day, 1367.4 mg of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ was added to 200 mL distilled water in a separate glass beaker with stirring. After the calcium sulfate was totally dissolved, the solution was added to the 3.8 L solution and mixed well. The combined solution was aerated vigorously with magnetic stirrer for an additional 24 h to dissolve the added chemicals and stabilize the medium. Then the prepared very hard water was diluted by distilled water at the ratio 1:6.5 for soft water, 1:4 for moderate hard water and 1:1.5 for hard water (Table 3). Right before sensory testing, 1.5 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 1L synthetic moderately hard water, 5.0 mg was dissolved in 1L synthetic hard water, and 14.9 mg was dissolved in 1L synthetic very hard water. The pH and hardness of each type of synthetic water were listed in Table 3.

Preparation of Sweetened Beverage

All samples were prepared daily to prevent degradation or precipitation of compounds. Clean glassware was rinsed thoroughly beforehand to remove any residual minerals. Iron-containing sweetened beverages were obtained by adding five sweeteners respectively into each of iron-containing solutions in Table 3 to form ace-K beverage (2.64×10^{-4} g/mL), honey beverage (0.0624 g/mL), saccharin beverage (0.0037 g/mL), sucralose beverage (9.5×10^{-5} g/mL)

and sucrose beverage (0.05 g/mL). The concentrations of above five sweeteners were established based on our previous study (Leitch et al., 2015), which identified sweet equivalence of different sweeteners to a 5% sucrose in water solution (w/w).

Table 3. Preparation of iron-containing synthetic water using reagent grade chemicals¹

Water Type	Reagent Added (mg/L) ²					Final Water Quality		
	FeSO ₄ ·7H ₂ O	NaHCO ₃	CaSO ₄ ·2H ₂ O	MgSO ₄ ·7H ₂ O	KCl	pH ³	Hardness ⁴	TDS (mg/L)
Soft (Control)	0.0	21.2	45.6	15.4	3.8	7.2-7.4	33	68.6
Moderately Hard	1.5	53.0	114.0	38.5	9.6	7.4-7.6	82	172.2
Hard	5.0	105.9	227.9	77.0	19.1	7.6-7.8	163	345.6
Very Hard	14.9	158.9	341.9	115.5	28.7	8.0-8.2	245	522.4
	Fe ²⁺	Na ⁺	Ca ²⁺	Mg ²⁺	K ⁺	pH ³	Hardness ⁴	
Soft (Control)	0.0	5.8	10.6	1.5	2.0	7.2-7.4	33	
Moderately Hard	0.3	14.5	26.5	3.8	5.0	7.4-7.6	82	
Hard	1.0	29.0	53.0	7.5	10.0	7.6-7.8	163	
Very Hard	3.0	43.5	79.5	11.3	15.0	8.0-8.2	245	

¹Source: Burlingame et al., 2007 and Smith et al., 2002.

²Add food-grade reagent grade chemicals to distilled water.

³Equilibrium pH after 24 h of aeration.

⁴Expressed as mg/L CaCO₃ (DES, 2008).

3.2.3 Participant Recruitment & Pre-screening Study

This study was approved by the Virginia Tech Institutional Review Board (IRB #15-399, Appendix B) prior to beginning the sensory test. Participants were faculty, staff and students from Virginia Tech, who were 18 years or older and did not have a taste disorder that would contribute additional variables to the experimental design. Initially, a pre-test was performed on all participants by simultaneously serving three samples, in a triangle test format, including one ferrous sulfate solution (0.3 mg Fe/L) and two distilled

water (in random order). Participants were asked to taste the sample from left to right and select the one considered as metallic flavor. Twenty-five individuals (20 females, age range 20-59) correctly identified ferrous sulfate solution and were recruited as panelists for the following sensory tests.

3.2.4 Testing Conditions

Testing was completed in individual booths in the sensory evaluation laboratory. All experimental testing was completed under red lighting to mask any yellow color variation induced by increasing concentrations of ferrous ions in the solutions. Samples were served at room temperature. Participants consumed regular drinking water and unsalted soda crackers to cleanse palate between samples.

3.2.5 Acceptance & Emotional Response on Iron-containing Sweetened Beverage

Once passed the pre-test, panelists were presented with one water sample at a time (six water samples in total). The six water samples with random three-digit number were: soft water, 5% sucrose in soft water (w/w), hard water with iron (3mg Fe/L), 5% sucrose in hard water with iron (w/w), hard water without iron, 5% sucrose in hard water without iron (w/w). Immediately following consumption, panelists rated their acceptability score at the top of the modified EsSense™ paper ballot (King et al., 2010) and selected all applicable emotional terms from the list of 43 terms, as described in our previous work (Leitch et al., 2015).

3.2.6 Pairwise Ranking Test on Synthetic Water

To detect the effect of mineral concentrations on metallic flavor in drinking water, a pairwise ranking test was used to evaluate the metallic taste of each synthetic water (Meilgaard *et al.*, 2007). A complete block design was employed on the four types of synthetic water including soft water (S), moderate hard water (HM), hard water (HH) and very hard water (VH). The 6

comparisons were: S x HM; S x HH; S x VH; HM x HH; HM x VH; HH x VH. Panelists were given one comparison at a time and asked to select the sample that tasted more metallic within the pair. If there was no difference between two samples, they had to answer their best guess. Panelists took a large portion of the sample into mouth, swirled it around and expectorated into a cup. To minimize fatigue, participants could eat crackers to clear their palate at any time. The six pairs of samples were presented randomly to 25 panelists and a different random three-digit number was assigned to each sample. The 25 panelists also completed pairwise ranking test on iron-containing sweetened beverages in the following five testing days.

3.2.7 Pairwise Ranking Test on Iron-containing Sweetened Beverage

Experiment 1. Determining the influence of water chemistry on perception of sweetness of different sweeteners

A pairwise ranking test was used to evaluate the effect of minerals in water on sweetness of different sweeteners (Meilgaard *et al.*, 2007). Each of the five sweeteners was chosen to combine with all 4 synthetic waters [soft water (S), moderate hard water (HM), hard water (HH) and very hard water (VH)] on each testing day. A complete block design was employed for each tested sweetener: S x HM; S x HH; S x VH; HM x HH; HM x VH; HH x VH. The 6 pairs were presented randomly to 25 panelists and each sample was assigned with a different random three-digit number. Panelists were given one comparison at a time and asked to select the sample that was sweeter within the pair. If there was no difference between two samples, they had to answer their best guess. Panelists took a large portion of the sample into mouth, swirled it around and expectorated into the appropriate cup. To minimize fatigue, participants could eat crackers to clear their palate at any time.

Experiment 2 Determining if sweeteners reduce the perception of metallic flavor of iron-containing sweetened beverage

A pairwise ranking test was used to assess the metallic flavor of synthetic waters sweetened by different sweeteners (Meilgaard *et al.*, 2007). Each of the four synthetic waters (soft water, moderate hard water, hard water and very hard water) was chosen to combine with all 5 sweeteners on each testing day. Sucrose was used as control to compare with each of the other 4 sweeteners in each type of water: sucrose × sucralose, sucrose × saccharin, sucrose × ace-K, sucrose × honey. The 4 pairs of sample were presented randomly to 25 panelists and each sample was assigned with a different random three-digit number. Panelists were given one comparison at a time and asked to select the sample that had higher metallic taste. If there was no difference between two samples, they had to answer their best guess. The panelists took a large portion of the sample into mouth, swirled it around and expectorated into the appropriate cup. To minimize fatigue, participants could eat crackers to clear their palate at any time. The sensory evaluation started with soft water combinations in order to determine whether sweeteners themselves produced metallic flavor. In each testing day, two pairwise ranking tests were tested by each participant: one test from Experiment 1 (6 pairs of samples) and another test from Experiment 2 (4 pairs of samples). At least half an hour was scheduled between the two tests for all participants each day.

3.2.8 Data Analysis

Data from pairwise ranking test on synthetic waters and iron-containing sweetened beverage-experiment 1 was analyzed by Friedman's T statistic according to Meilgaard *et al.* (2007). Tukey's test ($\alpha=0.05$) was used to calculate the minimum significant difference. Experiment 2 was assessed by counting the number of agreeing responses citing one sample

more frequently within a pair, and then compared with the critical number of correct response in a two-sided directional difference test ($n=25$, $\alpha=0.05$) (Meilgaard et al., 2007).

Hedonic scores of water samples were assessed using one-way ANOVA followed by Tukey's test ($\alpha=0.05$) for mean separation. Emotional terms were analyzed by counting their selection frequency for each water sample. Terms selected for more than 20% frequency for at least one water sample within group were classified as "frequently selected" terms (Leitch et al., 2015). Among frequently selected terms, terms with less than 8% difference between tested water samples within group were considered as "shared" terms; terms exhibiting significant difference ($\alpha=0.05$) between tested water samples and with greater than 12% selection frequency than other samples within group were classified as "unique" terms. Cochran's Q test was used to assess the minimum significant difference ($\alpha=0.05$) for each emotional term between tested water samples. Statistical analysis was performed using JMP (JMP vs 10, Cary, NC) and SPSS (Windows, vs 20.0, SPSS inc., Chicago, IL).

3.3 Results and Discussion

3.3.1 Sweet-Metallic Interaction (Pairwise Ranking Analysis)

3.3.1.1 Metallic flavor of synthetic waters

Metallic flavor of the four synthetic waters was detected to establish if the hardness influenced perception. Metallic perception of four types of synthetic waters did not show any significant differences ($p>0.05$) from each other. However, HSD value of very hard water (3 mg Fe/L) was 17.0 when comparing with soft water, which was between alpha of 0.05 (HSD=18.15) and 0.1 (HSD=16.2). This suggested that metallic taste of very hard water was detectable by certain number of panelists. Although all the selected panelists had successfully identified metallic taste caused by iron (0.03 mg Fe/L) in distilled water in pre-test, their sensitivity of

metallic taste could be changed due to the appearance of other minerals in water with hardness. Hoehl et al. (2010) reported that metallic sensation was affected most by mineral content in water. Recognition thresholds of iron (II) sulfate in tap water (11.22 $\mu\text{mol/L}$) were significantly higher than in deionized water (5.37 $\mu\text{mol/L}$) (Hoehl et al., 2010).

Taste threshold concentration (TTC) of minerals in water is 100-300 mg/L for calcium (WHO, 2011), 30-60 mg/L for sodium (USEPA, 2003), and 200-300 mg/L for chloride (Ricter and MacLean, 1939; Lockhart et al., 1995). Magnesium concentrations above 10 mg/L in water caused an offensive and bitter taste (Zoeteman et al., 1978). In this work, minerals added in distilled water, including calcium (10.6-79.5 mg/L), sodium (5.8-43.5 mg/L), magnesium (1.5-11.25 mg/L) and chloride (1.8-13.7 mg/L), were all below the reported taste threshold. The low concentration of potassium (2.0-15.0 mg/L in this work) was unlikely to influence the water taste (Platikanov et al., 2013). On the other hand, Platikanov et al. (2013) found that the most important factor that influenced water taste and preference was the overall level of mineralization (TDS). Panelists disliked very high mineralized water (TDS > 800 mg/L) and very low mineralized water (TDS level was 30-40 mg/L or lower). In the current study, TDS levels of all synthetic waters were between 68.6-522.4 mg/L, which explained the lack of significant difference between four tested waters.

Individual mineral content appeared to be an influential factor on taste of water mainly in moderate level of TDS (200-400 mg/L) (Platikanov et al., 2013). In moderate TDS level, water was preferred if it contained relatively high pH (7.5-8.1) and relatively high concentrations of Ca^{2+} (84.7-290.1 mg/L), Mg^{2+} (20.2-50.0 mg/L), SO_4^{2-} (around 130.4 mg/L) and HCO_3^- (around 178.7 mg/L) ions. In contrast, water with high concentrations of Na^+ (> 53.9 mg/L) and Cl^- (> 79.3 mg/L) was disliked by panelists (Platikanov et al., 2013). In our study, concentrations of

Ca^{2+} , Mg^{2+} , SO_4^{2-} and HCO_3^- in all four synthetic waters were within the acceptable range based on above data. In addition, water hardness (total concentration of Ca^{2+} and Mg^{2+} expressed as equivalent CaCO_3) between 25 and 100 mg/L were difficult to discriminate for panelists (Byrne et al., 2009). Water hardness as high as 200 mg/L was not significantly ($p>0.05$) different from moderate hard water (50-100 mg/L) in taste (Byrne et al., 2009). Therefore, water hardness in this study did not obviously influence the taste of synthetic waters.

In the current work, iron (II) sulfate was used to generate metallic sensation in synthetic water and we initially evaluated metallic flavor perception in the synthetic waters. There was no significant differences in metallic perception of synthetic waters with different levels of hardness ($p>0.05$) among all pairs, thus we were able to use the four synthetic waters for our study on the taste interactions with sweeteners.

3. 3.1.2 Effect of mineral ions on sweetness of sweetened synthetic water

Our hypothesis was that if no interaction occurred between sweeteners and minerals, the perception of sweetness would not be different among the four water conditions. However, sweetness perception was affected by mineral composition/water hardness and varied for the different sweeteners. As shown in Table 4, sweetness of sucralose in hard and very hard water was significantly lower ($p<0.05$) than in soft water. Similarly, hard water also reduced ($p<0.05$) sweetness perception of sucrose in soft and moderate hard water. Surprisingly, sucrose in very hard water tasted as sweet as in soft and moderate hard water, and was significantly sweeter ($p<0.05$) than in hard water. For both ace-K and honey, hard water generated significantly sweeter taste ($p<0.05$) than soft water in combination with the sweeteners. Honey in moderately hard water was less sweet than in soft or hard water. Mineral content did not affect the sweetness of saccharin in all four synthetic waters.

The decrease of sweetness of some sweeteners in mineral water might be explained by physio-chemical interactions, which affect taste intensity by changing composition or chemical structure of food compounds. Weak attractive forces (e.g. hydrogen, hydrophobic bonding) between different taste compounds will result in altered structures and/or form precipitation, which lead them to taste weaker or even tasteless (Keast and Breslin, 2002a). Although no precipitation was observed in this study, minerals might still interact with sweeteners through intermolecular forces and decrease their sweetness. Another possibility is minerals reacting with saliva proteins to form precipitation in the mouth, which served as a physical barrier between sweetener and taste buds/receptor; this could reduce the sweetness perception of sweeteners. In the current study, both sucrose and sucralose were found to have significantly less sweetness ($p < 0.05$) in hard water (Table 4). Our results are consistent with the data described by Hoehl et al. (2010), in which higher mineral content in water increased the recognition thresholds of sucrose; sucrose threshold was detected as 3.55 mmol/L in spring water (lower mineral content) but 5.13 mmol/L in tap water (higher mineral content). Although sucrose was reported to be able to significantly decrease maximum intensity and total duration for astringency, astringency was not found to affect sweetness of sucrose (Ishikawa and Noble, 1995). Therefore, sweet-astringency interaction did not influence sweetness in this study.

Table 4. Rank sum values of synthetic waters and sweetened water (experiment 1) according to pairwise ranking test (n=25) followed with Turkey's test ($\alpha=0.05$)

	Soft water ¹	Moderate hard water	Hard water	Very hard water	HSD value ²
No sweetener (Control)	119 ^a	116 ^a	113 ^a	102 ^a	
Sucrose (0.05 g/mL)	103 ^b	98 ^b	132 ^a	99 ^b	
Ace-K (2.64×10 ⁻⁴ g/mL)	113 ^a	106 ^{ab}	89 ^b	106 ^{ab}	
Honey (0.0624 g/mL)	123 ^b	145 ^a	90 ^c	128 ^{ab}	18.15 ($\alpha=0.05$)
Saccharin (0.0037 g/mL)	104 ^a	117 ^a	112 ^a	114 ^a	
Sucralose (9.5×10 ⁻⁵ g/mL)	103 ^b	109 ^{ab}	127 ^a	126 ^a	

^{a,b,c} Means within each row with different superscripts are significant different ($p<0.05$). Lower rank sum value means the sample is sweeter.

¹Water was synthesized by adding food grade chemicals into commercial distilled water, as described in Table 3.

²HSD value was calculated according to Meilgard et al. (2007). If the difference in rank sum of any two synthetic waters were bigger than the HSD value in the same row, then these two water samples showed significant differences ($p<0.05$) in taste perception.

Mechanisms for sweetness enhancement in mineral water were not consistent. One explanation for this phenomenon was the existence of subthreshold or synergistic side tastes that many compounds elicit. Food compounds that potentially contribute tastes to water can have an effect as a complex mixture in a perceptible way. For example, different brands of mineral waters were distinguishable in taste, even though their mineral contents were only different in the parts per billion ranges. This was due to the subthreshold minerals and salts combining additively to generate distinguishable tastes (Stevens, 1995). However, whether minerals increased or decreased the sweetness perception depends on their composition and concentration in water. The existence of side tastes was always spurious in enhancement of taste quality at moderate or high concentration mixtures (Kroeze, 1982). For example, the sweet taste of dilute NaCl could significantly increase the sweetness in NaCl-sucrose mixture (Breslin, 1996). The

existence of Na^+ (43.5 mg/L) in very hard water might contribute to the enhancement of sweetness when combined with sweeteners. The concentration exceeded its taste threshold for younger people as reported by Stevens (1996); the threshold of sodium in water was 30 mg Na/L for 18-29 year age group and 131 mg Na/L for 66-90 years old. Since 18 out of 25 panelists were between 18-29 years old in this study, Na^+ might lead to the salty flavor in very hard water and increase sweet perception by synergistic side effects.

Another explanation for the enhancement of sweetness in mineral water was sweet-metallic taste interaction. Metallic taste is a combination of multiple sensations, thus sweet-metallic taste interaction was a comprehensive result of sweet-bitter, sweet-astringent, and sweet-salt interactions. Sweet-bitter interaction was originated from the closely related transduction mechanisms and chemical structure between sweet and bitter compounds. Although sugar was always considered as “sweet”, sugar esters and halogenated sugars can be intensively bitter. In addition, numerous sweeteners and bitter compounds share the same structures including peptides, oximes, urea, terpenes, guanidines and saccharin derivatives (Walters, 1996). On the other hand, bitter and sweetness share the common transduction mechanism. A variety of sweet and bitter taste receptors might originate from a common ancestral chemoreceptor (Walters and Roy, 1996b). It was proven that both bitter and sweet tastes were mediated by G-protein-coupled receptors (GPCRs). GPCRs is a large family of receptors that detected chemical signals outside the cell and then transformed them to inside signals to trigger the action of GTP-binding protein (G-protein) (Walters and Roy, 1996). Activated G-protein stimulated one or more second messengers which controlled the opening or closing of specific ion channels. The increase in electrical potential in cytoplasm of the cell corresponding to the availability of certain ion channels ultimately leads to the enhancement of taste (sweet or bitter) intensity through the nerve

system (Walters, 1996). Thus, although sweeteners and bitter compounds have their own receptors, they all activate G-protein and follow the same transduction system eventually. This mechanism explained compounds that tasted both sweet and bitter such as saccharin and ace-K. In this study, sweetener-iron mixture produced similar results as a synergistic effect. The mixture of sweeteners and ferrous sulfate could activate, in total, more receptors and G-proteins. Then more second messengers are triggered to activate more ion channels, resulting in an increasing number of nerve signals which lead to the enhancement of taste intensity (Schiffman, 1995; Walters, 1996). However, it is unclear if this effect boosts both bitter and sweetness or only one of them. More study is needed to clarify whether this synergy effect depends on the ratio and types of sweeteners/bitter compounds.

In addition, the very hard water also had more bicarbonate, which was described as “sweet” (Matia, 1995) when in high concentration in water. The bicarbonate might contribute to sweetness independent of sweeteners (Matia, 1995).

Whether one taste could be increased/decreased in a food matrix was variable upon the concentration of taste compounds within the mixture. It was reported that binary taste of bitter-sweet mixture was variably affected at the low concentration/intensity. At medium and high concentration/intensity, sweetness was generally suppressive of bitter (Breslin, 1996). In the current study, sweetness of sucrose was observed to decrease in hard water (1mg Fe/L) but was significantly enhanced in very hard water (3mg Fe/L). Binary taste interaction was affected at different concentrations for different taste compounds. According to Table 4, the sweetness of sucralose (9.5×10^{-5} g/mL) was observed to be suppressed when iron concentration was more than 1 mg Fe/L in synthetic water. However, sweetness of both honey (0.0624 g/mL) and ace-K (2.64×10^{-4} g/mL) were significantly enhanced ($p < 0.05$) for the same water (1 mg Fe/L).

Since metallic sensation has a significant odor component and should be treated as flavor rather than taste (Ömür-Özbek and Dietrich, 2011), taste-odor interaction should also be considered when overall evaluating the sweet-metallic interaction. Many studies have shown that odors can enhance, suppress or have no effect on tastes in food products (Caporale et al., 2004; Labbe et al., 2006). For example, fruit aroma resulted in a decrease in astringency and bitterness, as well as an increase in sweetness of red wines (Sánchez-Navajas et al., 2010). Ethyl butyrate (fruit aroma) can increase the sweetness in sucrose-sweetened solution (Hornung and Enns, 1994). Furthermore, taste-aroma interactions are not necessarily associated with the concentration of odors or taste compounds. Labbe et al. (2007) found sweetness of sucrose-sweetened solution can be enhanced by subthreshold levels of an odorant. This effect can be explained by an “on-off” taste modulation that occurs in odorant stimulation at subthreshold level. Although effects may be inconsistent in some cases (Sánchez-Navajas et al., 2010), it still presented the ability in inhibiting off-flavor and potentially used to suppress metallic smell in mineral water. Future studies may test the effect of commercial odorants, such as benzaldehyde, ethyl butyrate and vanillin, in reducing metallic flavor caused by ferrous sulfate in water.

3.3.1.3 Effect of mineral ions on metallic flavor of sweetened synthetic water

To compare the effect of commercial sweeteners on the metallic flavor of mineral water, five sweeteners (sucrose, honey, ace-K, saccharin, and sucralose) were dissolved in one of the synthetic waters on each testing day. The intensity of metallic flavor of water sweetened by each sweetener was compared with sucrose as a pair. In soft synthetic water (control group), ace-K sweetened water had significantly higher ($\alpha=0.05$) metallic flavor than sucrose (Table 5). Moderate hard and hard water further strengthened this metallic taste perception, in which ace-K presented even more obvious ($p<0.01$) metallic flavor than sucrose did. However, very hard

water reduced the perceptible difference of metallic taste between sucrose and ace-K sweetened water. Similarly, saccharin-sweetened water also showed significantly higher metallic flavor ($p < 0.01$) than sucrose in soft and moderate hard water, while hard and very hard water decreased this taste difference. There was no significant difference in metallic taste between honey and sucrose sweetened water in soft, moderate hard and hard water. Nevertheless, high concentration of minerals in very hard water reacted with honey to generate significantly higher ($p < 0.05$) metallic flavor. Comparing with sucrose, sucralose-sweetened water did not show any significant difference of metallic flavor in all types of synthetic waters.

Table 5. Number of responses on sweetened synthetic water with higher metallic flavor (experiment 2) according to pairwise ranking test ($n=25$) and the corresponding critical value ($\alpha=0.05$)

	Pair 1 ¹		Pair 2		Pair 3		Pair 4	
	Sucrose ²	Ace-K	Sucrose	Honey	Sucrose	Saccharin	Sucrose	Sucralose
Soft water ³ (control)	6	19*	8	17	2	22**	9	16
Moderate hard water	4	21**	10	15	5	20**	9	16
Hard water	4	21**	8	17	8	17	8	17
Very hard water	8	17	7	18*	8	17	11	14
Critical number ⁴	18 ($\alpha=0.05$), 20 ($\alpha=0.01$)							

* Means the sample within a pair had significantly higher metallic flavor ($p < 0.05$); ** means the sample within a pair had significantly higher metallic flavor ($p < 0.01$).

¹Four pairs of samples were presented to panelists randomly on each testing day.

²Concentration of sweeteners used in this study: sucrose, 0.05 g/mL; ace-K, 2.64×10^{-4} g/mL; honey, 0.0624 g/mL; saccharin, 0.0037 g/mL; sucralose, 9.5×10^{-5} g/mL. Sweetness was established as equal sweet to sucrose as reported by Leitch et al. (2015).

³Water was synthesized by adding food grade chemicals into commercial distill water, as described in Table 3.

⁴Critical number was found in Table 17.12 (Meilgard et al. 2007). The critical number is the minimum number of responses required for significance at the stated α -level for 25 respondents. If the number of responses was greater than or equal to the selected critical number, there was significant difference between two samples at the stated α -level.

In our previous work, the five sweeteners presented the same sweetness in drinking water (Leitch et al., 2015). However, in this study, their function in inhibiting metallic flavor was varied in the same type of synthetic water (Table 5).

Ace-K and saccharin-sweetened water were chosen as higher metallic taste than sucrose in soft and moderately hard water. Ace-K and saccharin compounds can produce metallic/bitter taste themselves when reaching certain concentration (threshold varied) (Rader et al., 1967; Larson-Powers and Pangborn, 1978; Ott et al., 1991). The generation of metallic aftertaste was through activation of bitterness receptors including TAS2R family (hTAS2R43 and hTAS2R44) and TVR1. TAS2R family was able to stimulate bitter/metallic taste while blocking the expression of sweet taste in cells (Kuhn et al., 2004). TRPV1 was correlated with the metallic aftertaste of saccharin and ace-K (Riera et al. 2007). Higher concentration of Cu^{2+} , Zn^{2+} and Fe^{2+} were also found to be able to activate TRPV1 (Riera et al. 2007), which might further strengthen the metallic flavor of saccharin/ace-K sweetened mineral water.

In very hard water, ace-K and saccharin did not cause any significant difference ($p > 0.05$) in metallic flavor comparing with sucrose (Table 5). This result demonstrated that metallic taste produced from higher contents of mineral ions could cover the aftertaste of ace-K and saccharin. Correspondingly, it indicated that the metallic aftertaste of ace-K might be stronger than saccharin, since under the same condition (hard water), the significant metallic flavor ($p < 0.05$) of ace-K was still present while in saccharin it was not. In addition, ace-K was reported to have a delayed bitter aftertaste (Ott et al., 1991), which might strengthen the intensity of its metallic flavor and extend its duration. According to previous studies (Horne et al., 2002; Kuhn et al., 2004), variety of bitterness sensitivity of panels was also excluded because saccharin and ace-K had a common mechanism for bitter taste reception and transduction.

As shown in Table 5, honey-sweetened water exhibited significantly higher ($p < 0.05$) metallic flavor than sucrose only in very hard water. Generally, main taste compounds in honey were from sugars, such as fructose and glucose. The quantity and types of acids and amino acids present in honey may also influence its aroma and flavor (Bogdanov et al., 2008). Polyphenols, which are traditionally considered as antioxidant components in honey, are reported to cause bitterness and astringency in foods such as tea, red wine and several types of fruits (Lesschaeve and Noble, 2005). Therefore, polyphenols might contribute a side taste to honey and potentially change the sensory characteristics of honey in certain conditions. In addition, honey contains a variety of minerals (Fe, Mn, Pb, Cd, Zn, Cu, Cr, Ni), which are derived from natural resource and air or soil contamination of anthropogenic sources. It has been reported that metal ions constitute a large proportion of minerals found in honey, especially Fe ranging 0.136-9.852 mg/kg (mean 1.390 mg/kg), Cu ranging 0.051-3.317 mg/kg (mean 0.656 mg/kg), Zn ranging 0.016-4.133 mg/kg (mean 1.041 mg/kg), and Mn ranging 0.125-12.354 mg/kg (mean 2.063 mg/kg) (Bogdanov et al., 2007). The abundance of metal ions in honey increased the mineral levels of honey-sweetened water samples in this study, which might explain the significant enhancement of metallic taste of honey in very hard water. Furthermore, subthresholds and side tastes of other minerals in honey or very hard water may be able to substantially increase the taste intensity at high concentration mixtures (Kroeze, 1982).

3.3.2 Acceptability and emotional responses on sweetened synthetic waters

To investigate whether sweet-metallic taste interaction in sweetened beverage was acceptable by consumers and emotional profiles generated from it, acceptance test and emotion responses were detected on synthetic water with and without the addition of sucrose and iron.

Very hard water without iron was used as control to the binary taste interaction between iron and sucrose.

3.3.2.1 Acceptance test

As shown in Table 6, very hard water containing high concentrations of Ca^{2+} , Mg^{2+} , Na^+ , K^+ , and Cl^- did not show any significant differences ($p > 0.05$) in hedonic score compared with soft water. After the addition of Fe^{2+} (3mg Fe/L), however, hedonic score of very hard water was significantly reduced ($p < 0.05$). The decrease of the acceptability was consistent with the result in 3.3.1.1, in which certain number of panelists had detected the metallic flavor in very hard water. This result also demonstrated that metallic flavor in synthetic water was mainly from ferrous ions and reduced the acceptability of drinking water. Our result was also in agreement with Hoehl's study that iron (II) sulfate could best be matched to metallic sensation in deionized water (Hoehl et al., 2010).

Table 6. Mean hedonic scores¹ for synthetic waters with and without addition of iron and sucrose

Water Sample²	Mean	SD	95% CI	Median
Soft water	5.7 ^{ab}	1.4	5.1-6.3	5.5
Soft water + sucrose	6.0 ^a	2.2	5.1-6.9	7
Very hard water (0mg Fe/L)	5.9 ^a	2.0	5.1-6.8	5
Very hard water (0mg Fe/L) + sucrose	4.5 ^b	1.3	4.0-5.1	6.5
Soft water	5.7 ^a	1.4	5.1-6.3	5.5
Soft water + sucrose	6.0 ^a	2.2	5.1-6.9	7
Very hard water (3mg Fe/L)	4.1 ^b	1.6	3.4-4.8	5
Very hard water (3mg Fe/L) + sucrose	5.5 ^a	1.6	4.8-6.3	5.5

^{a-b}Means water samples with different letters in each column were significantly different ($p < 0.05$) from each other in mean hedonic score.

¹n=25; 1= extremely dislike; 9= extremely like.

²Water was synthesized by adding food grade chemicals into commercial distill water, as described in Table 3. Hedonic scores for all samples were normally distributed.

On the other hand, taste perception of sweetened very hard water (3mg Fe/L) showed similar acceptability with soft water and sweetened soft water. Although a metallic aftertaste might be left after consuming the sweetened very hard water (3mg Fe/L), Fe^{2+} still stimulated the enhancement of sweetness by metallic-sweet taste interaction. As shown in Table 6, the sweetness of sweetened very hard water (3mg Fe/L) was the same as sweetened soft water, which might explain why the sweetened very hard water (3mg Fe/L) was acceptable for consumers. In addition, consumer's rating of liking on a taste mainly depends on its bitterness rather than sweetness according to study (Kamerud and Delwiche, 2007). That is, consumers who rated a food high in sweetness were not always more likely to rate it high for liking. Correspondingly, if a food was tasted more than "moderate" in bitterness, its liking rated by consumers was neutral or lower (Kamerud and Delwiche, 2007). Both bitterness and astringency were reduced by sucrose in beverages (Keast, 2008; Ishikawa and Noble, 1995). Therefore, the addition of sucrose in very hard water (3mg Fe/L) might obviously reduce the metallic/bitter taste of ferrous ions, which significantly increased its liking rate (Table 6). In contrast, very hard water without iron generated off-flavor when in combination with sucrose and significantly decreased ($p < 0.05$) the taste acceptance. In water with higher hardness, even if all the other minerals (except iron) were not at threshold, the subthreshold of these mineral ions still contributed salty, sweet or bitter tastes and a "slippery" mouthfeel to water (Whelton and Dietrich, 2004). It was reported that water hardness might contribute salty, slightly metallic and sour taste, and "chalky", "mineral" or drying mouth feel to water (Byrne et al., 2009). According to the study of Byrne et al. (2009), taste perception of hard water (> 200 mg/L) was significantly ($p < 0.01$) different from soft water (< 25 mg/L). In addition, Lawless et al. (2004) found that even though sucrose could partially mask the bitterness of calcium chloride, calcium salts were also

observed to suppress the sweetness of sucrose. Therefore, water hardness might add off-flavor to sucrose-sweetened water and reduce the sweetness of sucrose probably by blocking its sweetness receptors.

3.3.2.2 Emotional response

To further understand consumer response to sweet-metallic taste interaction, emotional profiles generated from sweetened/unsweetened synthetic water were analyzed by selecting all applicable emotional terms using a modified EsSenseTM paper ballot (King et al., 2010; Leitch et al., 2015). Seventeen emotional terms were classified as frequently selected terms ($\geq 20\%$ selection frequency) for synthetic waters including soft water, very hard water without iron and very hard water with iron, and 15 frequently selected terms for these three synthetic waters mixed with sucrose. Selection frequency of frequently selected emotional terms for each sample is summarized in Appendix A. Cochran's Q test was performed to identify the significant differences ($\alpha=0.05$) between sweetened/unsweetened water samples for each frequently selected term.

Among unsweetened synthetic waters, there were 5 frequently selected emotional terms identified as shared terms ($\leq 8\%$ difference among samples) including *content*, *energetic*, *friendly*, *good* and *steady*. These five terms did not exhibit any significant difference between unsweetened synthetic waters according to Cochran's Q test (Appendix A). Emotional terms including *good-natured*, *happy*, *pleasant* and *satisfied* were identified as unique terms for very hard water (0mg Fe/L), while *bored* and *disgusted* were distinct for very hard water (3mg Fe/L). Unique emotional terms for very hard water (3mg Fe/L) explained its low acceptability for consumers, since the feeling of *disgusted* might be caused by metallic taste of iron ions.

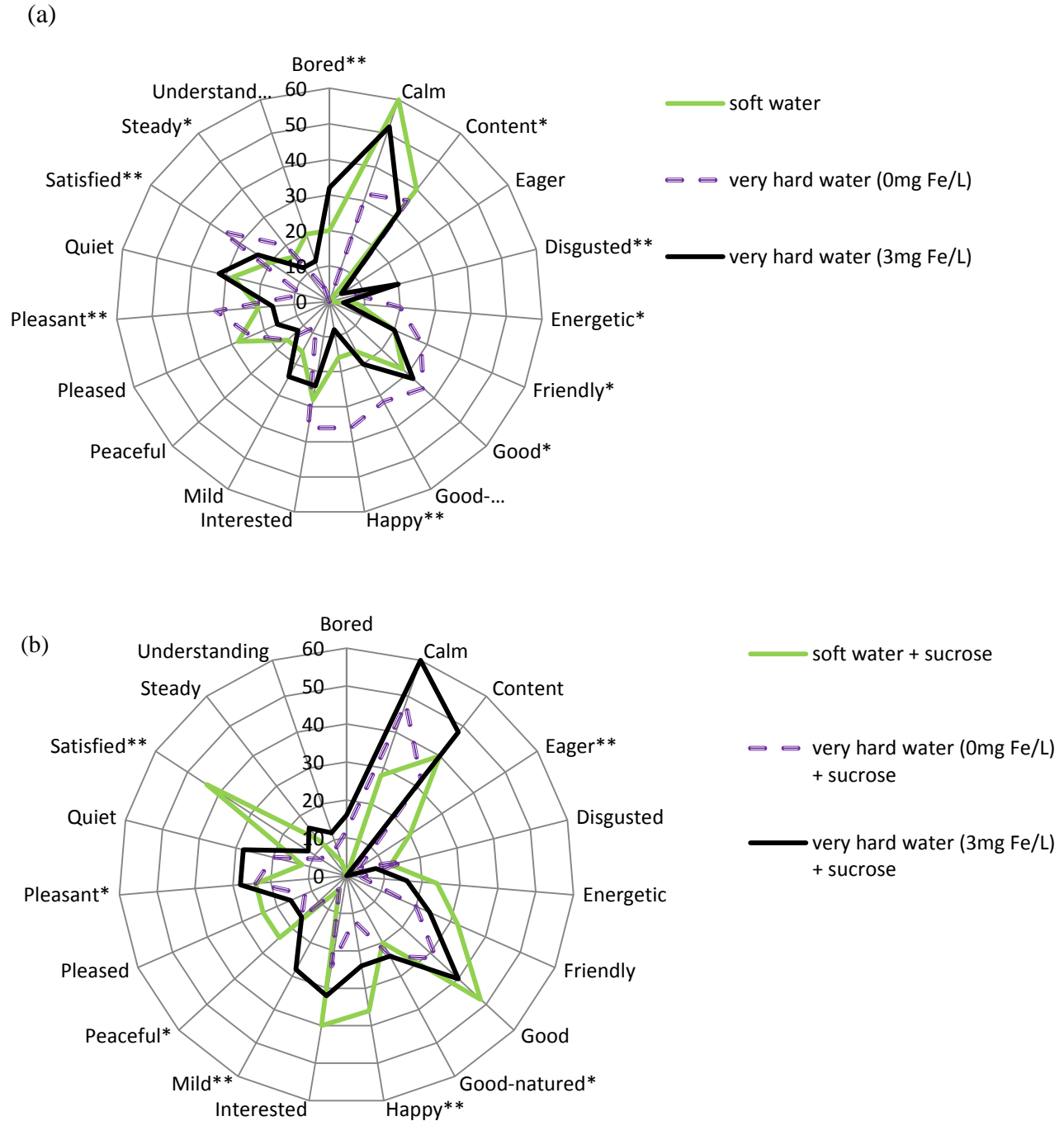


Figure 2. Frequency distribution of emotion terms frequently selected by panelists (n=25) for: (a) soft water, very hard water (0mg Fe/L) and very hard water (3mg Fe/L); (b) soft water + sucrose, very hard water (0mg Fe/L) + sucrose, and very hard water (3mg Fe/L) + sucrose. The displayed emotional terms were more than 20% of selection frequency for at least one water sample within group. Terms denoted with an asterisk were shared terms which had a difference in frequency $\leq 8\%$ among three samples in the group. Terms denoted with two asterisks were unique terms which used for one sample having a difference in frequency $\geq 12\%$ than the other two samples in the group.

Although soft water and very hard water without iron did not present any significant difference in acceptability, emotional response for these two waters was obviously different. As shown in Fig.2(a), emotion distribution of very hard water (0mg Fe/L) was more toward to positive terms such as *good-natured*, *happy*, *pleasant* and *satisfied*, while emotional terms selected for soft water were more neutral words such like *calm* and *content*. The production of delightful taste of very hard water (0mg Fe/L) might be due to its appropriate mineral composition. Even though several minerals could cause off-flavor in drinking water such as metal ions and sodium in high concentration, lacking of minerals was worse for taste perception of water due to its low TDS (<30 mg/L). In a study about taste preference of tap and commercial bottled waters, soft water was found to have a plastic taste according to sensory tests and could not be differentiated from distilled water (Dietrich and Burlingame, 2015). Since minerals such as Na^+ , Cl^- , K^+ , Ca^{2+} , Mg^{2+} , HCO_3^- are able to produce salty or sweet taste in water at low concentrations, they were always added back to keep certain concentration in bottle water to improve taste perception.

For sweetened synthetic waters, *peaceful*, *pleasant* and *good-natured* were identified as shared terms, which appropriately represented the effect of sucrose on taste perception in this group. As expected, emotional responses of sweetened soft water were mostly distributed among positive terms and *eager*, *happy*, and *satisfied* were identified as unique terms for sweetened soft water (Fig.2). Sweetened very hard water (3mg Fe/L) had similar sweetness and acceptability with sweetened soft water. However, it presented higher emotional response in neutral terms such as *calm*, *content*, *quiet* and *mild* than sweetened soft water and *mild* was determined as unique term for sweetened very hard water (3mg Fe/L). Sweetened very hard water without iron showed similar distribution of emotional terms with sweetened very hard water with iron but

with less frequency. In addition, sweetened very hard water (3mg Fe/L) exhibited much higher selection frequency in *energetic* and *mild* than sweetened very hard water without iron, which was considered as the result of sweet-metallic taste interaction corresponded with the enhancement of sweetness.

Since sweet-metallic interaction did not affect the sweetness and acceptability, sweetness can be applied to mask inherent metallic flavor of medicine, supplements or functional foods. When attempting to mask metallic taste with sweeteners, combination of different sweeteners was reported to be a promising method (Walters and Roy, 1996). The combination increased the possibility to block bitter receptors through competitively occupying the transduction system. Also, sweeteners with longer sweetness duration such as thaumatin and glycyrrhizin were more effective in masking the aftertaste of metallic sensation.

3.4. Conclusion

Effect of water quality on taste perception of sweetened beverages and corresponding emotional response underlying sweet-metallic taste interaction were illustrated in this study. Ferrous ion was the main factor to cause metallic flavor in hard water, and led to bored and disgusted feelings for consumers. However, acceptability and emotional response of iron-containing water could be improved through sweet-metallic taste interaction by adding sweeteners. Other minerals potentially influenced taste perception of drinking water at subthreshold concentrations. In contrast to iron, they could decrease the acceptability of sucrose sweetened water when reaching high concentrations (very hard water).

In this work, sweet-metallic taste interaction exhibited variable effects according to different types of sweeteners and different iron concentrations (metallic intensity) in water. Sweetness of sucrose (0.05g/mL) sweetened beverage was significantly higher ($p < 0.05$) in very hard water

than in hard water, while ace-K (2.64×10^{-4} g/mL) and honey (0.0624 g/mL) had significant higher ($p < 0.05$) sweetness in hard water than other types of water. Ace-K (2.64×10^{-4} g/mL) and saccharin (0.0037 g/mL) were found to have metallic aftertaste themselves in soft, moderate hard and hard water. This aftertaste could not be discriminated from metallic taste generated from ferrous ions only when Fe^{2+} concentration as high as 3mg/L. Honey presented significant metallic flavor only when dissolving in very hard water. Sucralose exhibited similar taste perception with sucrose due to their similar chemical structure. Mild was identified as the unique emotional term for sweet-metallic interaction. Since metallic sensation has a significant odour component, effect of taste-odour interaction in influencing metallic flavor needs to be further studied. Future research may evaluate the effects of metallic smell by sipping sweetened mineral waters with and without nose plugged, and comparing the differences of sweet-metallic flavor before and after adding aroma compounds.

Emotional response tests provide more in-depth information than acceptance tests and revealed consumer preferences in their sensory experiences. Although sweet-metallic interaction did not reduce the acceptability, emotional profiles generated from it may not be desirable for consumers compared with beverage made from soft water. On the other hand, sweet-metallic interaction was able to mask inherent metallic taste of functional food/beverage, supplements or medicine that fortified by health beneficial components but with poor taste quality. It is the combination of sweet, bitter and other tastes determine the final perception of a beverage product.

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Chapter 4

Iron in Water Affects Milk Quality- Milk Protein Composition and Stability Changes Affected by Iron in Water Sources

Abstract Water makes up more than 80% of the total weight of milk. However, the influence of water chemistry on milk proteome has not been extensively studied. The objective was to evaluate interaction of water-sourced iron (low, medium and high level) on milk proteome and implications on milk oxidative state and mineral content. Protein composition, oxidative stability, and mineral composition of milk were investigated under conditions of iron ingestion through bovine drinking water (infused) as well as direct iron addition in commercial milk in two studies. Four ruminally-cannulated cows each received aqueous infusions of ferrous lactate at doses of 0, 200, 500 or 1,250 mg of Fe/d. For comparison, ferrous sulfate solution was directly added into commercial retail milk with the same concentrations: control (0 mg Fe/L), low (2 mg Fe/L), medium (5 mg Fe/L), and high (12.5 mg Fe/L). For milk both from abomasal infusion of ferrous lactate and direct addition of ferrous sulfate, iron concentration as low as 2 mg Fe/L was able to cause oxidative stress on dairy cattle and infused milk respectively. Abomasal infusion affected the expression of both caseins and whey proteins in the milk while direct addition mainly influenced caseins. Although abomasal iron infusion did not significantly affect oxidation state and mineral balance (except iron), it induced oxidized off-flavor and partial degradation of whey proteins. Direct iron addition to milk led to lipid oxidation during storage at 4°C. Oxidation level was positively associated with the concentration of added iron. Minerals (Mg, P, Na, K, Ca, Zn) in milk were not affected with the added iron in milk. This study indicated that a small amount of iron contamination in bovine drinking water at the farm, or incidental iron addition from potable

water sources causes oxidation, impacts milk protein composition and stability and affects final milk quality.

Key words: iron, protein, oxidation, milk synthesis

4.1 Introduction

Cow's milk and the related dairy products are nutritious foods containing numerous essential nutrients, especially milk proteins which serve as an excellent source for essential amino acids. Milk proteins are comprised of caseins (80%) and whey proteins (20%). Caseins are well known to carry calcium and phosphate with many bioactive functions as well as contributing to efficient digestion (Haug et al., 2007). Whey proteins possess a variety of nutritional and biological properties, thus are widely used in reducing the risks of diseases such as cancer (Gill and Cross, 2000; de Wit, 1998), inflammation (Clare et al., 2003), chronic stress-induced disease (Ganjam et al., 1997), and HIV (Oona et al., 1997; Micke et al., 2002). Bovine whey proteins perform their biological functions through constituents including β -lactoglobulin (mediate and transport immunoglobulins during colostrum formation), α -lactalbumin (lactose synthase component and possible antimicrobial/anticancer activity), immunoglobulins (serving as antibodies to protect the mammary gland from infection), serum albumin (anti-mutagenic, anticancer, and immunomodulation activity), lactoferrin (iron-binding, iron transport, antimicrobial/anti-inflammatory/anticancer activities, immune system modulation), and lactoperoxidase (antimicrobial and antioxidant properties) (Alonso-Fauste et al., 2012; Haug *et al.*, 2007; Swaisgood, 1995; Leveux and Ollier, 1999; Loimaranta et al., 1999; Adlerova et al., 2008).

Quality and yield of bovine milk proteins are determined by genetics of dairy cows, hormones, dietary energy, and lactation environment (Bionaz et al., 2012). Recently, excess iron ingestion was found to impact cow's health through the formation of an oxidation environment

and interfere with absorption of other minerals (Hansen et al., 2010); this may contribute to increase in mastitis, bacterial infection, retained fetal membranes and decrease in immunity (Linn, 2008; Standish et al., 1971; Bullen et al., 1978). Declined health condition of dairy cattle immediately depressed their milk production and altered milk composition, including loss of milk yield (Gröhn et al., 2004), decrease of lactose and fat content (Bansal et al., 2005), and increase of sodium, chloride and electrical conductivity (Bruckmaier et al., 2004). However, effect of iron-induced oxidative stress on milk protein synthesis of lactating dairy cows has not been studied yet.

Dairy cattle drink 90 to 150 L of water each day when producing milk (Feng et al., 2013). A common resource for bovine drinking water is groundwater, where water can dissolve iron and other minerals as it percolates through soil and rock, and hold the minerals in solution. The iron concentration of ground water in the southwest portion of Virginia is commonly between 0.01-0.3 mg/L (Ayotte et al., 2011). However, in the upper Midwest and northeast of United States where is high in dairy production, the iron concentration in ground water is generally much higher (≥ 0.3 mg/L) (Ayotte et al., 2011). The U.S. Environmental Protection Agency (USEPA) reported that Fe concentration in ground water of many regions in USA exceeds the secondary maximum contaminant levels (0.3 mg Fe/L), as set by that agency (USEPA, 2015). Due to the large amount of water consumed by dairy cows per day, ferrous Fe in milligrams-per-liter concentrations makes water a potentially significant source of iron intake for cows. In addition, although direct addition of water during fluid milk processing is not legal, incidental contamination related to use of potable water in equipment cleaning on the farm or in the processing plant may occur. In addition, potable water is used as an ingredient in the manufacture of many dairy-based products, providing an avenue for the incidental addition of

iron and contact with milk proteins. However, there is limited information about effect of iron ingestion either *in vivo* or *in vitro* on quality of milk proteins.

This study is part of a larger interdisciplinary study involving the implications of water-sourced iron on milk quality. Feng et al. (2013) described the experimental conditions of the *in vivo* study on phosphorus absorption in lactating dairy cows. Concurrently, Mann et al. (2013) evaluated the subsequent effect of milk synthesized under the experimental conditions on fluid milk sensory quality. Our objective in this study was to evaluate the interaction of water-sourced iron (low, medium and high level), as determined through *in vivo* (water provided to dairy cattle) and *in vitro* (direct addition to whole processed milk) delivery, on milk proteome and implications on other milk quality parameters including oxidative state and mineral content. Our null hypothesis was that no differences in milk proteome, oxidative status, and mineral content would be observed in either the *in vivo* or the *in vitro* approach. Our alternative hypothesis was that both ways of iron ingestion would affect milk proteome, affect milk mineral content, and would contribute to changes in oxidative stability. Milk used for the *in vivo* portion of this study was sourced from this interdisciplinary study.

4.2 Material and Methods

4.2.1 Milk Samples Collected from Individual Farm Cows (*in vivo*)

Experiment Design and Raw Milk Collection. Protocols and procedures of this experiment were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) (12-027-DASC). Details of the *in vivo* experimental study are reported in Feng et al. (2013). In brief, four ruminally cannulated early lactation (second lactation) cows (2 Holstein and 2 Holstein × Jersey cross) were isolated from external water sources to eliminate outside variables, housed in individual stalls, and placed on a standard basal diet and water source (Feng et al.,

2013). Abomasal infusion of ferrous lactate was performed as described in our previous work (Feng et al., 2013). Treatments (abomasal infusion of 0, 200, 500, or 1,250 mg of Fe/d as ferrous lactate solution) were imposed in a 4 × 4 Latin square design with 14-d periods. Treatments were formulated to approximate 0, 2, 5, or 12.5 mg of Fe/L concentrations in drinking water, assuming water intake of 100 L/d. The infusion treatment design is summarized in Table 7 and Table 8. Milk samples were collected from the evening milking of each cow at day 13 of each period, allowing for 6 days of total infusion time before milk collection, as described by Feng et al. (2013).

Table 7. Concentration of ferrous lactate infused in bovine drinking water and expected ingestion by cow, and concentration of ferrous sulfate added in commercial whole milk

Treatment	Individual Farm Cow Diet			Commercial Whole Milk
	Infusion concentration, mg Fe/L	Infused ferrous dose, mg Fe/d	Equivalent drinking water concentration ¹ mg Fe/L	Ferrous added mg Fe/L
Control	0	0	0	0
Low	200	200	2	2
Medium	500	500	5	5
High	1250	1250	12.5	12.5

¹Assumes cow ingests 100 L water per day (Feng et al., 2013).

Table 8. Latin Square Design (Feng et al., 2013) for *in vivo* study. Iron intake¹ by abomasal infusion for each cow in each period²

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

¹Four treatments of iron intake are: control (0 mg/d), low (2 mg/d), medium (5 mg/d) and high (12.5 mg/d).

²Period of infusion: daily infusion of 1L of ferrous lactate solution with assigned iron concentration for 7 days; there is a wash-out period of 7 days before each infusion period.

³Jersey × Holstein (4541, 4543); Holstein (4558, 4559).

Milk Processing. Details of the milk processing are described in full in Mann et al. (2013). In brief, raw milk was collected in 5-gallon stainless steel milk cans and immediately transported to the Food Science and Technology Dairy Processing Laboratory, and stored in a cooler (Tonka, Hopkins, MN) at 4 °C. Milk from each period for each cow was processed separately. After preheating to 55-60 °C, milk was separated into cream and skim milk using a pilot plant separator (Elecrem separator, model IG, 6400rpm, Bonanza Industries Inc., Calgary, Alberta, Canada). Cream then was added back to skim milk to achieve 3.18 ±0.04 % milkfat. The homogenization of milk was conducted 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 min and microbial quality was assessed by aerobic plate count standard methods using aerobic count petrifilm (3M Petrifilm, Microbiology Products 3M Health Care, ST Paul MN). Processed milk was packaged in translucent food grade high density polyethylene gallon packages, which were pre-sanitized with a chlorine (100 mg/kg) rinse (dH₂O) (Mann et al., 2013). Samples of the processed milk were then immediately frozen at -80 °C for subsequent analyses.

4.2.2 Milk Samples Collected from Commercial Retail Milk (in vitro)

Milk [3.25% milk fat, pasteurized (HTST), Kroger] was purchased from the local supermarket in 1.89 L package. Milk samples were collected from the top, middle and bottom layer of the bottle as three replicates used for each iron treatment. Stock ferrous sulfate solution (200 mg Fe/L of water) was prepared by adding 0.25g food-grade ferrous sulfate (FeSO₄•7H₂O, Spectrum, Gardena, CA) into 250 mL double-distilled water. To compare with the iron treatments used for milk with abomasal infusion of ferrous lactate (in vivo), the same four ferrous concentrations were used in commercial retail milk with direct addition of ferrous sulfate.

The concentrations were achieved by adding stock ferrous solution in each 10 mL milk: 0 mL in 30 mL milk for control treatment (0 mg Fe/L of milk), 0.1 mL in 29.9 mL milk for low treatment (2 mg Fe/L of milk), 0.25 mL in 29.75 mL milk for medium treatment (5 mg Fe/L of milk) and 0.62 mL in 29.38 mL milk for high treatment (12.5 mg Fe/L of milk). Milk samples were immediately covered by aluminum foil to avoid light and stored at 4°C. Before protein analysis, milk samples were centrifuged at 16,000 ×g for 15 min at 4°C and upper fat layer was discarded. Three replications were completed for this study, following a complete block design.

4.2.3 Protein Analysis by Two-Dimensional Gel Electrophoresis (2-DE)

Chemicals. Urea, Trizma base, glycerol, iodoacetamide, CHAPS, protease inhibitor cocktail and sodium dodecyl sulfate were purchased from Sigma-Aldrich (St. Louis, MO), 2-D clean-up kit, 2-D quant kit, immobiline dry strip pH 4-7 (11 cm) and IpG buffer (pH 4-7) were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Criterion Tris-Hcl gels (10-20 %) were purchased from BIO-RAD (Hercules, CA).

Protein Preparation. Frozen (-80°C) processed milk samples were thawed on ice for about 2 hours before use, and then mixed thoroughly by vortex mixer. Thawed samples were further centrifuged at 16,000 ×g for 15 min at 4°C. The upper fat layers, which contained cell debris (somatic cells and bacteria) and most of the casein, were then discarded (Alonso-Fauste et al., 2012).

Proteins from skim milk were extracted by 2-D clean-up kit (GE Healthcare, Pittsburgh, PA) following the kit instruction (GE Healthcare, Pittsburgh, PA). Extracted protein pellet was dissolved in rehydration buffer containing 8 M urea, 2 M thiourea, 2 % CHAPS (w/v), 1.8 mM DTT, 1 % IPG buffer (v/v) (Bio-Rad, Hercules, CA) and 0.01 % bromophenol blue (Alonso-

Fauste et al., 2012). Protein concentration was determined with 2D-Quant Kit (GE Healthcare, Pittsburgh, PA) following the protocol given in the brochure and using BSA as a standard.

Two-Dimensional Electrophoresis. Precast 11-cm strips, pH range 4-7 (GE Healthcare, Pittsburgh, PA) were rehydrated in the presence of 200 µg of milk protein at 20 °C for 12 h. Isoelectric focusing was carried out using an Ettan IPGphor 3 Cell (GE Healthcare, Pittsburgh, PA) for 10 kVh using the following voltage program: (1) from 0 V to 200 V over 0.5 h; (2) from 200 V to 500 V over 0.5 h; (3) from 500 V to 1000 V over 1 h; (4) from 1000 V to 8000 V over 1 h; (5) held at 8000 V for 5 h (Yamada et al., 2002). The IPG strips then were incubated in the first equilibration buffer [1% DTT (w/v), 375 mM Tris-HCl (pH 8.8), 6 M urea, 20 % glycerol (v/v), 2 % SDS (w/v), 0.01 % bromophenol blue (w/v)] for 20 min, and then followed by the second equilibrium buffer [2.5% iodoacetamide (w/v), 375 mM Tris-HCl (pH 8.8), 6 M urea, 20 % glycerol (v/v), 2 % SDS (w/v), 0.01 % bromophenol blue (w/v)] for another 20 min (Alonso-Fauste et al., 2012). Strips were then transferred on top of a 10–20 % gradient Criterion Tris-HCl polyacrylamide gels (BIO-RAD, Hercules, CA) and coated by 0.5% low-melt agarose (w/v) containing 0.01 % bromophenol blue (w/v). SDS-PAGE was carried out using a charged cell (Bio-Rad, Hercules, CA) for 15 min at 35 mA and then 1.5 h at 70 mA. Gels were stained by Coomassie blue for 21 h and then destained for 4 h.

In-gel Trypsin Digestion and Mass Spectrometry Identification. Protein identification was conducted by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometry analysis, which is slightly modified according to the method of Chevalier et al. (2006). Protein spots were excised from 2-DE gels by hand with a spot picker. The cut pieces were transferred into clean 0.6 mL tubes and destained using a mixture of 25 mM ammonium bicarbonate and HPLC-grade

acetonitrile (1:1, v/v) about 2 h with constant shaking at room temperature. After carefully removing the liquid in the tube, the gel pieces were washed again by the above mixture. Then 25 mM HPLC-grade acetonitrile was added into the gel pieces and incubated for 15 min with constant shaking at room temperature. Gel pieces then were thoroughly dried by vacuum concentrator (Kansas City, Missouri). Protein digestion was carried out by addition of 0.065 μ g of trypsin and incubated on ice for 15 min, and then on a heat block at 37 °C overnight.

For mass spectra analysis, 1 μ L of each digest was transferred to a freshly-polished MALDI plate and covered with freshly-prepared matrix containing 4 mg/mL α -cyano-4-hydroxycinnamic acid, 50% CH₃CN, 0.1% TFA (v/v), 0.1% formic acid (v/v), and 5 mM (NH₄)Cl. Data collection was performed by an Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Framingham, MA). The MASCOT search engine software (Matrix Science, Boston, MA) was used to search the NCBI database. The parameters for searching and identifying matches were adjusted as follows: mass tolerance of 30 ppm, a minimum of five peptides matching to the protein, carbamidomethylation of cysteine as fixed modification oxidation of methionine as variable modifications, and one missed cleavage allowed. MASCOT parameters were regulated as follows: parent ion mass tolerance of 20 ppm, fragment ion mass tolerance of 100 ppm.

4.2.4 Oxidative Stability

Thiobarbituric acid reactive substance (TBARS) was used as an indicator of oxidative stress in milk and was calculated as malondialdehyde (MDA) value in sample Mann et al., 2013). To test the oxidative stress of milk caused by iron, TBARS of commercial milk samples were detected in the first day after adding ferrous sulfate and the eighth day after storing in cooler at 4°C respectively. For TBARS analysis, each milk sample was diluted with deionized water at 1:2 ratio. Then, 1 ml of diluted milk samples and MDA standards (0 mg/L, 0.18 mg/L, 0.36 mg/L

and 0.54 mg/L) were mixed with 2 mL of solution I (0.375% thiobarbituric acid, 0.506% SDS, 9.370% acetic acid) and 0.1 mL of solution III (antioxidant and chelator solution). After digestion for 60 min at 95 °C in water bath, milk samples were immediately cooled in an ice bath, followed by mixing with 2 mL of pyridine/n-butanol mixture (1:15) and centrifuged for 15 min at 3000 × g. The absorbance of the supernatant was measured with a spectrophotometer (Milton Roy Spectronic 21D Spectrophotometer, Milton Roy Company, Rochester, NY) at 532 nm. TBARS was reported as the concentration of MDA of each milk sample as calculated based on a standard curve made by means of a linear regression on absorbance values and concentration of MDA standard (Mann et al., 2013).

Mineral Analysis

Sodium, magnesium, calcium, potassium, copper, iron, zinc and inorganic phosphorus concentrations of each milk sample were measured by emission spectroscopy using Inductively Coupled Plasma (ICP) technique (Thermo Electronic Corporation, X-Series ICP-MS, Waltham, MA) (Menegário et al., 2001). Frozen milk samples (-80°C) were thawed on ice for about two hours. Thawed samples were centrifuged at 18500 ×g for 15 min to reduce viscosity and remove debris. Sample digestion was modified from the previous studies (Menegário et al., 2001; Watanabe et al., 2005) by diluting each milk sample at 1:10 (v/v) with 4 % nitric acid. Iron standard (1mg Fe/L) was prepared by mixing 4.75 mL of FeSO₄.7H₂O with 250 µL nitric acid, and reagent blank was prepared by adding 250 µL of nitric acid into 4.75 mL of nanopure water.

Data Analysis

Milk from individual cows while in the control treatment served as the baseline control for each treatment condition for each analysis. Milk from the commercial milk source, without the direct addition of iron, served as the baseline control for the *in vitro* study. Changes of milk

protein composition were analyzed by PDQuest software v.7.3.1 (Bio-Rad, Hercules, CA). Protein spots that had a least a 2-fold change in intensity were considered as differences among treatments in each replicated group. Protein concentration (intensity) was determined as the percentage of total valid spots volume on respective gels. *P*-value was calculated based on Wilcoxon test and $p < 0.05$ was used as cutoff for significance. Differences of milk minerals and oxidation levels between iron treatment (*in vivo* abomasal infusion of ferrous lactate or *in vitro* direct addition of ferrous sulfate) were analyzed by one-way ANOVA (analysis of variance). One-way ANOVA was performed by statistical software programs JMP 10.0. For all analyses, *p* values less than 0.05 were considered statistically significant.

4.3 Results and Discussion

4.3.1 Effect of Abomasal Infusion of Ferrous Lactate on Milk Protein Synthesis of Individual Farm Cows (in vivo study)

Total protein concentration in the milk of individual farm cows abomasally-infused with ferrous lactate was $3.37\% \pm 0.16$ (Feng et al., 2013), which fell within normal range (2.97%-3.74%) as reported by Lundín et al. (1997). After in-gel image analysis, a total of 69 protein spots with expression changes (≥ 1.1 -fold) in spot intensity were detected in the milk of individual farm cows along with the increasing abomasally-infused ferrous lactate. These milk protein spots were further excised, in-gel tryptic digested and analyzed by MALDI-TOF-TOF mass spectrometry. Identified protein spots are listed in Table 9 and marked on each 2-DE image with consistent spot ID number.

Table 9. Mass spectrometric identification of differentially expressed proteins in milk with abomasal infusion of ferrous lactate and direct addition of ferrous sulfate during treatments

Spots ¹ ID	Protein name	Accession No.	Mascot score	Matched peptides	Protein MW ² (kDa)	Protein PI ³
1,2,3,4,5,10,19, 21,22,23,34,35, 37,41,42,43,44, 45,52,55,59,60, 67,68,69	α_{s1} -Casein	gi 225632	292	4	27.3	4.6
6,7,8,9,	α_{s2} -Casein precursor	gi 27806963	217	4	24.3	5.2
11,12,13,14,15	Serum albumin	gi 472346475	118	2	66.4	5.6
16	Lactoferrin	gi 85700158	341	6	78	8.7
17	Lactoperoxidase	gi 27806851	338	5	81	8.8
18,50	IgG1 heavy chain	gi 7547266	209	2	36	7.3
20,38,39,53, 54,66	β -Lactoglobulin	gi 6729725	554	6	17	4.8
24,26,31,34,36, 37,47,48,49,59, 67	β -Casein	gi 162805	171	3	40.3	6.6
25,51	IgM heavy chain	gi 108750	209	2	49	5.6
27,28,29,30,31, 32,33,62	κ -Casein	gi 162811	238	5	21	6.3
46	α -Lactalbumin	gi 68	212	4	17	4.8

¹Identified proteins using the National Center Biotechnology Information nonredundant amino acid sequence database.

²Molecular weight (in theory).

³Isoelectric point (in theory).

Identified milk protein spots in this study contained all classes of casein (α_{s1} -, α_{s2} -, β -, and κ -) and whey proteins (β -lactoglobulin, α -lactalbumin, serum albumin, immunoglobulins, lactoferrin, and lactoperoxidase), which is in agreement with previous studies (Jensen et al., 2012; Senda et al., 2011; Chevalier et al., 2009; Alonso-Fauste et al., 2012). Position of these protein spots also roughly corresponded to the reported milk protein proteome (Chevalier et al., 2009; Jensen et al., 2012) analyzed with a similar 2-DE method (pH 4-7 IPG strip, 12%/12.5% SDS-PAGE).

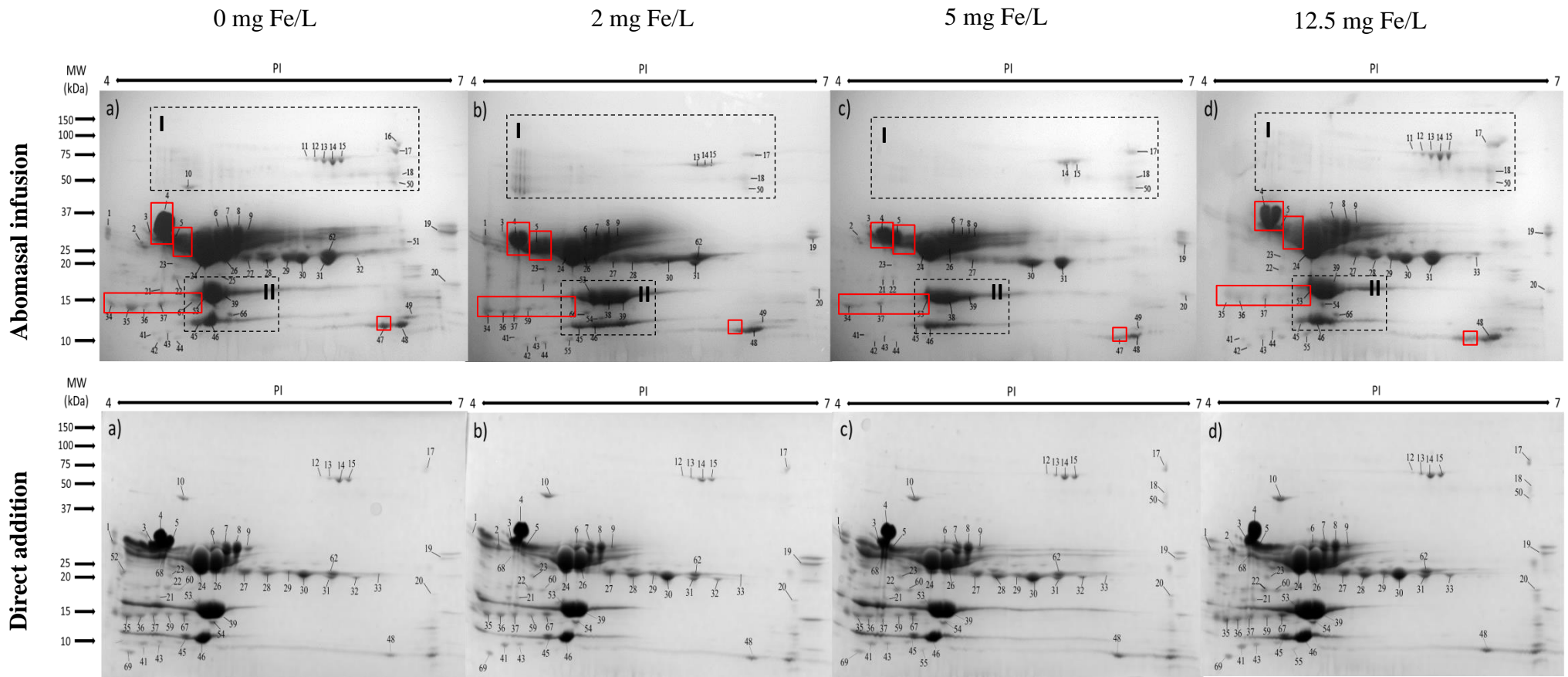


Figure 3. Protein composition of milk from a representative individual farm cow with abomasal infusion of ferrous lactate and commercial retail milk with direct addition of ferrous sulfate, during (a) control (0 mg Fe/L), (b) low (2 mg Fe/L), (c) medium (5 mg Fe/L), and (d) high (12.5 mg Fe/L) treatment. Each 2-DE gel (pH 4-7) was loaded with 200 μ g protein and stained by Coomossie Blue. Area I and area II are whey proteins that changed among different individual farm cows associated with different iron treatments.

Figure 3 (top row) illustrates the representative proteome of milk (from Cow A) as illustrated with increasing concentration of ferrous lactate infusion. Based on Wilcoxon test, milk protein spots with significant ($p < 0.05$) fold changes (≥ 2 -fold) in intensity during abomasal infusion of ferrous lactate were grouped (dotted (whey proteins) or lined (casein proteins) boxes) in Fig.3. Compared with control group (0 mg Fe/L), spot 4 (α_{s1} -casein) and spot 47 (β -casein) were significantly decreased in intensity at low treatment (2 mg of Fe/L), medium treatment (5 mg of Fe/L) and high treatment (12.5 mg of Fe/L). In addition, spot 34,36,67 (β -casein) and spot 5 (α_{s1} -casein) constantly decreased ($p < 0.05$) with the increasing levels of abomasally-infused ferrous lactate.

As shown in Fig.3, one protein may have several spots separated along the IEF pH strips, which means additional spots with different isoelectric points were originated from major milk proteins caused by alterations such as proteolysis, post-translational modification, and interaction/association with major milk proteins. For example, spot 34, 36, 67 were low molecular weight degradation products of β -casein. Proteolysis may be induced by the activity of principal indigenous milk protease, plasmin, or other indigenous/exogenous protease activities in milk (Chevalier et al., 2009).

The decreased expression of caseins found in this work might due to the iron-induced oxidative stress. When iron exceeds the demand of dairy cattle, there will be an increase in the concentration of reactive oxygen and nitrogen species ($\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, and $\text{NO}\cdot$ etc.), which is known as oxidative stress (Puntarulo, 2005). Iron-induced oxidative stress is reported to influence the activity of hormones in living systems, such as the development of insulin resistance (Rains and Jain, 2011). Milk protein synthesis has a high requirement of energy, and one important way of generating greater dietary energy during milk protein synthesis is the increase of insulin secretion

(Reynolds et al., 1994). Yield of milk protein showed strong and positive relationship with the concentration of insulin in dairy cows (Winkelman and Overton, 2010). The interruption of insulin signal resulting from an oxidation environment will fail to activate STAT5-ELF5, which is critical in controlling gene expression and translation during milk protein synthesis (Menziez et al., 2009 and 2010), thus decreasing the yield of milk proteins.

Another consequence of oxidative stress is the accumulation of oxidized protein. When free iron is available, which is determined by the concentrations of iron-binding proteins (such as lactoferrin, transferrin) and iron-responsive factors (controlling the binding and release of iron from iron-binding proteins), derivatives of protein oxidation such as H_2O_2 can generate the even more toxic OH by iron-catalyzed cleavage through the Fenton reaction (Berlett and Stadtman, 1997). It has been demonstrated that the primary oxidative damage to proteins was metal-catalyzed oxidation (Stadtman and Berlett, 1998; Berlett and Stadtman, 1997). To minimize the damage (protein aggregation and cross-linking) induced by protein oxidation, a series of antioxidant defenses mechanisms are activated within dairy cattle. Most oxidized proteins are degraded by proteolytic pathways to minimize protein aggregation and remove potentially toxic protein fragments (Berlett and Stadtman, 1997). We suggest that this might explain the proteolysis of β -casein fragments (spot 34,36,67) along with the increasing levels of abomasal infused ferrous lactate.

As expected, variations in milk proteome among the four individual farm cows were found in this work. Remarkably, whey proteins among different cows showed a large variation during abomasal infusion of ferrous lactate. Whey protein spots of interest were grouped by dashed line in Fig. 3, and the grouped area I and II were excised and compared within each individual farm cow under each abomasal infusion treatment (Fig. 4 and Fig. 5).

As shown in Fig.4, there was an obvious decrease (≥ 2 -fold) of IgG1 heavy chain, IgM heavy chain, lactoperoxidase and lactoferrin in the milk of cow A at low, medium and high abomasal infusion treatments compared with control. Serum albumin proteins were obviously decreased (≥ 2 -fold) at low and medium abomasal infusion treatment in cow A. Serum albumin and lactoperoxidase in cow C illustrated a decrease (≥ 2 -fold) only at low abomasal infusion treatment. However, all the immunoglobulins (IgG1 heavy chain and IgM heavy chain) in cow C were increased (≥ 2 -fold) in intensity at medium and high abomasal infusion treatments. For cow B and cow D, all immunoglobulins were relatively stable during abomasal infusion treatments.

Immune proteins such as lactoperoxidase, lactoferrin, immunoglobulins and serum albumin are well known to be increased in expression associated with mastitis, which suggested their role in the innate nonspecific defense system (Madureira et al., 2007). In addition, increasing levels of lactoferrin were demonstrated as oxidative stress-related parameters due to its antioxidant activity based on Fe-binding ability (Thome et al., 1996). In this study, lactoperoxidase, lactoferrin and immunoglobulins in cow C illustrated an obvious increase (≥ 2 -fold) associated with higher amounts of abomasal infused ferrous lactate. The increase of immune defense proteins might be the direct response to the iron-induced oxidative stress in cow C.

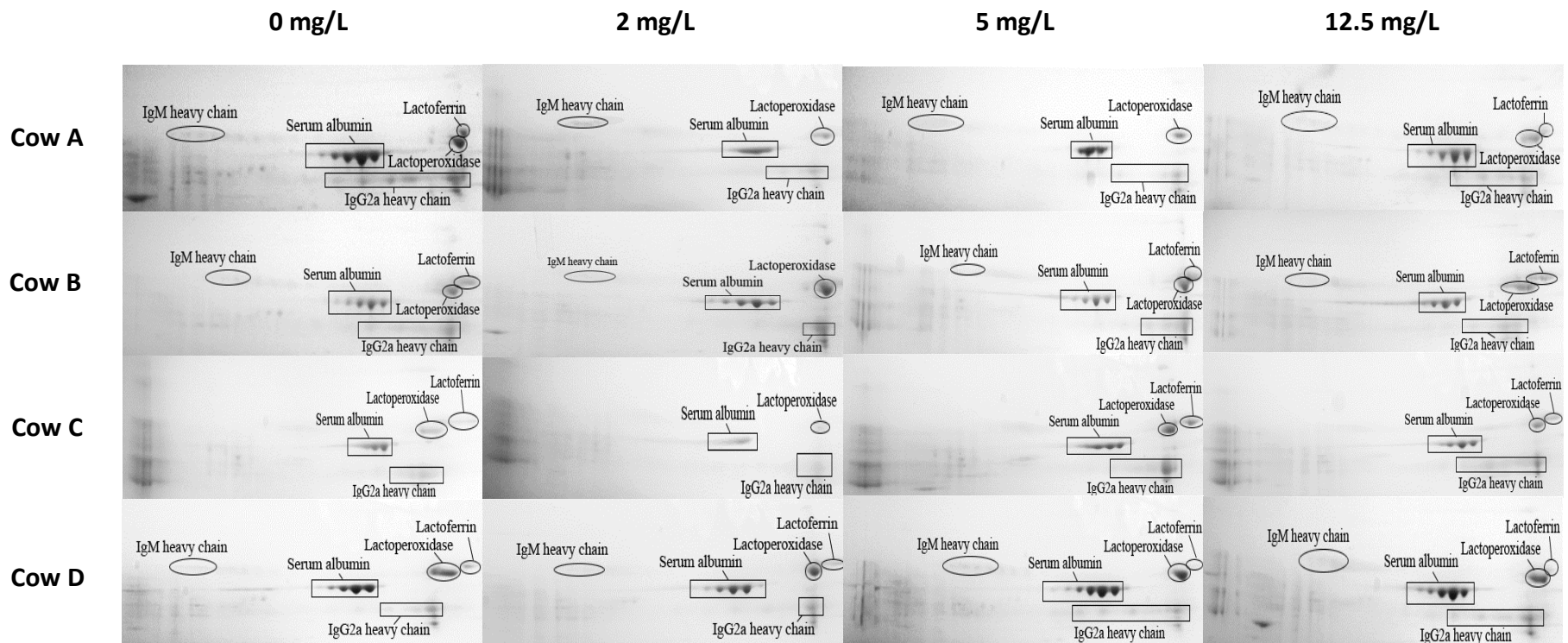


Figure 4. Comparison of changes in protein spots in are I of Fig.3 during (a) control (0 mg Fe/L), (b) low (2 mg Fe/L), (c) medium (5 mg Fe/L), and (d) high (12.5 mg Fe/L) treatment. Each 2-DE gel (pH 4-7) was loaded with 200 μ g protein and stained by Coomossie Blue.

In contrast, immune defense proteins showed an overall decrease (≥ 2 -fold) at low abomasal infusion treatment for both cow A and cow C compared to the control. Dairy cows have a moderate but consistent increase of the genes coding for lactoperoxidase and lactoferrin through lactation (Bionaz et al., 2012). The up-regulated expression of these two typical immune proteins indicated an increase in the innate immune response of the mammary gland during lactation (Loor et al., 2011). Therefore, a constant increase in lactoferrin production may have functioned to bind ferrous ions in abomasally-infused dairy cows, which worked together with lactoperoxidase (antioxidant) to fight against the iron-induced oxidative stress in the cow. As a consequence, the expression of lactoperoxidase and lactoferrin in the synthesized milk might be decreased for cows with greater susceptibility to oxidative stress as these proteins are utilized for protection. It is possible that, due to the self-repairing systems/self-antioxidant defense of dairy cows, the immune system could be triggered and produced higher amount of defense milk proteins in correspondence with increasing oxidative stress. The ability of a more robust self-repairing systems/self-antioxidant defense in dairy cows also might explain the relatively stable concentration of immune proteins in cow B and cow D during abomasal infusion of ferrous lactate.

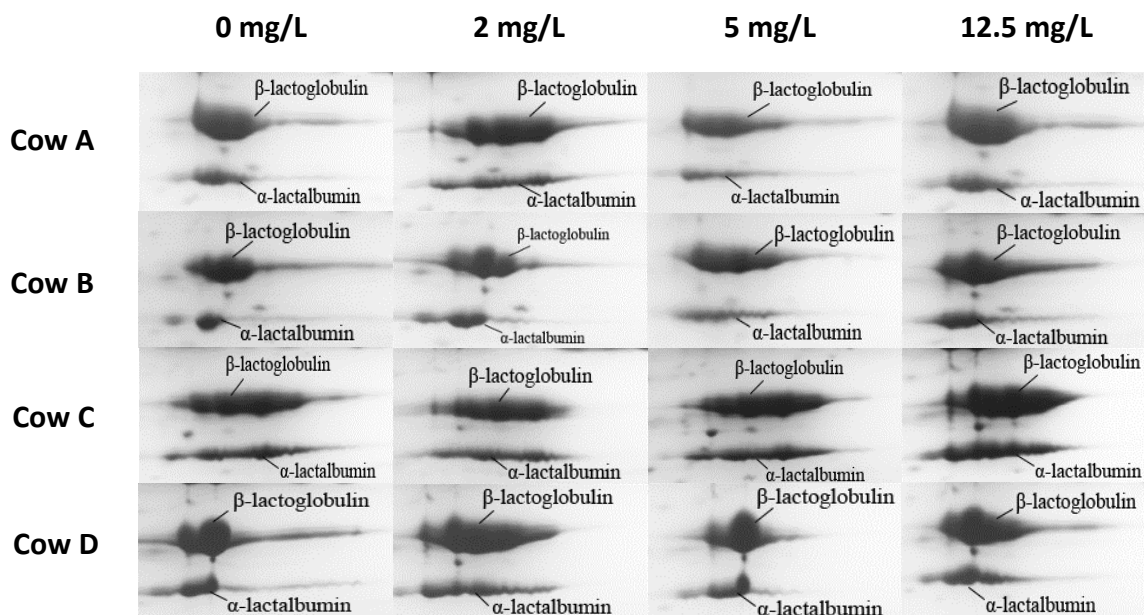


Figure 5. Comparison of changes in protein spots in are II of Fig.3 during (a) control (0 mg Fe/L), (b) low (2 mg Fe/L), (c) medium (5 mg Fe/L), and (d) high (12.5 mg Fe/L) treatment. Each 2-DE gel (pH 4-7) was loaded with 200 μ g protein and stained by Coomossie Blue.

As shown in Fig.5, post-translational modification of α -lactalbumin and β -lactoglobulin was both found in cow A and cow C at low abomasal infusion treatment, and in cow B at medium abomasal infusion treatment. Cow C seemed to experience a post-translational modification of α -lactalbumin and β -lactoglobulin under all abomasal infusion treatments. It was reported that some types of post-translational modification are due to oxidative stress (Grimsrud et al., 2008). Although the mechanism of post-translational modification of α -lactalbumin and β -lactoglobulin caused by oxidative stress are not well studied yet, post-translational modification of proteins was reported to affect the activity/function of most eukaryote proteins (Mann and Jensen, 2003). Therefore, post-translational modification and degradation of whey proteins might reduce nutritional value in bovine milk and the related dairy products.

4.3.2 Effect of Direct Addition of Ferrous Sulfate on Protein Composition of Commercial Retail Milk

To compare with milk proteome induced by abomasal infusion of ferrous lactate, changes of protein composition caused by direct (*in vitro*) addition of ferrous sulfate was analyzed on commercial retail milk. Total protein concentration in the commercial retail milk was 3.51% \pm 0.09, which fell within normal range (2.97%-3.74%) as reported by Lund \acute{e} n et al. (1997). Identified protein spots were listed in Table 9 and marked on each 2-DE image with consistent spot ID number.

For milk with direct addition of ferrous sulfate, a large number of protein spots were found differentially expressed associated with the increasing concentrations of ferrous sulfate (Fig.3; bottom row). Compared with control group, low iron treatment (2 mg Fe/L) induced a decrease (≥ 2 -fold) in α_{S1} -casein (spots 1, 2, 3, 5, 52, 59), α_{S2} -casein precursor (spot 6, 8, 9), β -casein (spot 24), and β -lactoglobulin (spot 39), but an increase (≥ 2 -fold) in α_{S1} -casein (spot 4, 19) and β -casein (spot 48). Medium iron treatment reduced the intensity (≥ 2 -fold) of α_{S1} -casein (spot 3, 5, 37, 43, 52), α_{S2} -casein (spot 8), and β -casein (spot 24, 31), but enhanced (≥ 2 -fold) α_{S1} -casein (spot 4, 19), and β -casein (spot 48). High iron treatment decreased (≥ 2 -fold) expression of α_{S1} -casein (spot 2, 3, 5) and α_{S2} -casein precursor (spot 7, 8), while increasing (≥ 2 -fold) the expression of α_{S1} -casein (spot 4, 10, 19, 41), β -casein (spot 48), κ -casein (spot 28, 29, 30) and β -lactoglobulin (spot 20).

Compared with whey proteins, a large number of casein spots were decreased (≥ 2 -fold) in the intensity in commercial retail milk with direct addition of ferrous sulfate. Degree of protein degradation in processed fluid milk varies with multiple factors, such as number of heat-stable proteinase (Dogan and Boor, 2003), storage time and somatic cell counts (SCC) in the raw milk

(Barbano et al., 2006). Proteolysis may serve as the primary pathway for the large degree of protein degradation induced by the addition of ferrous ions. As the response to iron-induced oxidative stress, heat-stable proteolytic enzymes were considered to be the primary enzyme for protein hydrolysis in UHT-processed commercial retail milk (Grufferty and Fox, 1988; Fairbairn and Law, 1986). These proteinases can be native milk alkaline proteinase (e.g. plasmin) (Grufferty and Fox, 1988) and extracellular bacterial proteinases produced by microorganisms (e.g. psychrotrophic) growing in raw milk before processing (Fairbairn and Law, 1986). For example, plasmin is able to rapidly cleave β -casein into the smaller β -casein and other polypeptide fragments, and milk proteins most susceptible to plasmin activity were β -casein, α_{s2} -casein and α_{s1} -casein (Eigel et al., 1979; Bars and Gripon, 1989; McSweeney et al., 1993).

In addition, when reaching optimum degree of oxidative damage of one protein, proteolytic activity would be decreased along with the increase of oxidation level (Shanely et al., 2002). It was found that heavily oxidized proteins are extensively cross-linked and aggregated, and unable to be degraded by proteinases (Pacifci et al., 1993, Giulivi et al., 1994). This statement was further demonstrated by our study; more protein spots were observed in proteolysis in low iron treatment than high iron treatment. For example, spots 1, 59 (α_{s1} -casein), α_{s2} -casein precursor (spot 6, 9), and spots 39 (β -lactoglobulin) were only found degraded (decreased in intensity) in lower iron treatments.

Proteolysis of milk often results in bitter and astringent off-flavors (Harwalkar et al., 1993). Degradation of amino acids in proteolytic milk would further produce putrid flavors (Shipe et al., 1978). The production of off-flavor in proteolytic milk might explain the sensory differences observed in processed milk in our previous study (Mann et al., 2013). Besides, proteolysis of milk proteins reduced flavor and nutritional value in the related dairy products, such as infant

formula, dietetics, and other functional foods (de Wit, 1998). Since concentrations of α_{s1} -, β -, κ -casein and β -lactoglobulin, and the proportion of κ -casein to total casein were found to be significant for the cheese yield (Wedholm et al., 2006), the degradation of α_{s1} -, β -, κ -casein and β -lactoglobulin found in milk with direct addition of ferrous lactate might affect the quality of cheese product if used in cheese industry. In addition, casein hydrates in proteolysis milk were degraded to either lower molecular weight peptides or protein fragments with altered isoelectronic points, which further reduced cheese yield that based on the PI value (\sim pH 4.6) of casein.

4.3.3 Effect of Abomasal Infusion of Ferrous Lactate and Direct Addition of Ferrous Sulfate on Milk Oxidative Stability

Oxidative stability of milk with direct addition of ferrous lactate was evaluated by the thiobarbituric acid reactive substances (TBARS) assay, which estimates volatile aldehydes. Oxidation level is positively related to TBARS value. As shown in Table 10, there was no significant difference ($p > 0.05$) in aldehyde concentration between control (0.26 ± 0.02 mg/kg) and low iron treatment (0.27 ± 0.01 mg/kg) on commercially processed milk after one day storage (light-protected, 4°C). However, TBARS value was significantly increased ($p < 0.05$) in medium iron treatment (0.31 ± 0.01 mg/kg) and kept rising significantly ($p < 0.05$) in high iron treatment (0.40 ± 0.02 mg/kg). After 8 days of storage, in general aldehyde concentration of milk, as estimated by TBARS, in all treatments was higher than that of first day of ferrous sulfate addition. Medium and high iron treatment still lead to significant higher ($p < 0.05$) oxidation level than milk without Fe addition, but there were no significant differences in TBARS value between low and medium iron treatment. This result indicated that oxidation occurred in iron-added milk during storage at 4°C even with light protection.

Table 10. TBARS values, expressed as malondialdehyde concentration (mg/kg; mean \pm SE), as indication of oxidation on commercial retail milk with direct addition of ferrous sulfate¹

Treatment ²	Fresh Milk (mg/kg)	Day 1 of storage (mg/kg)	Day 8 of storage (mg/kg)
		$\bar{X} \pm SE^3$	$\bar{X} \pm SE$
Control	0.22 \pm 0.02	0.26 \pm 0.02 ^a	0.27 \pm 0.02 ^a
Low		0.27 \pm 0.01 ^a	0.29 \pm 0.01 ^{ab}
Medium		0.31 \pm 0.01 ^b	0.33 \pm 0.02 ^b
High		0.40 \pm 0.02 ^c	0.44 \pm 0.01 ^c

¹Thiobarbituric Acid Reactive Substances (TBARS), expressed as malondialdehyde concentration.

²Concentrations of iron treatments in milk with direct iron addition were: control (0 mg Fe/L), low (2 mg Fe/L), medium (5 mg Fe/L), and high (12.5 mg Fe/L).

^{a,b,c} Means within each column with different superscripts are different ($p < 0.05$).

Oxidation of commercial retail milk in this study was primarily caused by the addition of ferrous sulfate, since all the milk samples were covered by foil for light protection during 8 days of storage. Oxidation can detrimentally impact the nutritional value and flavor of milk. Since total aldehydes, including malondialdehyde (MDA), are secondary oxidative products from milk oxidation and primarily contribute to oxidation induced off-flavors (Alvarez, 2009), a much more reliable method to identify oxidized milk was sensory evaluation. In our previous work (Mann et al., 2013), it was suggested that 3 mg/L ferrous sulfate solution resulted in slightly oxidized milk that showed sensory differences when comparing with fresh milk. This result suggested that even slightly oxidized milk produced off-flavor in milk and was able to be distinguished from non-oxidized milk in sensory.

In this study, oxidation in milk caused by ferrous ions showed an increase after 8 days of storage, which demonstrated ferrous ions present in fluid milk in early shelf-life can contribute to decreasing quality and flavor during storage. Although metal-binding proteins in milk including lactoferrin, casein, caseinophosphopeptides (CPP) and casein hydrolysates can work as metal chelators and effectively bind with iron (Bouhallab and Bouglé, 2004), their amounts in milk may be too low to bind with all the added ferrous ions available under the conditions of our

treatments. It was found that most chelators took effect as prooxidant once their concentrations are lower than that of iron (Mahoney and Graf, 1986; D áz and Decker, 2004).

Oxidative stability of milk from abomasal iron-infused cows was analyzed in our previous work (Mann et al., 2013). Overall TBARS value of milk was not significantly changed along with the increase of iron infusion, although milk from 3 out of 4 cows showed a general increase in aldehydes as estimated by TBARS. After storage for one week, TBARS value in milk still did not show any significant changes. Although TBARS analyses did not show any oxidation based on estimate of aldehydes, sensory tests indicated oxidative off-flavor occurred in milk with abomasal infusion of ferrous lactate (Mann et al., 2013).

4.3.4 Effect of Abomasal Infusion of Ferrous Lactate and Direct Addition of Ferrous Sulfate on Mineral Contents in Milk

Minerals that were listed in the standard reference for milk in National Nutrient Database (USDA, 2016) were quantified by ICP-MS in this work, including sodium, magnesium, calcium, potassium, copper, iron, zinc and inorganic phosphorus. As expected, iron concentration in milk significantly increased ($p < 0.05$) according to the increase of ferrous sulfate addition (Appendix C). However, all the other minerals did not show any obvious changes during this process, which indicated that other minerals in milk did not interact with the increasing iron. Since Fe concentration in regular processed milk ranged from 0.17 to 1.45 mg/L (Murthy et al., 1972), iron addition in low treatment (2 mg Fe/L) resulted in excessive Fe compared to regular commercial milk.

Mineral content in milk with abomasal infusion of ferrous lactate were reported in our previous work (Mann et al., 2013). Concentrations of Fe, Cu, P and Ca in milk did not present significant differences ($p > 0.05$) according to the increase of iron intake (935 ± 26 mg/kg Ca,

0.040 ±0.001 mg/kg Cu, 0.220 ±0.010 mg/kg Fe, 847 ±27 mg/kg P). The low variation of these minerals may be due to the iron-binding proteins that chelated extra iron or the self-repairing system of cows that inhibited oxidation. The infusion was of much shorter duration (14 days vs 45 days for iron treatment) and less dietary Fe supplementation (705 mg of Fe/kg of DM vs 1,000 mg of Fe/kg of DM) than Standish's study (Standish et al., 1971). The overall Fe and Ca contents of milk with abomasal infusion of ferrous lactate were lower than commercial retail milk. This can be explained by the difference from different breed of cows or the influence of high iron intake on cows. The Ca:P ratio was around 5:3 in milk with direct addition of ferrous sulfate in this work and around 3:3 in milk with abomasal infusion of ferrous sulfate (Mann et al., 2013). The Ca:P molar ratio (~4:3) is one of the most important factors in cheese making, and calcium phosphate plays an integral part in curd formation and stabilization (Mekmene et al., 2009). Either high or low Ca:P ratio in milk might decrease the quality and yield of final cheese products.

4.4 Conclusions

Both abomasal infusion of ferrous lactate and direct addition of ferrous sulfate as low as 2 mg Fe/L influence the milk proteome. Abomasal infusion of ferrous lactate affects both caseins and whey proteins in milk of lactating dairy cows, although the influence was varied among individual farm cows along with different concentrations of infused iron. Direct addition of ferrous sulfate mainly affects the caseins in purchased processed milk. Abomasal infusion of ferrous lactate did not significantly affect oxidation state in milk of individual farm cows. In contrast, iron-induced oxidation occurred in milk with direct addition of ferrous sulfate during storage at 4°C. Neither abomasal infusion nor direct addition of iron significantly influence ($p < 0.05$) the mineral composition in milk, except for increasing levels of Fe. A small amount of

iron contamination in bovine drinking water or milk processing plant can cause changes in milk protein composition and oxidation in final milk product, which decreases the quality, sensory profile, and nutritional value in milk and the related dairy products.

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Chapter 5

Effect of Lactoferrin on Metallic Flavor Induced By Chemotherapy and By Ferrous Sulfate Solution

Abstract Cancer patients receiving chemotherapy often experience taste alterations, commonly described as metallic flavor, which have a negative impact on food intake and nutrition condition. However, the frequent taste disturbance symptom in cancer patients was under-recognized and currently there was no effective treatment for this problem. In this study, we demonstrated the effect of lactoferrin supplementation on reducing taste disturbance symptom and investigated the related mechanism by detecting changes of constituents in saliva. Sixteen cancer patients with self-reported taste disturbance since chemotherapy and ten healthy subjects were recruited in this study. Both cancer and healthy participants took lactoferrin supplements 3 tablets per day (250 mg/tablet) for 30 days. Saliva samples were collected at baseline, 30 days after lactoferrin supplements daily intake, and 30 days after lactoferrin supplementation ended. To compare with chemotherapy induced metallic taste disturbance, ferrous sulfate solution (1 mg Fe/L) was used to generate metallic flavor in sensory level for both cancer patients and healthy subjects. Metallic taste intensity was evaluated by all participants at each sample collection. Taste and smell function during lactoferrin supplementation of cancer patients were assessed by a self-perceived taste and smell abnormality questionnaire. Salivary proteome and minerals were analyzed at each time point of lactoferrin supplementation. Our study found that lactoferrin supplementation significantly decreased ($p < 0.05$) total abnormality score in cancer patients, and this effect lasted at least 30 days after the treatment was ended. Although lactoferrin supplementation did not effectively reduce ($p > 0.05$) the metallic taste intensity stimulated by ferrous sulfate solution (1 mg Fe/L), it significantly ($p < 0.05$) decreased the salivary Fe in oral for both healthy subjects and

cancer patients. The production of metallic taste perception both induced by chemotherapy and ferrous sulfate solution might be associated with the decreased expression of low-abundance proteins (pH 5.5-8.5, MW 25-75kDa), which were mainly immune proteins in saliva. Lactoferrin supplementation may improve metallic flavor perception through recovering low-abundance salivary proteins, decreasing the detection threshold of ferrous ions, and binding iron ions to reduce iron-induced oxidative stress.

5.1 Introduction

Most cancer patients receive chemotherapy as treatment during the course of their disease. A secondary effect of the condition and/or treatment is taste and odor disturbance, experienced by a large proportion of this population (Kokal, 1985; Capra et al, 2001; Johnson, 2001, Bernhardson et al., 2008; Zabernigg et al., 2010). Symptoms of taste and odor disturbance in cancer patients after chemotherapy include abnormality in taste acuity (McDaniel and Rhodes, 1998; Johnson, 2001; Epstein et al., 2002), disorder in olfactory perception (McDaniel and Rhodes, 1998), food aversions (Kokal, 1985; Mattes et al., 1987; Capra et al., 2001), and dry mouth syndrome (xerostomia) (Berk et al., 2005). As a consequence of taste and odor disturbance, many cancer patients suffer poor appetite, weight loss, decreased energy and diminished nutrition condition, which are detrimental to clinical rehabilitation (Bernhardson et al., 2007; Hutton et al., 2007; Sánchez-Lara et al., 2010; Zabernigg et al., 2010).

The prevailing disturbance symptom grieving cancer patients is the perception of a persistent metallic flavor and/or aftertaste with or without food intake (Wickham et al., 1999; Boltong et al., 2012). Metallic flavor is an infused sensation of retronasal metallic odor with weaker bitter, salty and astringent taste perceptions (Ömür-Özbek and Dietrich, 2011). In food sensory evaluation, ferrous sulfate (FeSO_4 , food-grade) is proposed as a reference standard for generating metallic

flavor (Civille and Lyon, 1996; Mirlohi et al., 2011). A low level of chemotherapy can stimulate metallic taste (McDaniel and Rhodes, 1998; Capra et al., 2001; Comeau et al., 2001). Metallic taste has been experienced in 16% of lung cancer patients (n=352) and 32.2% of patients (n=196) with colorectal, breast, head and neck, stomach, lung, and other cancers (Newell et al, 1998). In a study among patients with various cancer types, 29 of 37 (78%) patients described their perceived taste change as metallic after at least two cycles of various chemotherapy treatments (Rehwaldt et al., 2009). Metallic flavor commonly occurs during the chemotherapy, and can last several hours, several weeks, or even several months after the completion of the treatments (Rhodes et al., 1994; Bernhardon et al., 2007; Steinbach et al., 2009).

Chemotherapy may cause metallic flavor symptom by destroying taste and olfactory receptor cells (McDaniel and Rhodes, 1998; Capra et al., 2001; Comeau et al., 2001).

Chemotherapy may cause metallic sensation simply by the bitter/metallic taste of chemotherapeutic drugs (Epstein et al., 2002), or by producing carbonyls through lipid oxidation between metal-containing compounds (such as food and medicine) and oral epithelial cells (Lawless et al., 2004). The turnover rate of healthy human taste bud cells is 10 days whereas the life span of olfactory receptor cells is about 1 week (McEntire and Pixley, 2000; Nahikian-Nelms et al, 1990). However, most cancer patients recover their normal taste acuity within one-half to one year after treatment (Sandow, 2006; Yamashita et al, 2006 a and b).

Although the metallic taste disturbance is widespread and is a frequent complaint of cancer patients, there are no established therapies that are reliable to prevent or treat this problem. Oral glutamine has been reported to significantly reduce the severity of sensory and motor neuropathy in 50 breast cancer patients treated with high-dose paclitaxel (Vahdat et al., 2001). However, for 52 cancer patients who were receiving taxane-containing chemotherapy, neither glutamine

(30g/day in 2-3 doses) nor placebo (maltodextrin) decreased subjective taste disturbances or altered taste perception associated with taxane-containing chemotherapy (Strasser et al., 2008). Currently, treatments that may temporarily reduce metallic taste perception are recommended to cancer patients, with common suggestions including using plastic utensils instead of metallic silverware (Hong et al., 2009), eating cold food (Rehwaldt et al., 2009), adding strong herbs and spices (Boltong et al., 2012), and using sweeteners or other taste enhancers. A dietary supplement based on *Synsepalum dulcificum*, which is known as “miracle fruit”, has been demonstrated to temporarily mask the metallic taste caused by chemotherapy (Wilken and Satiroff, 2012). After chewing the miracle fruit supplement for 5min, five of eight patients reported the metallic taste disappeared for 20-30 min. All 8 patients reported the taste of food was improved during the use of the supplement and their food intake was increased. However, long-term effective treatment methods are still urgently needed to improve the prolonged and recurring metallic taste disturbance of cancer patients.

To relieve metallic flavor symptom, iron-binding proteins that can effectively reduce iron-induced metallic flavor are garnering attention. Lactoferrin is widely known as an immune protein and naturally found in mucosal secretions including tears, saliva, seminal fluid, vaginal secretions, breast milk and intestinal mucus in human (Levay and Viljoen, 1995). Acting as an iron-binding protein, lactoferrin reduces metal-induced oxidative stress as a chelating antioxidant (Mulder et al., 2008; Burrow et al., 2011). In addition, lactoferrin is a key protein acting during the first step of inflammatory response against infection and severe inflammation in the human body (Nuijens et al., 1996; Baveye et al., 1999). Due to its anti-inflammatory and antioxidant functions, lactoferrin is involved in a wide range of biological activities such as prevention of cell injury and tissue damage, antimicrobial defense, immune modulation, cellular growth and

differentiation, and cancer prevention (Bellamy et al, 1992; Wright and Gallin, 1979; Varadhachary et al, 2004; Spadaro et al, 2007).

Recently, lactoferrin was found to effectively decrease metallic flavor stimulated by ferrous sulfate solution in healthy subjects. In our previous study (Ömur-Özbek et al., 2012), a post-rinse of the oral cavity with a lactoferrin solution (10.4 mg/L) after ferrous iron (1mg Fe/L), completely removed the metallic flavor for all 19 participants (10 females, age 19-53 years). Clinical activity of talactoferrin (3g/day for 12 weeks) in other studies was well tolerated without any significant toxicity (Jonasch et al., 2008).

Human saliva is an attractive early detection biofluid, since it contains a large array of immune salivary proteins that are informative of the host defense system. Salivary proteome analysis is a well-established strategy for the discovery of biomarker for human diseases, such as lung cancer (Xiao et al., 2012), oral cancer (Jou et al., 2010), Sjögren's syndrome (Hu et al., 2007), and type 2 diabetes (Wu et al., 2009). Moreover, proteomics can also reveal the changes of specific salivary proteins that may indicate the organism activities such as mineralization of dental hard tissues, oral mucosa protection, and interactions with oral microorganisms (Mandel, 1987; Scannapieco, 1994; Tabak, 1995). However, few studies have investigated the relationship between salivary protein composition and taste perception. Lorenz et al. (2011) reported that the stimulation of 6-gingerol (pungent) significantly decreased abundance of several salivary proteins. Nevertheless, salivary proteins that may serve as indicators for metallic taste and act as defense proteins to inhibit taste disturbance have not been studied yet.

In this study, effects of lactoferrin supplements on reducing metallic taste disturbance of cancer patients and metallic flavor perception stimulated by ferrous sulfate solution were investigated. As comparison, lactoferrin supplements intake was also performed on healthy

subjects. Salivary proteome analysis conducted by 2-DE was used to detect the relationship between salivary proteins and metallic taste perception. In addition, concentration of salivary minerals, metallic taste intensity, and self-reported taste abnormality along with lactoferrin supplement treatment were also recorded and compared. We hypothesize that lactoferrin could be clinically used to alleviate taste disturbances in cancer patients.

5.2 Materials and Methods

Human Subjects Recruitment, enrollment and management of cancer patients in this study was completed at the Comprehensive Cancer Center of Wake Forest University. This study was approved by the Institutional Review Board at the Medicine School of Wake-Forest (cancer patients recruitment and data collection; CCCWFU 98112) and Virginia Tech (healthy subjects recruitment, data collection and data analysis; VT IRB 14-880, Appendix D). Sixteen cancer patients (seven females, ages 33-76 years with median age of 63) with self-reported taste disturbance were recruited for this study. The self-reported taste disturbances developed since the initiation of oxaliplatin-based therapy or a pre-existing, treatment-induced taste disturbance that subjectively worsened since initiating oxaliplatin-based therapy from Comprehensive Cancer Center of Wake Forest University. A wide variety of cancer types were included in this study, including rectal carcinoma (2), colon carcinoma (6), pancreas (4), breast (5), brain tumor (3), mycosis fungoides (1), lymphoma (2), myeloma (1), and other (1). Demographic information of cancer patients included age, sex, height, weight, diagnosis (types of cancer), disease stage, chemotherapy history prior to enrollment including agents, doses, schedules, tobacco history (never, current, ever smoker, and amounts if actively smoking), and concurrent medications were recorded by Wake Forest Medical School (Appendix G). Eligible cancer patients in this study had histologically or cytologically confirmed cancers with expected life span of at least 3 months,

normal taste perception before the development of cancer, and were currently undergoing chemotherapy following the recruitment of this trial. Any dose or schedule of chemotherapy reagents administration was allowed as long as patients had self-reported taste disturbance. Exclusion criteria included difficulty in producing abundant saliva according to the subjective history of extreme dry mouth syndrome, HIV-Positive test result, pregnant or breastfeeding, untreated gastrointestinal reflux disease, untreated diabetes mellitus, active thrush, active oral infection, or active mucositis.

Additionally, 11 healthy subjects (seven females) with age ranging 45-71 years (median age of 59) were recruited from the local community including New River Valley region, and students, faculty and staff of Virginia Tech. Demographic information collected for healthy subjects included self-reported age, sex, height, weight, tobacco history (never, current, ever smoker and amounts if actively smoking), milk and iron allergy conditions, dental health history, and concurrent medications/supplements. All enrolled healthy participants had no chronic oral or general health problems, no milk allergy, were nonsmokers, and not pregnant. Data was recorded by the Virginia Tech researchers (Appendix H).

Lactoferrin Treatment Both cancer patients and healthy subjects were provided with lactoferrin supplement tablets, with directions to take one tablet three times a day for 30 days. Lactoferrin tablets (250mg/tablet) used in this study were purchased from Jarrow Formulas Inc. (Los Angeles, California). A GRAS notification released on January 1, 2012 states that Bioferrin 2000 was processed with Good Manufacturing Practices as per the Federal Code of Regulations by Glanbia Foods, Inc. for the intended conditions of use.

Salivary Collection Saliva samples from cancer patients and healthy subjects were collected at baseline (before starting lactoferrin treatment), after 30 days with daily intake of lactoferrin

tablets (30-day lactoferrin treatment), and 30 days after stopping intake of lactoferrin tablets (30-day post lactoferrin treatment). All participants were required not to consume any food or beverage (except water) and not to smoke at least one hour prior to saliva collection. Before collecting saliva samples, participants were instructed to rinse their mouth using purified drinking water (Kroger®). After a 1-minute rest, they sipped 2 mL of purified drinking water as the control sample, swished it around their mouth for 15-20 sec. Without swallowing, participants expectorated saliva into a clean sample collection tube until approximately 4 mL of saliva was collected. After a short rest period (2-3 minutes), subjects sipped 2 mL of ferrous sulfate solution (1mg Fe/L) prepared by food-grade $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Spectrum, New Brunswick, NJ) and swished around their mouth. Without swallowing, they expectorated their saliva into a clean sample collection tube until approximately 4 mL sample was collected. Subject's oral pH was measured using a pH indicator strip (Cen-med/Fisher M95883) before and after each sample collection. Saliva samples were kept at -80°C prior to assay.

Chemosensory Assessment Chemosensory assessment in this study including taste and smell abnormalities (cancer group) and pre- and/or post-ferrous taste intensity (cancer and healthy group) was conducted at baseline, 30-day lactoferrin treatment, and 30-day post lactoferrin treatment.

Smell and Taste Questionnaire. Taste and smell function of cancer patients were assessed by a self-perceived taste and smell abnormality questionnaire as described in our previous study (Mirlohi et al., 2015). In the taste section of the questionnaire, cancer participants were initially asked to rate their individual taste abnormalities as “insignificant,” “mild,” “moderate,” “severe,” or “incapacitating” from five aspects as shown in Appendix E. Then, they would answer “yes” or “no” on four additional questions about their taste abnormality when experiencing salt, sweet,

sour, and bitter. This tool yields a taste complaint score (0-10) on the basis of subject responses to nine questions addressing changes to the sense of taste. One point was added for each reported taste complaint and additional one point would be added if a rating of “severe” or “incapacitating” was reported on the severity of the taste abnormality question. Similarly, a smell complaint score (0-6) was generated by adding one point for a taste abnormal response to each of five questions addressing self-perceived changes to the sense of smell. An additional one point would be assigned to a severity rating of “severe” or “incapacitating” for smell abnormality questions. The total chemosensory complaint score (0-16) was calculated by adding the taste and smell complaint scores.

Pre- and/or Post-ferrous Taste Intensity. Metallic taste intensity induced by weak ferrous solution was conducted on both cancer patients and healthy people along with the saliva sample collection (Appendix F). As described above, all participants sipped 2 mL of ferrous sulfate solution (1mg Fe/L) and swished around in their mouth for 15-20 sec. Without swallowing, they expectorated their saliva into a clean sample collection tube and then immediately rated the perceived metallic flavor intensity as “no perception”, “weak intensity”, “moderate intensity”, “strong intensity”, or “very strong/unbearable intensity”. Correspondingly, a five-point (0-5) scale was applied to score the metallic intensity, in which 0 means “no perception” and 5 means “very strong/unbearable intensity”. In addition, participants were required to describe the perceived flavor.

Salivary Minerals Analysis Saliva samples were first thawed on ice for about 2h. Then, 500 μ L of each saliva sample was diluted with 4.25 mL deionized water followed by digesting with 250 μ L trace metals grade nitric acid (TraceMetal™ Grade, Fisher, St. Louis, MO) at room temperature (Menegário et al., 2001; Watanabe et al., 2005), which resulted in a final dilution

ratio at 1:10 (v/v). Reagent blank was prepared by adding 250 μ L nitric acid into 4.75 mL deionized water. Concentration of salivary minerals including iron, magnesium, potassium, copper, zinc, sodium, calcium, phosphorus, sulphur, and chlorine of each diluted saliva sample were measured by emission spectroscopy using Inductively Coupled Plasma (ICP) technique (Thermo Electronic Corporation, X-Series ICP-MS, Waltham, MA).

Salivary Proteome Analysis Salivary proteome of both cancer patients and healthy subjects were analyzed by 2-DE technology (Hu et al., 2005; Jou et al., 2010; Goncalves et al., 2010; Xiao et al., 2012). Frozen saliva samples were thawed on ice for about 2 hours. Thawed samples were then mixed thoroughly by vortex mixer followed by centrifugation at 18500 \times g (4 $^{\circ}$ C) for 15 minutes to reduce viscosity and remove debris.

Proteins in each saliva sample were precipitated by adding a solution containing 10% trichloroacetic acid (TCA)/90% acetone/20mM dithiothreitol (DTT) (Hu, 2005) at twice the volume of saliva sample, and chilled overnight at -20 $^{\circ}$ C. The next day, the samples were placed in a chilled centrifuge (18500 \times g, 4 $^{\circ}$ C) for 15 min to pellet the protein. The pellet was washed a second time using a 20mM DTT/acetone wash and placed in the chilled centrifuge (18500 \times g, 4 $^{\circ}$ C) for 15 min to pellet the protein once again.

The protein pellet was then resuspended in the 2D cell lysis buffer containing 9 M urea, 2% CHAPS(w/v), 50 mM DTT, 0.5% IPG buffer (v/v), and 0.01% bromophenol blue. Saliva protein concentration was determined by 2D-Quant Kit (GE Healthcare, Pittsburgh, PA) following the protocol given in the brochure and using BSA as a standard. Each saliva sample was loaded (20 μ g) on a 11cm immobilized pH gradient strip (pH 3-11NL, GE Healthcare, Pittsburgh, PA), and then was carried out by GE Healthcare Ettan IPGphor 3 Cell (GE Healthcare, Pittsburgh, PA) using the following voltage program: (1) rehydrated for 15 hours; (2) held at 500V for 1 h; (3)

from 500 V to 1000 V over 1h; (4) from 1000 V to 6000 V over 2 h; (5) held at 6000 V for 7 min; (5) from 6000 V to 500V for 1 h.

Sample strips were then equilibrated with a two-step process: (1) rinsed with equilibration buffer containing 6 M urea, 4% SDS (w/v), 0.375 M Tris-HCl (pH 8.8), 20% glycerol (v/v), and 130 mM DTT for 15 min, (2) rinsed with equilibration buffer containing 6 M urea, 4% SDS (w/v), 0.375 M Tris-HCl (pH 8.8), 20% glycerol (v/v), and 130 mM iodoacetic acid (IAA) for 15 min. Then, sample strips were transferred into 11cm Criterion Precast 12.5% Polyacrylamide Gels (BIO-RAD, Hercules, CA). The gels were initially run at 35 mA for 15min and then at 70 mA until the dye front ran out of the gels. Gels were stained using Flamingo™ Fluorescent Gel Stain (BIO-RAD, Hercules, CA) following the manufacturer's instructions. For better visualization of low-abundance proteins, gels from one representative healthy subject were stained by PlusOne Silver Staining Kit (BIO-RAD, Hercules, CA). Gel images were scanned by Molecular FX Imager (BIO-RAD, Hercules, CA).

In-gel Trypsin Digestion and Mass Spectrometry Identification Based on the repeatability and stability of salivary protein composition shown on 2-D image, saliva samples of 10 cancer patients and 10 healthy subjects were selected for further analysis.

For matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometry analysis, selected spots were excised from 2-DE gels by hand with spot picker. To remove staining dye and other inhibitory chemicals, protein pellets were washed multiple times using a mixture of 25 mM ammonium bicarbonate and HPLC-grade acetonitrile (1:1, v/v). After centrifuging at 13,000 ×g for 2 min, pellets were washed with 25 mM HPLC-grade acetonitrile for 15 min with constant shaking at room temperature. The pellets were then dried thoroughly by vacuum concentrator

(Kansas City, Missouri). Protein digestion was carried out by adding 0.065 μg of trypsin and incubated on ice for 15 min, then followed by incubation at 37 $^{\circ}\text{C}$ overnight. The next day, 1 μL of each digest was transferred to a freshly-polished MALDI plate and covered with freshly-prepared matrix containing 4 mg/mL α -cyano-4-hydroxycinnamic acid, 50% CH_3CN , 0.1% TFA (v/v), 0.1% formic acid (v/v), and 5 mM $(\text{NH}_4)\text{Cl}$. Protein identification was performed by an Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Framingham, MA), which based on peptide fingerprint mass mapping (using MS spectra) and peptide fragmentation mapping (using MS/MS spectra). The MASCOT search engine software (Matrix Science, Boston, MA) was used to identify proteins from the National Center Biotechnology Information nonredundant *Homo sapiens* amino acid sequence database. The parameters for searching and identifying matches were adjusted as follows: enzyme of trypsin, 1 missed cleavage, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (M), peptide mass tolerance: ± 0.5 Da, fragment mass tolerance: ± 0.5 Da, mass tolerance of 30 ppm, peptide charge of 1+ and monoisotopic (Xiao et al., 2012). MASCOT parameters were regulated as follows: parent ion mass tolerance of 20 ppm, fragment ion mass tolerance of 100 ppm.

Data Analysis Changes in salivary protein composition before and after ferrous sulfate stimulation, and between different lactoferrin treatment stages (baseline, 30-day lactoferrin treatment, 30-day post lactoferrin treatment) were analyzed by PDQuest software v.7.3.1 (Bio-Rad, Hercules, CA). Protein concentration (intensity) was determined as the percentage of total valid spots volume on respective gels. Protein spots that had at least 2-fold change in intensity were considered as differences among treatments in each replicated group. *P* value was calculated based on Wilcoxon test and $p < 0.05$ was used as cutoff for significance. For the salivary mineral changes before and after ferrous sulfate stimulation, paired t-test was performed

on the mean responses within each (healthy/cancer) group. Paired t-test was used to compare the salivary mineral concentrations before and after ferrous iron stimulation, and t-test analyses were confirmed with the Wilcoxon test due to the variation of minerals among human subjects. One-way analysis of variance (ANOVA) and comparison of the means using Tukey HSD, were performed on data of salivary minerals associated with different stages of lactoferrin treatment and of quantitative assessment of smell and taste changes in cancer patients during lactoferrin treatment. To assess the significance of the pre/post-ferrous taste intensity differences in cancer patients, paired t-test was used for the mean response within the cancer group. T-test and one-way ANOVA was performed by statistical software programs JMP 10.0. For all analyses, *p* values less than 0.05 were considered statistically significant.

5.3 Results

5.3.1 Chemosensory Assessment for Healthy Subjects and Cancer Patients

To investigate the effect of lactoferrin supplement on reducing metallic flavor caused by iron stimulation in mouth, ferrous sulfate solution (1mg Fe/L) was used as stimulus in this study. Reported individual threshold of ferrous (Fe^{2+}) ion ranged from 0.003 to 3 mg Fe/L (Mirlohi et al., 2011). In this study, all cancer patients could identify metallic flavor from ferrous sulfate solution (1 mg Fe/L), and all the healthy subjects were able to discriminate ferrous sulfate solution (0.3 mg Fe/L) from water in the pretest.

For both healthy subjects and cancer patients, lactoferrin treatment at each stage did not have a significant influence ($p>0.05$) on metallic flavor intensity induced by iron stimulation (Table 11). The treatment at each stage also did not show any significant influence ($p>0.05$) on water perception for all cancer patients (Table 11).

The taste intensity of metallic flavor stimulated by ferrous sulfate solution was evaluated as significantly higher ($p < 0.05$) than the intensity of water by all 16 cancer patients at each time interval as shown in Table 11. However, for the 10 cancer patients for whom proteome analysis was completed, no significant difference ($p > 0.05$) in metallic flavor intensity was detected between water and ferrous sulfate solution. This result might be explained by the lactoferrin treatment that reduced the metallic flavor perception on cancer patients, since the corresponding total abnormality score of these 10 patients also showed a significant decrease ($p < 0.05$) (Table 12).

Results of self-reported smell and taste abnormality for cancer patients were also summarized in Table 12. For all 16 cancer patients, 30-day post-lactoferrin treatment presented significantly lower ($p < 0.05$) taste, smell and total abnormality scores than stage I (baseline) and stage II (30-day lactoferrin treatment). The same post-lactoferrin treatment effect on improving total abnormality score was also found in the 10 selected cancer patients whose saliva received proteome analysis. However, there was no significant difference ($p > 0.05$) of taste and smell abnormality score between different treatment stages.

Table 11. Results of sensory test of metallic flavor in cancer patients¹ and healthy subjects²

Lactoferrin treatment periods³	Intensity of water-rinse⁴	Intensity of ferrous-rinse⁵
All cancer patients		
Baseline	1.3 ± 0.6 ^{aA}	1.9 ± 1.0 ^{aB}
30-day treatment	1.4 ± 0.6 ^{aA}	2.6 ± 0.9 ^{aB}
30-day post treatment	1.5 ± 0.8 ^{aA}	2.4 ± 1.3 ^{aB}
10 cancer patients with proteome analysis		
Baseline	1.3 ± 0.7 ^{aA}	2.0 ± 0.8 ^{aB}
30-day treatment	1.4 ± 0.7 ^{aA}	2.5 ± 1.1 ^{aB}
30-day post treatment	1.5 ± 0.7 ^{aA}	2.2 ± 1.2 ^{aA}
Healthy subjects		
Baseline	-	3.2 ± 0.6 ^a
30-day treatment	-	3.3 ± 0.6 ^a
30-day post treatment	-	2.8 ± 0.7 ^a

¹Test results include all 16 participated cancer patients and the 10 cancer patients whose saliva had proteomic analysis.

²Ten healthy subjects participated in sensory evaluation test on metallic flavor perception after ferrous sulfate rinsed in mouth.

³Baseline means before lactoferrin supplementation treatment, 30-day treatment means taking lactoferrin supplements (750 mg lactoferrin/day) for 30 days, and 30-day post treatment means 30 days after lactoferrin supplementation treatment.

^{4,5}Intensity score was based on a 0-5 scale as described in Methods. 0 means no perception and 5 means very strong/unbearable intensity. Intensity of water-rinse means the evaluation of taste intensity after a water rinse in mouth by each participant. Intensity of ferrous-rinse means the evaluation of metallic flavor intensity after a ferrous sulfate solution rinse in mouth by each participant.

^{a-b}In each column, different superscripts (a-b) indicates significant differences (p<0.05) among different treatments.

^{A-B}In each row, different superscripts (A-B) indicates significant differences (p<0.05) in taste intensity between water-rinse and ferrous-rinse.

Table 12. Results of smell and taste questionnaire in cancer patients¹

Lactoferrin treatment periods²	Taste abnormality score³	Smell abnormality score⁴	Total abnormality score⁵
All cancer patients			
Baseline	6.5 ± 1.5 ^a	3.1 ± 2.3 ^a	9.5 ± 2.5 ^a
30-day treatment	5.8 ± 1.7 ^{ab}	2.1 ± 1.9 ^{ab}	7.9 ± 2.6 ^{ab}
30-day post treatment	4.5 ± 2.7 ^b	1.2 ± 1.6 ^b	5.8 ± 3.5 ^b
10 cancer patients with proteome analysis			
Baseline	6.3 ± 1.6 ^a	2.6 ± 2.5 ^a	8.9 ± 2.5 ^a
30-day treatment	5.4 ± 1.4 ^a	1.8 ± 1.9 ^a	7.2 ± 2.1 ^{ab}
30-day post treatment	4.0 ± 2.9 ^a	1.1 ± 1.4 ^a	5.1 ± 3.5 ^b

¹Test results include all 16 participated cancer patients and the 10 cancer patients whose saliva was used to perform proteomic analysis.

²Baseline means before lactoferrin supplementation treatment, 30-day treatment means taking lactoferrin supplements (750 mg lactoferrin/day) for 30 days, and 30-day post treatment means 30 days after lactoferrin supplementation treatment.

^{3,4}Score was based on a 0-10 scale as described in Methods and obtained by self-evaluation of each cancer patient. 0 means insignificant taste/smell abnormality and 10 means severe taste/smell abnormality.

⁵Total score was the sum of taste abnormality score and smell abnormality score.

^{a-b}In each column, different superscripts (a-b) indicates significant differences (p<0.05) among different treatments.

5.3.2 Concentration of Salivary Minerals in Healthy Subjects and Cancer Patients

Salivary minerals of all participants before and after ferrous sulfate stimulation were compared at baseline, 30-day lactoferrin treatment, and 30-day post lactoferrin treatment, respectively (Table 13). Paired t-test was used to compare the salivary mineral concentrations before and after ferrous iron stimulation, and t-test analyses were confirmed with the Wilcoxon test due to the variation of minerals among human subjects. After data analysis, only Na at 30-day post treatment and Ca at 30-day treatment showed different results between t-test and Wilcoxon test: Wilcoxon showed significant differences while t-test did not show significant changes. All the data shown in Table 13 presented the results analyzed by Wilcoxon test.

For the healthy group, mineral concentration for 9 tested minerals (Na, Mg, P, S, Cl, K, Ca, Cu, Zn) did not show any significant differences (p>0.05) before and after ferrous sulfate

stimulation at baseline and 30-day post lactoferrin treatment; only Fe increased in concentration ($p < 0.05$) as expected with ferrous sulfate rinse at baseline (Table 13). However, during and after lactoferrin supplementation treatment, there was no significant difference ($p > 0.05$) in concentration of salivary Fe before and after iron stimulation. This result suggested that lactoferrin supplementation effectively reduced the oral concentration of salivary Fe when exposed to ferrous sulfate solution (1 mg Fe/L), and this effect even last until 30 days after the treatment. Concentrations of P, Cl, K, and Ca increased ($p < 0.05$) after the ferrous sulfate rinse at the end of the 30-day lactoferrin supplementation.

Table 13. Comparison of salivary minerals before and after iron stimulation at each stage of lactoferrin supplementation treatment in healthy subjects and cancer patients¹

Minerals	Minerals concentration (mg/L; mean ± standard deviation (95% CI))											
	Baseline				30-Day Treatment				30-Day Post Treatment			
	Water-rinse ²		Ferrous-rinse ³		Water-rinse		Ferrous-rinse		Water-rinse		Ferrous-rinse	
Healthy group												
Na	42.3	±31.1 ^a	38.8	±29.9 ^a	48.5	±30.9 ^a	47.7	±30.7 ^a	53.9	±42.2 ^a	45.3	±21.0 ^a
Mg	1.57	±0.680 ^a	1.36	±0.600 ^a	1.73	±0.704 ^a	1.62	±0.644 ^a	1.68	±0.57 ^a	1.49	±0.34 ^a
P	61.8	±28.5 ^a	61.3	±29.8 ^a	65.5	±25.6 ^a	73.2	±26.3 ^b	65.2	±25.4 ^a	69.6	±25.3 ^a
K	291	±122 ^a	286	±119 ^a	302	±79.1 ^a	333	±77.0 ^b	286	±84.4 ^a	308	±65.3 ^a
Ca	15.0	±7.53 ^a	13.9	±5.00 ^a	17.0	±4.83 ^a	18.2	±4.75 ^b	16.1	±5.74 ^a	16.7	±4.94 ^a
S	0.0597	±	0.0566	±0.0205 ^a	0.0454	±	0.0523	±	0.0497	±	0.0491	±
	0.0287 ^a				0.0132 ^a		0.0167 ^a		0.0225 ^a		0.0177 ^a	
Cl	0.209	±	0.197	±0.0885 ^a	0.206	±	0.227	±	0.218	±	0.218	±
	0.0885 ^a				0.0495 ^a		0.0422 ^b		0.0941 ^a		0.0458 ^a	
Fe	0.0816	±	0.147	±0.109 ^b	0.0299	±	0.101	±0.112 ^a	0.00975	±	0.0545	±
	0.0863 ^a				0.0567 ^a				0.00682 ^a		0.0297 ^a	
Cu	0.00670	±	0.0248	±0.0585 ^a	0.00682	±	0.00595	±	0.0180	±	0.00480	±
	0.00600 ^a				0.00745 ^a		0.00468 ^a		0.0481 ^a		0.00514 ^a	
Zn	0.0749	±0.140 ^a	0.0420	±0.0931 ^a	0.0602	±0.142 ^a	0.0738	±	0.0142	±	0.0221	±
							0.0839 ^a		0.00700 ^a		0.0392 ^a	
Cancer group												
Na	92.7	±49.2 ^a	57.3	±21.5 ^a	89.2	±54.9 ^a	78.6	±60.9 ^a	198	±199 ^a	57.5	±34.4 ^b
Mg	1.91	±1.42 ^a	0.976	±0.300 ^a	1.35	±0.32 ^a	0.85	±0.19 ^b	3.62	±2.20 ^a	1.09	±0.294 ^b
P	69.9	±58.0 ^a	33.0	±13.8 ^a	36.3	±13.3 ^a	27.8	±15.2 ^b	102	±71.3 ^a	27.9	±15.8 ^b
K	397	±200 ^a	243	±98.5 ^a	251	±92.6 ^a	201	±86.8 ^a	622	±367 ^a	208	±80.8 ^b
Ca	19.5	±13.5 ^a	10.3	±2.79 ^a	15.1	±7.27 ^a	10.8	±5.23 ^b	26.6	±11.9 ^a	11.3	±3.79 ^b
S	0.0790	±0.0666 ^a	0.0494	±0.0441 ^a	0.0332	±0.0243 ^a	0.0246	±0.0194 ^a	0.104	±0.0613 ^a	0.0369	±
											0.0274 ^b	
Cl	0.272	±0.155 ^a	0.144	±0.0637 ^a	0.212	±0.106 ^a	0.179	±0.0837 ^a	0.550	±0.383 ^a	0.160	±
											0.0730 ^b	
Fe	0.195	±0.0501 ^a	0.640	±0.470 ^b	0.0654	±0.0798 ^a	0.519	±0.658 ^a	0.204	±0.162 ^a	0.214	±0.179 ^a

Cu	0.113 ±0.0505 ^a	0.0104 ±0.00763 ^a	0.0107 ±0.0110 ^a	0.00916± 0.00566 ^a	0.0102± 0.00794 ^a	0.0253 ± 0.0182 ^a
Zn	1.38 ±2.11 ^a	0.554 ±0.959 ^a	1.01 ±1.92 ^a	0.622 ±1.00 ^a	0.410 ±0.602 ^a	0.432 ±0.860 ^a

¹Saliva samples were collected from 10 healthy subjects and 10 cancer patients whose saliva was performed proteomic analysis.

²Saliva samples were collected after each participant sipped 2 mL of water and swished around in mouth.

³Saliva samples were collected after each participant sipped 2 mL of ferrous sulfate solution (1mg Fe/L) and swished around in mouth.

^{a-b}In each row, different superscripts (a-b) indicates significant differences (p<0.05) between water-rinse and ferrous-rinse.

Table 14. Comparison of salivary minerals between cancer patients and healthy subjects¹

	Minerals concentration (mg/L: mean \pm standard deviation (95% CI))											
	Baseline		One Month				Two Month					
	Healthy group	Cancer group	Healthy group	Cancer group	Healthy group	Cancer group	Healthy group	Cancer group				
Na	42.3	$\pm 31.1^a$	92.7	$\pm 49.2^b$	48.5	$\pm 30.9^a$	89.2	$\pm 54.9^a$	53.9	$\pm 42.2^a$	198	$\pm 199^a$
Mg	1.57	$\pm 0.680^a$	1.91	$\pm 1.42^a$	1.73	$\pm 0.704^a$	1.35	$\pm 0.32^a$	1.68	$\pm 0.57^a$	3.62	$\pm 2.20^b$
P	61.8	$\pm 28.5^a$	69.9	$\pm 58.0^a$	65.5	$\pm 25.6^a$	36.3	$\pm 13.3^b$	65.2	$\pm 25.4^a$	102	$\pm 71.3^a$
K	291	$\pm 122^a$	397	$\pm 200^a$	302	$\pm 79.1^a$	251	$\pm 92.6^a$	286	$\pm 84.4^a$	622	$\pm 367^b$
Ca	15.0	$\pm 7.53^a$	19.5	$\pm 13.5^a$	17.0	$\pm 4.83^a$	15.1	$\pm 7.27^a$	16.1	$\pm 5.74^a$	26.6	$\pm 11.9^b$
S	0.0597	$\pm 0.0287^a$	0.0790	$\pm 0.0666^a$	0.0454	$\pm 0.0132^a$	0.0332	$\pm 0.0243^a$	0.0497	$\pm 0.0225^a$	0.104	$\pm 0.0613^b$
Cl	0.209	$\pm 0.0885^a$	0.272	$\pm 0.155^a$	0.206	$\pm 0.0495^a$	0.212	$\pm 0.106^a$	0.218	$\pm 0.0941^a$	0.550	$\pm 0.383^b$
Fe	0.0816	$\pm 0.0863^a$	0.195	$\pm 0.0501^b$	0.0299	$\pm 0.0567^a$	0.0654	$\pm 0.0798^a$	0.00975	$\pm 0.00682^a$	0.204	$\pm 0.162^a$
Cu	0.00670	$\pm 0.00600^a$	0.113	$\pm 0.0505^a$	0.00682	$\pm 0.00745^a$	0.0107	$\pm 0.0110^a$	0.0180	$\pm 0.0481^a$	0.0102	$\pm 0.00794^a$
Zn	0.0749	$\pm 0.140^a$	1.38	$\pm 2.11^a$	0.0602	$\pm 0.142^a$	1.01	$\pm 1.92^a$	0.0142	$\pm 0.00700^a$	0.410	$\pm 0.602^a$

¹Saliva samples were collected from 10 healthy subjects and 10 cancer patients whose saliva was performed proteomic analysis. samples were collected after each participant sipped 2 mL of water and swished around in mouth.

^{a-b}In each row, different superscripts (a-b) indicates significant differences ($p < 0.05$) between healthy subjects and cancer patients.

Similarly, salivary Fe concentration in cancer patients did not significantly increase ($p>0.05$) with the ferrous sulfate rinse (1mg Fe/L) during and after lactoferrin supplementation (Table 13). However, Mg and P in cancer group decreased ($p<0.05$) during lactoferrin treatment; this is in contrast to observation in the healthy group. In addition, 30-day post lactoferrin treatment further lead to an overall decrease ($p<0.05$) on salivary minerals including Mg, P, S, Cl, K, and Ca.

Salivary minerals between healthy subjects and cancer patients at each stage of lactoferrin treatment were also compared (Table 14). Relative to healthy subjects, cancer patients had significantly higher ($p<0.05$) salivary Na at baseline, significantly higher ($p<0.05$) salivary Na and P during lactoferrin treatment, and significantly higher ($p<0.05$) Mg, S, Cl, K, and Ca after lactoferrin treatment.

Changes of salivary minerals (saliva samples were collected with water-rinse in mouth) associated with different stages of lactoferrin supplementation treatment in both healthy subjects and cancer patients were compared in Table 14. Healthy subjects did not show any significant difference ($p>0.05$) in salivary minerals before, during and after lactoferrin treatment. In contrast, salivary Mg and K in cancer patients significantly increased ($p<0.05$) after lactoferrin supplementation. Salivary Cu significantly ($p<0.05$) decreased during and after lactoferrin treatment in cancer participants.

5.3.3 Salivary Proteome Comparison between Healthy Subjects and Cancer Patients

Saliva of 10 cancer patients and 10 matched healthy control subjects, were loaded on each 2-DE gel at equal amount of protein (20 μ g) from each individual sample for 2-DE analysis; saliva from 4 cancer patients (10 μ g) with difficulty in producing abundant saliva at 30-day post lactoferrin treatment were not used for this analysis. After in-gel image analysis, a total of 102 salivary protein spots with at least a 2-fold expression differences in spot intensity were found in

the three comparisons (cancer patient/healthy subjects, pre-/post-ferrous sulfate rinse, pre-/post-lactoferrin treatment). Based on Wilcoxon test, 57 protein spots with significant fold changes ($p < 0.05$) during lactoferrin treatment were further excised, in-gel tryptic digested and analyzed by MALDI-TOF-TOF mass spectrometry. Identified protein spots were listed in Table 15 and marked on each 2-DE image with consistent spot ID number. The reference whole salivary proteome maps from one representative healthy subject and one representative cancer patient are shown in Fig.6A and B. Compared with healthy group at baseline, six protein spots in cancer group were significantly decreased ($p < 0.05$) including salivary α -amylase (spot 1,2,3,4,8) and hypothetical protein (spot 9), and seven protein spots in cancer group were significantly increased ($p < 0.05$) including transferrin precursor (spot 15,16,17,18), lactoferrin (spot 27), and β -Galactosidase precursor (spot 19,20).

Table 15. Mass spectrometric identification of differentially expressed proteins in the saliva of healthy subjects and cancer patients¹ during different stages of lactoferrin treatment

Spots ² ID	Protein name	Accession No.	Protein MW ³ (kDa)	Protein PI ⁴
1,2,3,4,8,24, 35,38,39,40,41, 43,44,50	α -Amylase, salivary	gi 178585	57.7	6.4
5,6,7,32,42, 46,49	Carbonic anhydrase VI precursor	gi 112693294	40.3	6.6
9	Hypothetical protein	gi 60219551	31.5	4.5
10,11,12,13,14	Prolactin-inducible protein	gi 4505821	9.1	5.3
15,16,17,18	Transferrin precursor	gi 553788	76.8	8.4
19,20	β -Galactosidase precursor	gi 179419	76.5	6.1
21	Fructose- bisphosphate aldolase	gi 312137	39.8	8.3
22	Glyceraldehyde-3- phosphate dehydrogenase	gi 182861	36.2	8.5
27	Lactoferrin	gi 85700158	84.5	9.3
29,30	Fibrinogen beta chain precursor	gi 399492	55.8	8.5
36,37	α -Enolase	gi 1167843	57.3	6.2
47	Proteinase inhibitor	gi 52001472	43.5	6.1
48,52,59	Zinc- α -2- glycoprotein	gi 4502337	33.9	5.5
54	Annexin A1	gi 119582950	40.2	6.6
55,56	Phosphatidylinositol transfer protein alpha isoform	gi 5453908	45.8	6.1
57,58	Immunoglobulin A secretory chain	gi 219109282	83.1	8.2
28,23,25,26,31, 33,34,45,51,53	Unidentified protein			

¹Saliva samples were collected from 10 healthy subjects and 10 cancer patients whose saliva was performed proteomic analysis.

²Identified proteins using the National Center Biotechnology Information nonredundant *Homo sapiens* amino acid sequence database and compared with previous publications (Hu et al., 2005).

³Molecular weight.

⁴Isoelectric point.

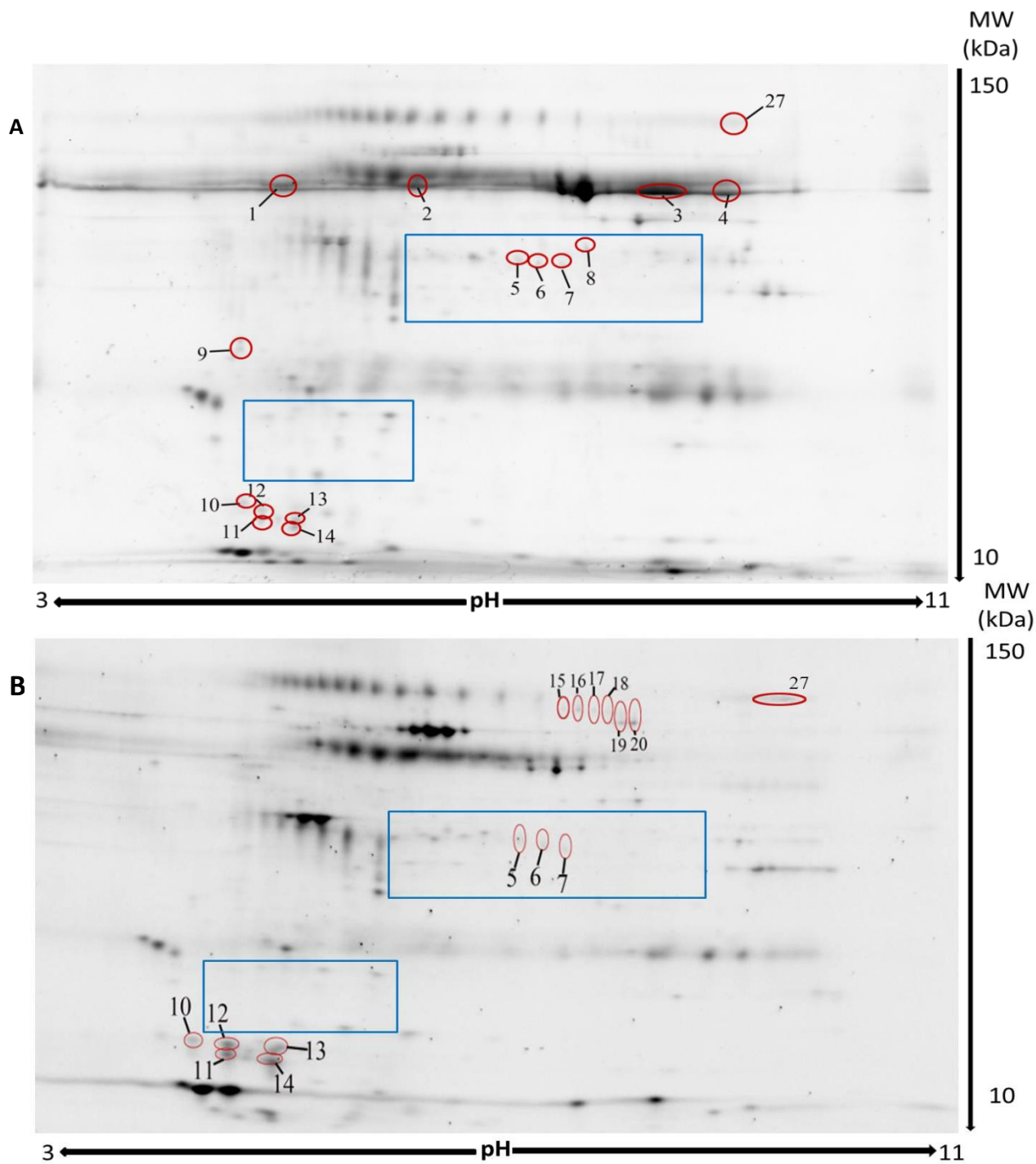


Figure 6. Reference 2D gel map of human saliva proteins from representative healthy subject (A) and cancer patient (B). 20 μ g protein of each sample was loaded on 11-cm IPG strip (pH 3-11NL) followed by SDS-PAGE separation on a 12.5% precast gel. Protein spots were visualized by FlamingoTM fluorescent stain. The circled spots were proteins with significant differences in abundance between healthy and cancer subjects. The protein spot ID numbers are consistent with those in Table 1. Protein spots showed an overall difference between healthy group and cancer group were grouped in the 2-DE images.

Human whole saliva proteome analyzed by the same 2-DE method (pH 3-10 IPG strip, 12%/12.5% SDS-PAGE) illustrated variability among previous published works (Vitorino et al., 2004; Hirtz, et al., 2005; Hu, et al., 2005), especially the relative location and intensity of low abundance proteins. This person to person variation was also found in the current study. For example, 4 out of 10 healthy subjects presented relatively higher intensity of carbonic anhydrase VI precursor (spot 5,6,7) while these spots were barely detected in the remaining 6 healthy subjects. However, even though salivary protein composition and intensity varied among human subjects, low-abundance protein spots, as grouped in Fig.6A, were still decreased in overall cancer group. These low-abundance protein spots, which concentrated between pH 5.5-8.5 and MW (molecular weight) 25-75 kDa, might be related with the loss of partial salivary function in cancer patients. The relationship of these proteins to metallic taste has not been described previously.

According to previous studies, different types of cancer resulted in different salivary proteome, such as lung cancer (Xiao et al., 2012), oral cancer (Jou et al., 2010), and breast cancer (Zhang et al., 2010). This might well explain the variation of salivary composition among cancer participants in this study, since a wide variety of cancers was recruited for this study. For example, compared with healthy subjects, 5 out of 10 cancer patients demonstrated a 2-fold decrease of spots 10,11,12,13,14 (prolactin-inducible protein); the remaining 5 patients illustrated an increase (1.5-2 fold) of this protein (Fig.6B). Compared to the healthy group, this variation might also be caused by different stages of chemotherapy, types of diet, or individual immune system in addition to type of cancer.

5.3.4 Salivary Proteome Comparison Before and After Ferrous Sulfate Stimulation in Healthy Subjects and Cancer Patients

To compare with metallic taste disturbance derived from chemotherapy, ferrous sulfate was used as oral stimulant to generate metallic flavor in both healthy subjects and cancer patients. Results of salivary proteome analysis before and after ferrous sulfate stimulation at each stage of lactoferrin treatment [baseline (I), 30-day lactoferrin treatment (II), 30-day post lactoferrin treatment (III)], are shown in Fig.7 for both healthy subjects and cancer patients.

For healthy subjects (Fig.7A and 7B), ferrous sulfate stimulation lead to a significant increase ($p < 0.05$) in the intensity of transferrin (spot 16,17,18) at stage I and III. Both lactoferrin and transferrin did not present any significant differences ($p > 0.05$) before and after stimulation at stage II during lactoferrin treatment. For cancer patients (Fig.7C and 7D), ferrous sulfate stimulation significantly increased ($p < 0.05$) the intensity of transferrin (spot 15,16,17,18) and lactoferrin (spot 27) at stage I. After iron stimulation, salivary lactoferrin and/or transferrin increased in their intensity, which suggested their defense capability in reducing oxidative stress by binding with excess metal ions in saliva.

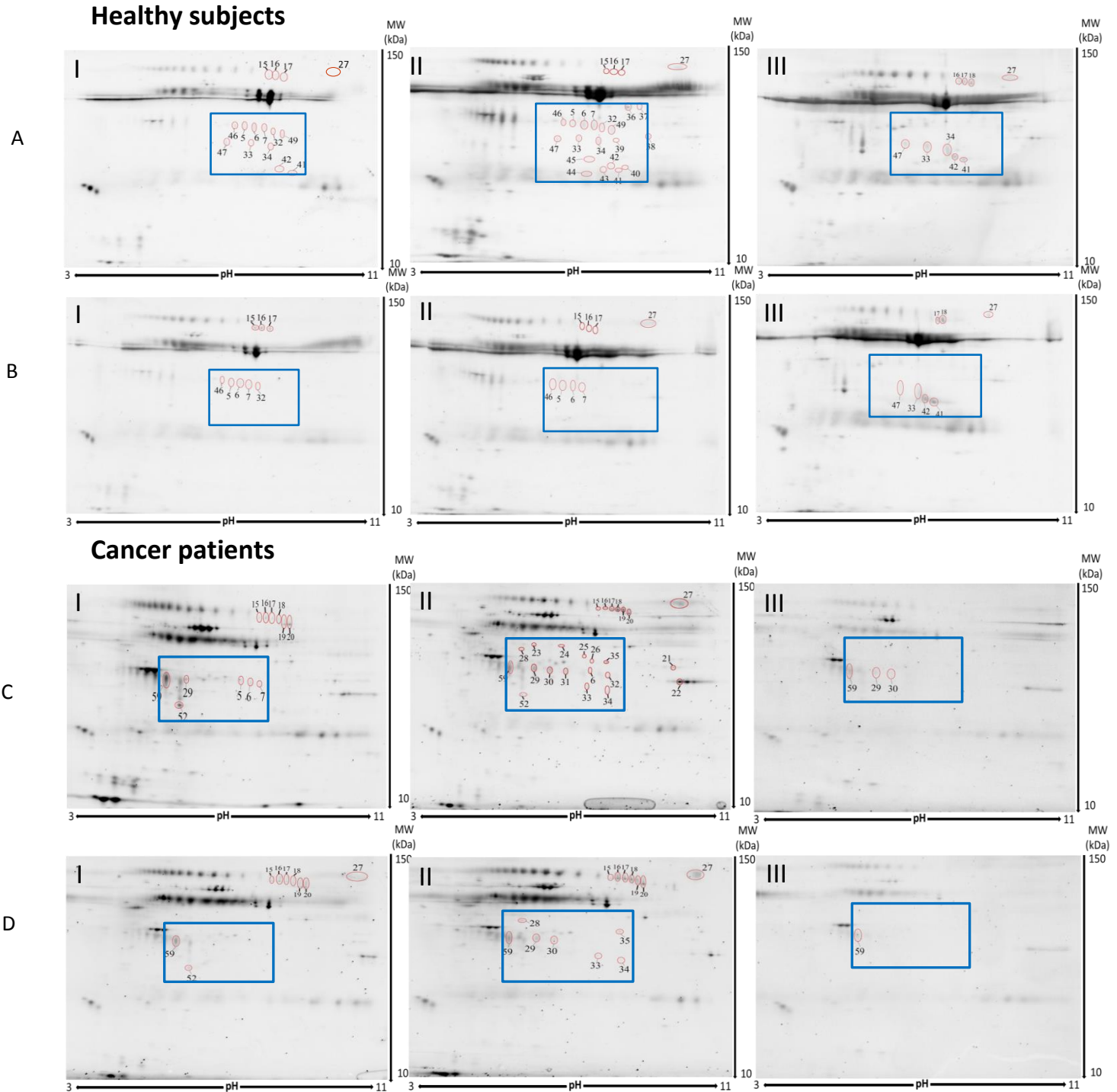


Figure 7. Protein proteome of saliva samples in a representative healthy subject with water-rinse in mouth (A) and ferrous sulfate-rinse in mouth (B), and a representative cancer patient with water-rinse in mouth (C) and ferrous sulfafate-rinse in mouth (D) at baseline (I), 30-day lactoferrin supplementation treatment (II), and 30-day post lactoferrin supplementation treatment (III). Low-abundance protein spots concentrated between pH 5.5-8.5 and MW (molecular weight) 25-75 kDa were grouped in each 2-DE image.

A large variation was observed among cancer patients at stage III. After lactoferrin supplementation treatment for 30 days, 4 out of 10 cancer patients demonstrated an overall decrease in the intensity of salivary proteins (Fig.7 CIII, DIII) compared with baseline. This result might be caused by the difficulty of generating saliva of these cancer patients due to the persistent chemotherapy. In contrast, the remaining 6 cancer patients showed an overall increase in the intensity of lactoferrin and several low-abundance proteins after ferrous sulfate stimulation, which might be due to the post effect of lactoferrin treatment. However, although a person to person variation existed in cancer group, ferrous sulfate stimulation resulted in an overall decrease of the low-abundance salivary proteins comparing with control (Fig.7).

5.3.5 Effect of Lactoferrin Treatment on Salivary Proteome of Healthy Subjects

Salivary proteins that were different in expression above 2-fold along with different stages of lactoferrin supplementation were summarized in Table 16. Lactoferrin treatment (750 mg/day, 30 days) did not cause any significant differences ($p > 0.05$) in the intensity of transferrin (spot 15,16,17,18) in saliva of healthy subjects at different treatment stages. As expected, there was a significant increase ($p < 0.05$) in abundance for salivary lactoferrin (spot 27) during lactoferrin treatment (stage II). No significant changes ($p > 0.05$) in the intensity of lactoferrin (spot 27) were observed between stage II and III, which suggested that the supplementary lactoferrin during stage II was elevated in saliva at least 30 days after the lactoferrin supplement intake ended.

Although a person to person variation existed in the low-abundance salivary proteins, as grouped in Fig.7 for healthy subjects, most of these protein spots were increased overall during the lactoferrin supplementation compared to the baseline. The elevated protein concentrations decreased overall after the supplementation ended to comparable levels as observed at baseline

(Fig.7 AIII, BIII). Most of these low-abundance spots were concentrated between pH 5.5-8.5 and MW (molecular weight) 25-75 kDa.

As shown in Fig.7 AII, lactoferrin treatment lead to a 2-fold increase in the intensity of α -enolase (spot 36,37), α -amylase (spot 38,39,40,41,43,44), carbonic anhydrase VI precursor (spot 42,46,49), and an unidentified protein (spot 45) in saliva of healthy subjects. However, most of these protein spots were not detected after the lactoferrin supplement intake ended (Fig.7 AIII). In addition, carbonic anhydrase VI precursor (spot 5,6,7,32) which existed in saliva of healthy subjects at baseline, were also not detected at stage III. Interestingly, α -amylase (spot 41), carbonic anhydrase VI precursor (spot 42), proteinase inhibitor (spot 47), and two unidentified proteins (spot 33,34) were observed an obvious increase (≥ 2 -fold) in their intensity at stage III of the representative healthy subject as shown in Fig.7.

5.3.6 Effect of Lactoferrin Treatment on Salivary Proteome of Cancer Patients

As shown in Table 16, lactoferrin treatment lead to a significant increase ($p < 0.05$) in the intensity of transferrin (spot 16, 17) and lactoferrin (spot 27) in saliva of all cancer patients. However, a variation in salivary proteome was observed among cancer patients at 30-day post lactoferrin treatment (stage III). Four out of 10 patients had an overall decrease in the intensity of salivary proteins at stage III; a higher taste abnormality score was also observed with stage II. In contrast, there was an overall increase in the intensity of salivary proteins for the remaining 6 cancer patients at stage III, which corresponds with improved taste abnormality scores. The 2-DE patterns of salivary proteome of two representative cancer patient models were shown in Fig.8 and the differently expressed proteins were summarized in Table 16.

Table 16. Differentially expressed proteins during and after lactoferrin supplementation treatment compared with baseline for both healthy subjects and cancer patients¹

Protein ¹	#Spot	Lactoferrin Supplementation Treatment ²	
		30-Day Treatment	30-Day Post Treatment
Healthy subjects			
Lactoferrin	27	↑	↓
α-Enolase	36,37	↑	↓
α-Amylase	38,39,40,41,43,44	↑	↓
Carbonic anhydrase VI precursor	42,46,49	↑	↓
Unidentified protein	45		↓
Cancer patients model I			
Anhydrase VI precursor	6,32	↑	↓
Prolactin-inducible protein	10,11,12,13,14	↓	↓
Transferrin	16,17	↑	↓
Fructose-bisphosphate aldolase	21	↑	↓
Glyceraldehyde-3-phosphate	22	↑	↓
α-amylase	24,35	↑	↓
Lactoferrin	27	↑	↓
Fibrinogen beta chain precursor	29,30	↑	↓

zinc- α -2-glycoprotein	48,52,59	↓	↑
Unidentified proteins	23,25,26,28,31 ,33,34	↑	↓
<hr/>			
Cancer patients model II			
Prolactin-inducible protein	13,14	↑	↑
Fructose-bisphosphate aldolase	21		↑
Transferrin	29	↑	↑
Lactoferrin	31	↑	↑
α -Amylase	38,41		↑
Carbonic anhydrase VI precursor	42		↑
Proteinase inhibitor	47		↑
α -Amylase	50	↑	↑
Zinc- α -2-glycoprotein	52		↑
Annexin A1	54	↑	↑
Phosphatidylinositol transfer Protein alpha isoform	55,56		↑
Immunoglobulin A secretory chain	57,58		↑
Unidentified proteins	33,34,53	↑	
Unidentified proteins	51		↑

¹Saliva samples were collected from 10 healthy subjects and 10 cancer patients whose saliva was performed proteomic analysis.

²Proteins listed in the table were showing ≥ 2 -fold change in expression, along with the direction of increase (\uparrow) or decrease (\downarrow).

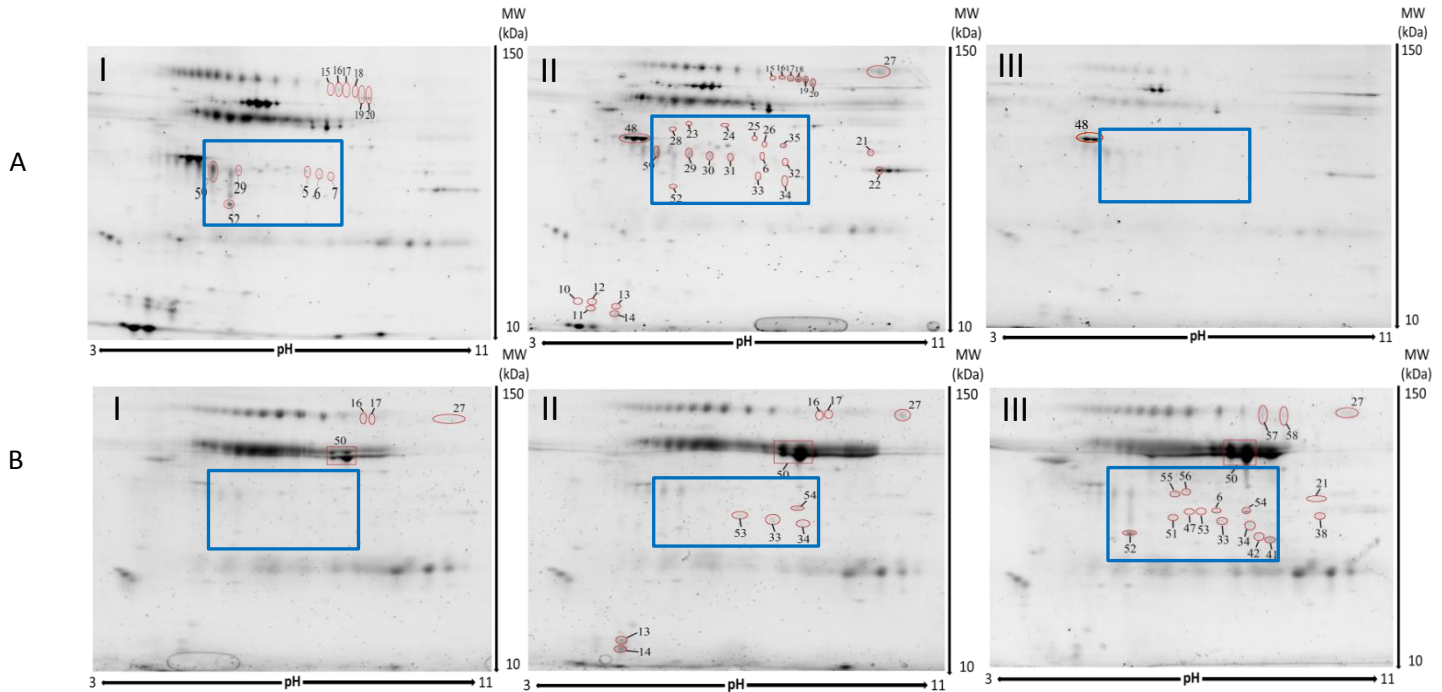


Figure 8. Comparison of salivary proteome between (A) cancer patient model I (taste abnormality score did not change before and after lactoferrin supplementation treatment) and (B) cancer patient model II (taste abnormality score was decreased after lactoferrin supplementation treatment compared with baseline) at baseline (I), 30-day lactoferrin supplementation treatment (II), and 30-day post lactoferrin supplementation treatment (III). Low-abundance protein spots concentrated between pH 5.5-8.5 and MW (molecular weight) 25-75 kDa were grouped in each 2-DE image.

Besides proteins that increased significantly ($p < 0.05$) among all cancer participants at stage II, cancer patient model I also demonstrated a decrease (≥ 2 -fold) in the intensity of prolactin-inducible protein (spot 10, 11, 12, 13, 14) and zinc- α -2-glycoprotein (spot 48, 52, 59) and an increase (≥ 2 -fold) in the intensity of fructose-bisphosphate aldolase (spot 21), glyceraldehyde-3-phosphate dehydrogenase (spot 22) compared with baseline. Low-abundance proteins including

carbonic anhydrase VI precursor (spot 6, 32), fibrinogen beta chain precursor (spot 29,30), α -amylase (spot 24,35) and several unidentified proteins (spot 23,25,26,28,31,33,34) also increased (≥ 2 -fold) in the intensity associated with the lactoferrin supplementation treatment (stage II). However, after lactoferrin treatment for 30 days (stage III), proteins including transferrin, lactoferrin and all the proteins spots stated above could not be detected except zinc- α -2-glycoprotein (spot 48) (Fig.8 AIII). This reduction of protein abundance was not only due to the decrease of total protein concentration in saliva of cancer patients who had the difficulty in generating saliva, since spots such as zinc- α -2-glycoprotein (spot 48) had a relative increase in its intensity percentage, it might be also caused by other effects such as the duration of chemotherapy on cancer patients.

For cancer patient model II, lactoferrin treatment increased (≥ 2 -fold) expression of α -amylase (spot 50), annexin A1 (spot 54), prolactin-inducible protein (spot 13,14), unidentified proteins (spot 53,33,34), as well as transferrin and lactoferrin (Fig.8 II). A post-effect of lactoferrin treatment was observed on cancer patient II at stage III. In addition to those proteins with increased expression at stage II, immunoglobulin A secretory chain (spot 57,58), phosphatidylinositol transfer protein alpha isoform (spot 55,56), fructose-bisphosphate aldolase (spot 21), α -amylase (spot 38,41), proteinase inhibitor (spot 47), carbonic anhydrase VI precursor (spot 42), zinc- α -2-glycoprotein (spot 52), and an unidentified protein (spot 51) increased (≥ 2 -fold) at stage III (Fig.8 BIII).

5.4 Discussion

5.4.1 Effect of Lactoferrin on Reducing Metallic Flavor Induced by Ferrous Sulfate

Stimulation

Production of metallic flavor commonly occurs when taste buds (taste receptors) contact with reducing metals (such as Fe and Cu), such as metal ions contained in water/beverage, iron-containing compounds in red meat, and food packaging made from metal materials. In this study, ferrous sulfate solution (1 mg Fe/L) produced metallic flavor for all the participants at each time point. Most healthy subjects evaluated the intensity of ferrous sulfate solution as weak-moderate metallic flavor (Table 11). The perception of metallic flavor is comprised of the taste of metal ions in the oral cavity and the retronasal odor of carbonyls (e.x. aldehydes and ketones) resulted from lipid oxidation catalyzed by metals (Ömur-Özbek et al., 2012). In our previous study (Ömur-Özbek et al., 2012), lactoferrin solution (0.13 μ M; 10.4 mg/L) was effective in reducing metallic flavor produced by ferrous iron solution (18 μ M; 1 mg/L). The elimination of metallic flavor occurred through chelating reaction of lactoferrin, which was demonstrated to be more effective in inactivating the metals than antioxidants Vitamin C and E (Ömur-Özbek, et al., 2012). In the current study, however, daily intake of lactoferrin supplements (750 mg/day) did not significantly ($p>0.05$) reduce metallic flavor intensity caused by ferrous sulfate stimulation (Table 11) for either healthy subjects or cancer patients. This result might be due to the relative low abundance of lactoferrin in whole salivary proteins. Although lactoferrin abundance in saliva was significantly increased ($p<0.05$) according to the daily intake of lactoferrin supplements, the increased amount was still not enough to bind all iron ions in the acute dose of 1 mg Fe/L of ferrous solution. In our current study, we tested a recurring intake over 30 days, using a supplement administration 3 times per day for 30 days. Ömur-Özbek et al. (2012) evaluated the

acute and immediate administration of lactoferrin in solution independently of the ferrous sulfate solution was consumed.

In a case study focused on salivary proteome changes before and after taste stimulation in healthy subjects (two women and one man, ages 26-28 years), it was reported that oral 6-gingerol (pungent taste) stimulation significantly increased abundance of salivary proteins including prolactin inducible proteins, zinc- α -2-glycoproteins, carbonic anhydrase VI, and SPLUNC2 (short palate, lung and nasal epithelium carcinoma-associated protein 2) (Lorenz et al., 2011); in addition, there was decreased protein abundance for annexin A1 (Lorenz et al., 2011). In the current study, we found an overall decrease in the intensity of low-abundance proteins after ferrous sulfate (metallic taste) stimulation in both cancer patients and healthy subjects. This difference may be caused by the different taste stimulation used in these two studies, even though metallic flavor has typically been described as pungent and bitter taste. The concentration of ferrous sulfate solution used in our study was 4.96 mg/L FeSO₄, which was far below the concentration of 6-gingerol (600 mg/L) in Lorenz's study (Lorenz et al., 2011), but sufficient to generate perception of metallic flavor. The relatively low concentration of ferrous sulfate solution might not be strong enough to stimulate the oral immune response, although all the participants could identify metallic flavor at an even lower concentration in pre-test (1.49 mg/L of FeSO₄). Furthermore, age range of participants in this study for both healthy subjects and cancer patients, was between 45-71 years of old. The age-related decline in immune system, immunosenescence, might exist in elderly participants in this study. Declining immune functions can result in increased risk of infectious disease and vaccine failure due to the decreased cell-mediated immune functions as well as reduced humoral immune response (Weiskopf et al., 2009; Targonski et al., 2007); this might explain the overall decrease of low-

abundance salivary proteins, partially of which were immune proteins, in the current work. The metallic taste disturbance also might be associated with age, since a significant proportion of elderly patients were found to have impaired salivary function (Dodds et al., 2000).

5.4.2 Effect of Lactoferrin on Reducing Metallic Flavor Induced by Chemotherapy

Metallic taste due to chemotherapy might originate from localized taste damage that resulted in a phantogeusia (Hong et al., 2009; Logan et al., 2008). Formation of taste phantoms is a result of inhibitory interactions between different taste nerves that mediated and transmitted taste input to the central nervous system (Yanagisawa et al., 1998). Once the input of one taste nerve is interrupted, it may present inhibition in signal transportation. As a consequence, neural signals from other taste nerves may be intensified, which might lead to metallic taste (Yanagisawa et al., 1998). Another mechanism of metallic taste symptom in cancer patients might be the composition of chemotherapeutic agents (Ijima et al., 2015). Chemotherapeutic agents containing the metal compound platinum, such as cisplatin and carboplatin, might cause metallic taste when secreted in saliva and binding with taste receptors (Aps et al., 2005). A decreased detection threshold for metals in cancer patients due to chemotherapy might also explain their increased sensitivity to metallic flavor, since using plastic utensils instead of metallic silverware during eating can reduce incidence or perception of metallic taste symptom (Hong et al., 2009; Sherry et al., 2002).

However, in our study, salivary proteome analysis suggested that the production of metallic taste in cancer patients receiving chemotherapy, also might be associated with the decreased expression of α -amylase, hypothetical protein, and low-abundance proteins which are mainly immune defense proteins in saliva. As stated above, α -amylase and hypothetical protein showed significant differences ($p < 0.05$) in expression between healthy subjects and cancer patients,

which suggested their decrease might be correlated with the taste abnormality in cancer patients. Low-abundance salivary proteins grouped such as α -enolase and carbonic anhydrase VI precursor were also decreased in overall intensity in cancer patients compared with healthy subjects. The absence of these low-abundance proteins in saliva of cancer patients at each stage of lactoferrin supplementation may suggest their correlation with metallic taste disturbance and taste abnormality.

Furthermore, the appearance of low-abundance salivary proteins (pH 5.5-8.5, MW 25-75kDa), occurred with lactoferrin supplementation treatment corresponded with an improved taste abnormality score in cancer patients, which further demonstrated their role in mediating taste. Although lactoferrin treatment brought out two different effects on cancer patients, intensity of metallic taste was related with the appearance of low-abundance salivary proteins. Taste abnormality scores for cancer patient I at stage I, II, III was 6, 5, 6, respectively. The slightly decreased taste abnormality scores at stage II might be due to a difference in use of the self-reported rating scale by cancer patients. However, lactoferrin supplementation period was still found to associate with an increase in the intensity of low-abundance salivary proteins including carbonic anhydrase VI precursor, fibrinogen beta chain precursor, and α -amylase, and a decreased expression of zinc- α -2-glycoprotein and prolactin-inducible protein. After lactoferrin treatment for 30 days (stage III), the overall protein concentration in saliva largely decreased and the taste abnormality scores of cancer patients were also back to original levels. In contrast, taste abnormality score for cancer patient II at stage I, II, III was 6, 6, 1, respectively. The improvement of taste ability at stage III might be associated with an increased level of immunoglobulin A secretory chain, phosphatidylinositol transfer protein alpha isoform, fructose-bisphosphate aldolase, proteinase inhibitor, carbonic anhydrase VI precursor and an unidentified

protein. However, the increase of α -amylase, annexin A1, zinc- α -2-glycoprotein, prolactin-inducible protein, and two unidentified proteins at stage II did not contribute to the improvement of taste abnormality score in cancer patient II. The decreased intensity of prolactin-inducible protein and zinc- α -2-glycoprotein were associated with an improved taste abnormality score in cancer patient I. Therefore, it was speculated that salivary proteins influencing an improvement (reduced metallic taste) in taste were fibrinogen beta chain precursor, immunoglobulin A secretory chain, phosphatidylinositol transfer protein alpha isoform, proteinase inhibitor, fructose-bisphosphate aldolase and carbonic anhydrase VI precursor. Among them, fructose-bisphosphate aldolase (spot 21) and carbonic anhydrase VI precursor (spot 6) were found in both models of salivary proteome in cancer patients.

Most of the mentioned low-abundance proteins are defense proteins in saliva and their abundance are usually increased in saliva of patients suffering from infectious disease and cancers, such as increased transferrin in oral squamous cell carcinoma (Jou et al., 2010), head and neck squamous cell carcinoma (Dowling et al., 2008), and oral cancer (Jou et al., 2010); increased lactoferrin in dental caries (Vitorino et al., 2006), Sjögren's syndrome (Hu et al., 2007), acute and chronic episodes of recurrent parotitis (Tabak et al., 1978), periodontal disease (Groenink et al., 1999), and diabetes (Harrison and Bowen, 1987); increased salivary α -amylase in gingivitis (He et al., 2011), aggressive periodontitis (Goncalves et al., 2010), and dental caries (Vitorino et al., 2006); increased IgA in dental caries, periodontal disease (Bachrach et al., 2008), oral cancer patient (Jou et al., 2010), and diabetes (Harrison et al., 1987); increased annexin A1 in lung cancer (Xiao et al., 2012); increased zinc- α -2-glycoprotein in aggressive periodontitis (Wu et al., 2009), lung cancer (Xiao et al., 2012), and female breast and male prostatic tumors (Diez-Itza et al., 1993); and increased carbonic anhydrase VI in non-invasive breast cancer

(Zhang et al., 2010). However, these defense proteins were low in cancer patients in the current study. This difference might be because the cancer participants in our study were all receiving chemotherapy, therefore their salivary proteome was different from patients whose saliva was analyzed for biomarker identification for certain disease before any treatment. In addition, although cancer chemotherapy is often considered as a strategy to treat cancer tumor cells, accumulated evidence has indicated that cytotoxic drugs also affect the immune system, damaging the immune cells and leading to tumor regression (Zitvogel et al., 2008). Thus, although decreased expression of some salivary defense proteins has been reported in untreated cancer patients, such as carbonic anhydrase VI in Sjögren's syndrome (Hu et al., 2007) and lung cancer (Xiao et al., 2012), chemotherapy is still considered as the main factor in this study that lead to the overall decrease of salivary defense proteins in cancer patients compared with healthy subjects.

However, daily intake of lactoferrin supplements (750 mg/day) for 30 days affected salivary proteome. The reduced low-abundance salivary proteins in many cancer patients were increased after 30 days of supplementation, which was associated with an improvement of taste abnormality scores. One possible mechanism of this reproduction might be that the increased abundance of lactoferrin in saliva triggers innate protective mechanisms in mucosal immunity and in nonimmune mucosal defense (Lorenz et al., 2011), which stimulated the production of other associated salivary defense proteins such as immunoglobulin A secretory chain (anti-bacteria and anti-viruses), α -amylase (anti-inflammation), annexin A1 (anti-inflammation), and prolactin-induced protein (anti-bacteria and anti-viruses) (Marcotte et al. and Lavoie, 1998; Goncalves et al., 2010; Cirino et al., 1989; Schenkels et al., 1994; Schenkels et al., 1997).

Mechanisms of defense salivary proteins in reducing metallic taste disturbance have not been fully studied yet. Based on the results of this study, we speculate that the defense proteins might contribute to taste improvement by repairing damaged taste receptors such as G-protein-coupled receptors (GPCRs), regulating the taste intensity through accurately detecting and transforming chemical signals from outside of the cells into inside nerve signals (Walters and Roy, 1996). Other low-abundance proteins also might be involved in mediating metallic taste perception. For example, carbonic anhydrase VI was associated with the conversion of salivary bicarbonate and microbe delivered hydrogen ions to carbon dioxide and water, a process that is crucial in maintaining cellular pH homeostasis (Karhumaa et al., 2001).

As the most effective agent in reducing metallic flavor produced by ferrous sulfate stimulation (Ömur-Özbek et al., 2012), lactoferrin was speculated as the most important factor in improving metallic taste disturbance of cancer patients in this study. Lactoferrin might take effect by binding with metal compound platinum existing in chemotherapeutic agents, or associate with other salivary proteins to repair taste receptors destroyed by chemotherapy. Furthermore, synthesis of lactoferrin in humans might also explain its repairing function. Lactoferrin is produced by activated microglia and dopaminergic neurons around the central nervous system under inflammatory process and/or oxidative stress, which contributed to the repair of neuropathological disorders such as Alzheimer's and Parkinson's diseases (Fillebeen et al., 2001). Thus, the supply of lactoferrin might assist in repairing and transmitting neural signals to the central nervous system, which relieved phantogeusia as well as reduced metallic taste intensity as a consequence. In addition, this mechanism also provided a good explanation of increased expression for salivary lactoferrin after iron stimulation in healthy subjects at baseline.

Salivary lactoferrin might serve as immune proteins in responding to iron-induced oxidative stress, and metallic taste disturbance caused by factors such as taste receptors damage.

Besides regulating metallic taste perception, lactoferrin might also contribute to the inhibition of tumor proliferation. Zinc- α -2-glycoprotein actively participated in tumor proliferation, and thus served as a sensitive biomarker of tumor stages (Gangadharan et al., 2007). In this work, zinc- α -2-glycoprotein had decreased expression during lactoferrin treatment while an increased expression after 30 days after lactoferrin supplementation. This result might further demonstrate the anti-cancer capability of lactoferrin as reported in previous studies (Rodrigues et al., 2008).

Salivary transferrin served as biomarker in oral cancer and other infectious diseases (Jou et al., 2010). However, different from immune proteins such as lactoferrin, correlation between increased transferrin expression and enlarged tumor size is due to the participation of transferrin in the rapid growth of tumor cells, rather than protection from tumor damage. Transferrin might contribute to the reduction of metallic taste symptom by binding with metal compound platinum in chemotherapeutic agents.

5.4.3 Relationship between Salivary Minerals, Metallic Flavor and Lactoferrin Supplementation

A large variation of salivary minerals among cancer participants was observed in this study. Different from our previous study (Mirlohi et al., 2015), only Na and Cl showed significantly higher ($p < 0.05$) concentration in cancer patients at baseline when compared with healthy subjects. However, post-lactoferrin supplementation effect induced significant higher ($p < 0.05$) concentration of Mg, S, Cl, K, and Ca in saliva of cancer patients compared with healthy subjects. In addition, Mg and K in cancer patients were increased in concentration along with the process

of lactoferrin supplementation. In a study about the effect of water minerals on recognition thresholds of metallic flavor (Hoehl et al., 2010), taste sensitivity of metallic flavor stimulated by ferrous sulfate was weakened when exposing to higher concentrations of other minerals such as Ca and Mg. In addition, the recognition thresholds of iron (II) sulfate in tap water (containing Ca, Mg, Na, K, Cl) were significantly higher than in deionized water (no minerals) (Hoehl et al., 2010). In this study, total abnormality score of cancer patients was significantly decreased along with the increased levels of other salivary minerals at 30-day post lactoferrin treatment.

Therefore, the increased levels of salivary minerals in cancer participants might decrease the detection threshold of ferrous ions, which resulted in the reduction of metallic taste perception.

Although lactoferrin treatment did not show any significant ($p>0.05$) effect on reducing metallic taste intensity in this study, lactoferrin supplementation still effectively reduced the concentration of salivary Fe after ferrous sulfate rinse for both healthy subjects and cancer patients. This result could be explained by the increased expression of lactoferrin in saliva due to lactoferrin supplements intake, in which lactoferrin bound with ferrous ions thus decreased the salivary Fe concentration. However, how lactoferrin treatment decreased salivary mineral concentrations in cancer patients but increased several of them in healthy subjects is still puzzling.

5.5 Conclusion

In this work, effects of lactoferrin supplements on metallic flavor intensity caused by ferrous ions stimulation and taste disturbance of cancer patients receiving chemotherapy were studied. Our results showed that lactoferrin supplements intake (750 mg lactoferrin/day, 30 days) significantly reduced ($p<0.05$) the taste and smell abnormality in cancer patient during the treatment and/or 30 days after the treatment. Although lactoferrin treatment did not effectively

reduce ($p > 0.05$) the metallic taste intensity stimulated by ferrous sulfate solution (1 mg Fe/L), it significantly ($p < 0.05$) decreased the salivary Fe in oral cavity for both healthy subjects and cancer patients. Production of metallic taste disturbance in cancer patients and mechanism of lactoferrin treatment were investigated through salivary proteome analysis. Our study found that the production of taste disturbance might be associated with the decreased expression of low-abundance proteins (pH 5.5-8.5, MW 25-75kDa) which were mainly immune proteins in saliva. Lactoferrin supplementation potentially helped to recover low-abundance salivary proteins, which are valuable in repairing damaged taste receptors, decreasing the detection threshold of ferrous ions, and binding iron ions to reduce iron-induced oxidative stress.

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Appendix A

Results of Selection Frequency and Cochran's Q Test of Sweetened/Unsweetened Synthetic Waters

Emotional Terms ³	Unsweetened synthetic waters ¹				Sweetened synthetic waters ²			
	S ^d	VH_0	VH_3	Q ⁴	S + sucrose	VH_0 + sucrose	VH_3 + sucrose	Q
Bored*	5	0	7	0.029	-	-	-	-
Calm	15	8	12	0.099	7	12	15	0.056
Content	10	9	8	0.794	10	8	12	0.449
Eager*	-	-	-	-	5	1	0	0.015
Disgusted*	0	2	5	0.042	-	-	-	-
Energetic	2	5	1	0.115	6	1	4	0.093
Friendly	5	7	5	0.695	8	5	6	0.529
Good	7	9	8	0.846	12	8	10	0.549
Good-natured	4	8	5	0.368	5	6	6	0.905
Happy*	4	9	2	0.020	9	3	6	0.086
Interested	4	9	6	0.305	10	6	8	0.336
Mild*	-	-	-	-	1	1	7	0.011
Peaceful	-	-	-	-	6	4	4	0.607
Pleased	7	6	4	0.558	6	3	4	0.497
Pleasant	5	8	4	0.307	6	6	7	0.920
Quiet	7	2	7	0.062	3	5	7	0.336
Satisfied*	5	9	6	0.444	11	2	3	0.002
Steady	4	5	3	0.741	-	-	-	-
Understanding	5	0	3	0.093	-	-	-	-

¹Unsweetened synthetic waters included soft water (S), very hard water (0mg Fe/L) (VH_0), and very hard water (3mg Fe/L) (VH_3).

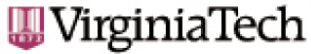
²Sweetened synthetic waters were mixtures of sucrose and each of the following synthetic waters including soft water (S), very hard water (0mg Fe/L)+sucrose (VH_0), and very hard water (3mg Fe/L) (VH_3). Total number of participants is 25.

³Emotional terms were frequently selected terms that were equal or greater than 20% selection frequency (n=43) in emotional response test using a modified EsSense™ paper ballot (King et al., 2010; Leitch et al., 2015). Terms marked with asterisk exhibited statistically significant differences (p<0.05) between samples for at least one group.

⁴Frequency value marked with “-“ means the emotional term did not belong to the frequently selected terms in the corresponding group.

Appendix B

IRB Approval Letter for Chapter 3



Office of Research Compliance
Institutional Review Board
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website <http://www.irb.vt.edu>

MEMORANDUM

DATE: April 16, 2015
TO: Susan E Duncan, Aili Wang
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)
PROTOCOL TITLE: Effect of Iron Concentration on the Metallic Flavor in Sweetened Beverage under Different Water Conditions
IRB NUMBER: 15-399

Effective April 16, 2015, the Virginia Tech Institutional Review Board (IRB) Chair, David M Moore, approved the New Application request 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 within 5 business days 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 responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Exempt, under 45 CFR 46.110 category(ies) 2,6**
Protocol Approval Date: **April 16, 2015**
Protocol Expiration Date: **N/A**
Continuing Review Due Date*: **N/A**

*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 federal 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 C

Results of Mineral Content in Commercial Retail Milk with Direct Addition of Ferrous Lactate

Treatment ²	Mineral Content ¹ (mg/kg)						
	$\bar{X} \pm SE^4$						
	Mg	P	Na	K	Ca	Fe	Zn
Control	118.8± 4.2 ^a	781.5± 26.8 ^a	422.1± 10.9 ^a	1701.5± 55.0 ^a	1250.4± 35.4 ^a	0.485± 0.05 ^a	4.43± 0.20 ^a
Low	117.5± 2.1 ^a	768.7± 4.3 ^a	417.9± 10.9 ^a	1678.4± 11.1 ^a	1238.4± 5.2 ^a	2.31±0. 40 ^b	4.25± 0.18 ^a
Medium	118.7± 1.0 ^a	749.3± 33.5 ^a	421.3± 3.9 ^a	1683.9± 12.0 ^a	1256.0± 10.6 ^a	4.58±0. 12 ^c	4.21± 0.13 ^a
High	116.0± 1.3 ^a	740.7± 9.0 ^a	412.9± 5.9 ^a	1651.7± 33.0 ^a	1233.4± 28.9 ^a	10.3±0. 34 ^d	4.52± 0.44 ^a
USDA reported values ³	60.5	739.8	779.7	1140.4	330.0	3.9	11.8

¹Mineral contents of milk samples were analyzed by inductively-coupled plasma spectroscopy (ICP) with a prior nitric acid digestion.

²Concentrations of iron treatments in milk with direct iron addition were: control (0 mg Fe/L), low (2 mg Fe/L), medium (5 mg Fe/L), and high (12.5 mg Fe/L).

³USDA reported value is from: <http://ndb.nal.usda.gov/ndb/foods/show/69>.

⁴In each column, different superscripts (a-d) indicates significant differences (p<0.05) among different iron treatments.

Appendix D

IRB Approval Letter for Chapter 5



Office of Research Compliance
Institutional Review Board
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website <http://www.irb.vt.edu>

MEMORANDUM

DATE: August 31, 2015
TO: Susan E Duncan, Aili Wang
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires July 29, 2020)
PROTOCOL TITLE: Effect of lactoferrin supplement on changes of protein composition in human saliva under ferrous sulfate treatment
IRB NUMBER: 14-880

Effective August 31, 2015, the Virginia Tech Institutional Review Board (IRB) Chair, David M Moore, approved the Continuing Review request 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 within 5 business days 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 responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Expedited, under 45 CFR 46.110 category(ies) 3,4,6,7**
Protocol Approval Date: **September 19, 2015**
Protocol Expiration Date: **September 18, 2016**
Continuing Review Due Date*: **September 4, 2016**

*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 federal 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 E

Smell and Taste Questionnaire

Patient # _____ Date _____ Timepoint (please circle): Baseline One month Two months ____

Taste Complaints: Please rate

Questions	Insignificant	Mild	Moderate	Severe	Incapacitating
I have noticed a change in my sense of taste					
A food tastes different than it used to					
I have a persistent bad taste in my mouth					
Drugs interfere with my sense of taste					
I would rate my abnormal sense of taste as					

Taste Complaints: Answer "yes" or "no"

Questions	Yes	No	If "Yes" then:
I am experiencing an abnormal sensitivity to salt			Salt tastes: Stronger ___ or Weaker ___
I am experiencing an abnormal sensitivity to sweet			Sweet tastes: Stronger ___ or Weaker ___
I am experiencing an abnormal sensitivity to sour			Sour tastes: Stronger ___ or Weaker ___
I am experiencing an abnormal sensitivity to bitter			Bitter tastes: Stronger ___ or Weaker ___

Smell Complaints: Please rate

Questions	Insignificant	Mild	Moderate	Severe	Incapacitating
I have noticed a change in my sense of smell					
A food smells different than it used to					
Specific drugs interfere with my sense of smell					
I would rate my abnormal sense of smell as					

Smell Complaints: Answer "yes" or "no"

Questions	Yes	No	If "Yes" then:
I have an abnormal sensitivity to odors			Odors are: Stronger ___ or Weaker ___

Appendix F

Post Ferrous Sample Questionnaire

Patient # _____ Date: _____

Timepoint (please circle): Baseline One month Two months

Specific questions for flavor during each saliva collection:

1. How do you describe the taste/flavor of the first sample

2. What is the intensity?

- No perception
- Weak intensity
- Moderate intensity
- Strong intensity
- Very strong / unbearable intensity

3. How do you describe the taste/flavor of the second sample

4. What is the intensity?

- No perception
- Weak intensity
- Moderate intensity
- Strong intensity
- Very strong / unbearable intensity

Appendix G

Demographic Information of Cancer Patients

Participant#	Height (Inch)	Weight (Pound)	Gender	Age	Tobacco use history (from registration form)	Chemo history (from registration form)
1	64	100	F	65	Current - 1 ppd + 45 years	Oxaliplatin 65 mg/m ² , 5 Fu 320 mg/m ² , Leucovorin 572 mg
2			M	67	From 1970 - 2013 20 Cig/day	Neoadj. Xeloda 1500 mg BID on Days of RT (11-29-12 thru 1-9-13) Xeloda 1800 mg BID 1-7-13 to 1-11-13 Oxaliplatin 100.5 mg (dose reduced from 130.65 mg) 4-29 - present Camptosar 281.4 mg 4-29-13 to present 5-FU 804 mg 4-29-13 to 6- 10-13
3	72	177	M	72	Remote Hx. 1ppd 25 pack - years quit 11-15-90	Xelox 3-12-12 + Avastin added C4 9-7-12 (9 cycles) FolFIRI (1 Regoravenib) 10-17-12 (7- 19-13 to 9-3-13 Break) Folox 12-17-13 to present
4	62	138.1	F	48	1 ppd, 28 pack-years Quit 8-28-68 1.5 ppd ; 7.5 pack-years	
5	69	146	M	70		5 FU 400mg/m ² Oxaliplatin 85 mg/m ² Oxaliplatin 65 mg/m ² , Irinotecan 140 mg/m ² , 5 FU 400 mg/m ² Bolus 5FU - 2400 mg/m ² CIV (ver 46 hrs) Leucovorin 400 mg/m ²
6	65	116	F	61	Never Smoked	Oxaliplatin 85 mg/m ² -> 65 mg/m ² -> 68 mg/m ² 5-FU 400 mg/m ²
7	70	186	M	67	Former - Quit 1972 (2.5 pack- years) 5 ppd	
8	70	221	M	76	Quit 10-2-64	Velcade 1 mg/m ² + Revlimid 10 mg/d Methotrexate 15 mg/week ; ISTO PAX 30.65 mg = 14 mg/m ²
9	70	209	M	53	Quit 10-2-64	Doxorubicin 10 mg/m ² , Vincristine 0.5 mg, Etoposide 50 mg/m ² , Cytosar 750 mg/m ²
10	71	164	M	55	Quit 1-1-2012	

11	66	170	F	60	Never	Docetaxel 75 mg/m ² , Cytosan 600 mg/m ² 1) Herceptin x 14 doses + Neratinib 200 mg/d decreased 160 mg/day + Taxol 80 mg/m ² -> Adriamycin 60 mg/m ² + Cytosan 600 mg/m ² 2) Tamoxifen [1212013] 3) Pertuzumab 420 mg / Docetaxel 75 mg/m ² / Herceptin started 5/2014
12	70.5	287	F	33	Never	Adriamycin 60 mg/m ² (x 4 cycles) Cytosan 600 mg/m ² (x 4 cycles) Current Taxol 80 mg/m ² Herceptin 2 mg/kg
13	62	142	F	65	Never	Abraxane 80 mg/m ²
14	64	137	F	68	1.5 ppd x 60 years	Temodar
15	65.2	117	M	51	5 ppd x 15 years	Temodar 75 mg/m ² - 100 mg/m ² ; Avastin 10 mg/kg & Dasatinib/Placebo 150 mg d
16	74	227	M	57	None	

Appendix H

Demographic Information of Healthy Subjects

Participant#	Age	Gender	Height (Inch)	Weight (pound)	Smoke History	Dental Health	Current Prescriptions	Anti-oxidants or vitamin supplements
1	48	Female	66	185	Never	metal dental fillings dental fillings and	No	No
2	59	Female	63.6	130	Never	applicances	No	Yes
3	53	Female	64.8	130	Never	metal dental fillings	No	Yes
4	59	Male	73.2	215	Never	No	No	Yes
5	55	Female	64.8	128	Never	metal dental fillings lots of metal dental	No	No
6	52	Female	62.4	126	Never	fillings	NO	No
7	71	Male	69.6	150	Never	gold fillings	no	no
8	44	Male	72	245	Never	fillings amalgam fillings/gold	No	No
9	61	Male	61.2	180	no	crowns	no	no
10	69	Male	66	190	no	fillings	no	yes
11	67	Female	60	160	no	fillings	no	yes