FORMULATION AND PHYSICAL, CHEMICAL AND SENSORY ANALYSIS
OF A NOVEL FLAXSEED-ENRICHED MILK-BASED BEVERAGE TO DELIVER
OMEGA-3 FATTY ACIDS

by

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ABSTRACT

Formulation and Physical, Chemical and Sensory Analysis of a Novel Flaxseed-enriched Milk-based Beverage to Deliver Omega-3 Fatty Acids

Clara Sueling Lau

An increased interest in functional beverages is occurring, and omega-3 fatty acids (FA) are one of the most commonly sought ingredients to fortify such beverages. Omega-3 FA produce beneficial health effects, likely due to their anti-inflammatory properties. The majority of current omega-3 FA-fortified products include marine-derived omega-3 FA sources, often producing undesired flavors due to lipid oxidation. Little research regarding incorporation of alpha-linolenic acid in functional beverage formulation has been conducted. Alpha-linolenic acid is less susceptible to oxidation and may be a candidate to deliver omega-3 FA into the diet via functional products.

Flaxseed is the richest plant source for alpha-linolenic acid; consumption may increase omega-3 FA intake and lower the omega-6:omega-3 FA ratio, thereby, attenuating inflammation. Finely ground flaxseed was, therefore, incorporated into a chocolate milk foundation (“flaxmilk”) to increase dietary omega-3 FA. An untrained consumer panel tasted and rated flaxmilk’s palatability using a 9-point hedonic scale. A score of “6.0” (“like slightly”) was targeted. A mean hedonic score of 6.35 was achieved, surpassing the targeted score and indicating an acceptable product.

Sensory and analytical analyses of flaxmilk were conducted and compared to standard chocolate milk. Flaxmilk was significantly different in most physical, chemical and sensory characteristics compared to chocolate milk.

A reduction in the omega-6:omega-3 FA ratio may attenuate inflammation; inflammation has been linked to osteoporosis. Thus, a secondary analysis of data collected from 202 women was conducted to estimate the dietary omega-6:omega-3 FA ratio and examine relationships between the omega-6:omega-3 FA ratio and total body and site-specific bone mineral density (BMD). The omega-6:omega-3 FA ratio had no appreciable association with any measure of BMD in the overall sample of women or in younger or older subsamples of women.
In summary, consumers found flaxmilk to be an acceptable product, despite sensory and compositional differences compared to chocolate milk. The relationship between the omega-6:omega-3 FA ratio and BMD remains unclear.
Dedication

Monday, April 16, 2007 will forever be remembered by all Virginia Tech students, Alumni, and our families as a day of great loss of life and innocence. In the darkness there have been many bright lights: the heroism; the incredible strength in the midst of chaos; the unity of the Hokie community and the support of the entire Nation.

My dissertation is dedicated to those who unfairly lost their lives on that tragic day:

Ross Abdallah Alameddine
Christopher James Bishop
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Ryan Christopher Clark
Austin Michelle Cloyd
Jocelyne Couture-Nowak
Kevin P. Granata
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Maxine Shelly Turner
Nicole White

“We will continue to invent the future through our blood and tears and through all our sadness... We are the Hokies...”

-Nikki Giovanni
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Michael: What comes before anything? What have we always said is the most important thing?
George Michael: Breakfast.
Michael: Family.
George Michael: Family, right. I thought you meant of the things you eat.

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Several academic and research advisors aided in the research and writing supporting the chapters of this dissertation. A brief description of their contributions are included here.

Prof. Sharon M. Nickols-Richardson - Ph.D., R.D. (Department of Nutritional Sciences, Pennsylvania State University) is the primary Co-Advisor and Co-Committee Chair. Dr. Nickols-Richardson provided intellectual guidance, assistance with study design, data collection, data interpretation and editorial direction.

Prof. Susan E. Duncan - Ph.D., R.D. (Department of Food Science and Technology, Virginia Tech) is the primary Co-Advisor and Co-Committee Chair. Dr. Duncan provided intellectual guidance, assistance with study design, data collection, data interpretation and editorial direction.

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Prof. Elena Serrano – Ph.D. (Department of Human Nutrition, Foods and Exercise, Virginia Tech) served as a committee member. Dr. Serrano provided input in study design, data interpretation and editorial direction.

Prof. Sean F. O’Keefe – Ph.D. (Department of Food Science and Technology, Virginia Tech) provided technical assistance and guidance for data collection and data interpretation.
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CHAPTER I
Introduction

Many health conscious individuals incorporate functional foods and beverages into their daily lives, with the hopes of maintaining or enhancing quality of life. As the costs of health care and prescription drugs increase, a growing trend towards self-medication with natural food-based ingredients occurs (Brouns and Vermeer 2000). The market for functional foods is growing, and the food industry is involved in an intense search for natural food-based ingredients that may positively affect health, disease prevention or management (Brouns and Vermeer 2000).

Omega-3 fatty acids are one of the food-based ingredients that is gaining widespread attention. In 2006, an estimated $2 billion was spent on retail sales of omega-3 fatty acid fortified products, representing a 111.5% growth in retail sales from 2005 (Packaged Facts 2007). It is predicted that retail sales will reach $7 billion in 2007, with an anticipated annual growth of 60.3% (Packaged Facts 2007). In the beginning of 2007, omega-3 fatty acids were identified as one of the “hottest food additives” of 2007 (Horovitz 2007).

These valuable lipids are essential fatty acids that must be obtained in the diet, because the human body cannot synthesize them from precursor substrates. Not only are omega-3 fatty acids essential nutrients, but they have also been linked to heart health benefits in a qualified health claim issued by the Food and Drug Administration in 2004 (Harris 2007; Packaged Facts 2007). In addition, an emerging body of research demonstrates potential benefits of an increased consumption of omega-3 fatty acids on attenuation of inflammatory diseases [i.e., osteoporosis (Chapter IV)]. Omega-3 fatty acids are found naturally in foods such as coldwater fish, walnuts and flaxseed.

The current American diet is composed of significantly greater amounts of foods containing omega-6 fatty acids (another essential fatty acid). The average intake of omega-6 fatty acids is approximately 10-fold greater than omega-3 fatty acids, producing an average 10:1 omega-6 to omega-3 fatty acid ratio. Simopoulos (2002), among other researchers, recommends alterations in the current American diet (i.e., increased fish consumption and decreased cereal grain-based products) to lower this
fatty acid ratio to achieve a 4-6:1 ratio. This may attenuate inflammation in the body and, thereby, effects of chronic inflammatory diseases.

Some foods and beverages have been fortified with omega-3 fatty acids to serve as vehicles to deliver these omega-3 fatty acids into the consumers’ diets and aid in lowering the omega-6 to omega-3 fatty acid ratio. Currently, many products utilize marine-derived omega-3 fatty acids that may develop a fishy or metallic off-flavor due to the high susceptibility to lipid oxidation (Sattar and deMan 1975; Let and others 2003, 2005; Djordjevic and others 2004; Venkateshwarlu and others 2004). In addition, many vegetarian and non-seafood consumers may be opposed to purchasing these food products. A less explored alternative is the use of plant-derived omega-3 fatty acids in the formulation of novel functional food and beverage products. Flaxseed is the richest plant source of omega-3 fatty acids, as well as an excellent source of fiber and lignans.

The use of milk as the base for functional beverages has been claimed to be ideal due to its inherent nutrient content (Boland and others 2001; Huth and others 2006) as well as sensory appeal and health promoting attributes (Miller and others 2000; McCabe and others 2004; Lanou and others 2005; St-Onge 2005; Zemel and others 2005; Huth and others 2006). The formulation of a flaxseed-fortified milk-based beverage may provide a delivery vehicle for the combined nutrients found in milk (i.e. calcium, protein, vitamin A, B₁₂, D, riboflavin, niacin, potassium and phosphorus) and flaxseed (i.e. omega-3 fatty acids, lignans, fiber and vitamin E). A formulation of a novel flaxseed-enriched chocolate milk-based beverage (“flaxmilk”) was successfully developed with consumer acceptance (Chapter III). The sensory, physical and chemical analysis of flaxmilk compared to a control chocolate beverage (same formulation without flaxseed) was conducted to explore the functionality of the added flaxseed in the chocolate milk foundation and to compare to a commonly accepted product (chocolate milk) over a 14-day storage period (Chapter IV). Differences between the beverages were identified. Chapter V provides a summary of the formulation, sensory and analytical evaluation of flaxmilk, intended for the audience of a food industry trade journal.

There is currently a gap in the knowledge of the effect of increased flaxseed consumption on inflammation of the body. It is unknown whether regular consumption
of flaxmilk would be beneficial to inflammatory diseases such as osteoporosis. Different food-based ingredients have been investigated and reviewed for their potential health benefits on bone, including omega-3 fatty acids (Chapter VI).

A secondary analysis of data regarding the association between the estimated dietary omega-6 to omega-3 fatty acid ratio and total body and site-specific bone mineral measurements was conducted in a sample of women (Chapter VII). Further directions are discussed in Chapter VIII.
References


Horovitz B. Omega-3 pours into cereal, orange juice, eggs, pet food. Jan 2, 2007. USA Today.


Introduction

This chapter serves to review essential fatty acids (FA), particularly the omega-3 (n-3) family, and the potential opportunity to supplement the current American diet with these n-3 FA to attenuate inflammation in the body via functional foods and beverages. Benefits as well as limitations of the formulation of these novel products enhanced with n-3 FA will be discussed.

Essential fatty acids

Essential FA are polyunsaturated FA, because they contain two or more carbon double bonds. There are two classes of essential FA: n-3 and omega-6 (n-6). Biosynthesis enzymes in the human body can only insert a carbon double bond at the 9th carbon or higher position (Albertazzi and Coupland 2002). Only plants, marine algae and phytoplankton are able to synthesize n-6 and n-3 FA from shorter chain fats, introducing these essential FA into the food chain (Albertazzi and Coupland 2002). Due to the double bond located at the 9th carbon in n-6 and n-3 FA, these FA are not synthesized de novo in the mammalian body and must be consumed in the diet. They are, therefore, classified as essential.

The parent compound in the n-6 FA family is linoleic acid (LA, 18:2 n-6), while the parent compound in the n-3 FA family is alpha-linolenic acid (ALA, 18:3 n-3) (Figure 2.1). Linoleic acid and alpha-linolenic acid are both 18 carbon FA, where LA has two carbon double bonds and ALA has three. The additional double bond creates significant differences between the two fatty acid compounds.

\[
\text{C – C – C = C – C – C = C – C – C = C – C – C – C – C – C – COOH}
\]
\text{Alpha-linolenic acid (18:3)}

\[
\text{C – C – C – C – C – C – C = C – C – C = C – C – C – C – C – C – C – C – COOH}
\]
\text{Linoleic acid (18:2)}

Figure 2.1. Graphic representation of the essential fatty acids: Alpha-linolenic acid and Linoleic acid
**Food sources**

Omega-6 FA can be found in most plant oils, meat and poultry products. Omega-3 FA are less ubiquitous and can be found in high amounts in fatty coldwater fish, walnuts and oilseeds. ALA is present in high levels (>50% of FA) in only a limited number of oilseeds such as flaxseed, mustard seed and cranberry seed. Walnuts are the only nut source with significant amounts of ALA. Canola and soybean oils are the most widely consumed ALA sources; however, they only provide modest levels (5-10% of FA) of ALA. Green vegetables have proportionally high amounts of ALA (30-60%); however, due to their low-fat nature, these vegetables are poor sources of ALA. Animals can store ALA in their fat tissue; therefore, grazing animals that consume ALA-containing plants have a high proportion of ALA in their meat carcasses (Cunnane 2003). It has been reported that wild game meat has an estimated \( n-3 \) FA content of 3% of total fat, while domestic or commercial meat is only 0.4% of total fat (Medeiros and others 1989). Fatty coldwater fish contain very low levels of ALA, but a high concentration of the ALA derivatives: eicosapentaenoic acid (EPA, 20:5 \( n-3 \)) and docosahexanenoic acid (DHA, 22:6 \( n-3 \)).

**Metabolism of essential fatty acids**

There are three metabolic fates for the essential FA: 1) incorporation into the cell membrane structure, 2) precursor to longer-chain FA, and 3) \( \beta \)-oxidation for energy.

*Incorporation into cell membrane structure*

The fluidity of the cell membrane is dependent on its lipid composition (Das 2006). Greater levels of saturated fats and cholesterol produce a more rigid membrane, whereas unsaturated FA provide fluidity within the phospholipid bilayer of the cell membrane and influence actions of the membrane-bound enzymes. The fluidity of the cell membrane affects the cell’s intercellular interaction, receptor expression and signal transduction (Puertollano and others 2006). For example, a more rigid cell membrane has a lesser number of insulin receptors, resulting in a decreased affinity for insulin and insulin resistance. Conversely, a fluid cell membrane has a greater number of insulin receptors enhancing insulin binding and action (Das 2006). The presence of essential FA in the brain is important for cognitive development. Fluid cell membranes contain...
more receptors, allowing the proper synaptic connections to take place in the developing brain (Das 2006).

**Precursor for longer-chained fatty acids**

The main metabolic role for the essential FA is to act as precursors to the longer-chain, more unsaturated FA (Figure 2.2). Linoleic acid and ALA compete for delta-6-desaturase which initiates the series of metabolic reactions involving desaturation and elongation to form longer-chain FA of 20 and 22 carbon atoms, specifically arachidonic acid (AA, 20:4 n-6), EPA and DHA (Albertazzi and Coupland 2002). These longer-chain FA play more specific roles in the body and are not considered essential, because they can be produced by the body from fatty acid substrates.

**omega-6 FA family**

- Linoleic acid (18:2n-6)
- Gamma-linolenic acid (18:3n-6)
- Dihommo-gamma-linolenic acid (20:3n-6)
- Arachidonic acid (20:4n-6)
- Adrenic acid (22:4n-6)
- 22:5n-6

**omega-3 FA family**

- Alpha-linolenic acid (18:3n-3)
- 18:4n-3
- 20:4n-3

Both essential fatty acid families require delta-6-desaturase to initiate the elongation process of the polyunsaturated FA. The essential FA can be stored in the phospholipid bilayer of the cell membrane until needed elsewhere. When FA are released from the cell membrane, the n-6 or n-3 FA become substrates for locally
produced eicosanoids (Saldeen and Saldeen 2004). Eicosanoids are short lived, very active and hormone-like compounds, serving as signaling molecules in response to hormonal stimuli (Benatti and others 2004; Wahrburg 2004). Eicosanoids include prostaglandins, leukotrienes and thromboxanes. Eicosanoids from n-6 FA sources such as the prostaglandins of the 2-series and leukotrienes of the 4-series promote cell proliferation, inflammation and blood clotting. Omega-3 FA derived eicosanoids such as prostaglandins of the 3-series and leukotrienes of the 5-series have an inhibitory effect on cell growth, anti-inflammation and blood thinning (Wahrburg 2004). Arachidonic acid and EPA both have the ability to convert into prostaglandins where AA produces pro-inflammatory prostaglandins and EPA produces anti-inflammatory prostaglandins. The presence of ALA displaces AA from the phospholipid pool of the cell membrane and impedes the formation of pro-inflammatory prostaglandins (Das 2006). Although delta-6-desaturase preferentially utilizes ALA over LA, generally more LA is available resulting in greater AA production compared to EPA. Yet, inflammation may be attenuated with dietary n-3 FA intake.

**β-oxidation for energy**

β-oxidation is a series of catabolic reactions occurring in the mitochondria where fatty acids are metabolized to produce energy. This is generally initiated by a large concentration of free fatty acids present in the human body. Previous studies have shown that LA and ALA are partly partitioned towards β-oxidation, reducing the availability of either FA to serve as precursors of the longer-chain FA. It has been estimated that 16-20% of ALA is expired as CO\(_2\) over a 12 hr period (Vermunt and others 1999; Brenna 2002). The use of ALA as an energy source occurs at higher efficiency in men than women, which is confirmed by the lower usage of fat than carbohydrates as an energy source in women. This reduces the availability of ALA in the conversion process into the longer-chain FA, especially in men (Burdge 2004). This is likely to limit the anti-inflammatory effect of ALA present in the diet. An increased intake of ALA may be necessary in order for the anti-inflammatory effect to be appreciable.
Recommended intakes

Although the United States (US) Department of Agriculture (USDA) has not established recommended intakes for the essential FA, the Institute of Medicine of the National Academies of Science has reported adequate intakes (AI) for the essential FA (National Research Council 2005). Adequate intakes represent median intake levels that prevent an essential FA deficiency. For men aged 19-50 years, the AI for n-6 FA is 14 g/day and for n-3 FA is 1.6 g/day. In men older than 50 years, 17 g/day of n-6 FA and 1.6 g/day of n-3 FA are recommended. For women aged 19-50 years, the AI for n-6 FA is 11 g/day and for n-3 FA is 1.1 g/day. In women older than 50 years, 12 g/day of n-6 FA and 1.1 g/day of n-3 FA are suggested. There are no established tolerable upper limits for these essential FA.

More specific n-3 FA recommendations for cardiac health have been reported by professional societies. The American Heart Association (AHA), the American College of Cardiology and the European Society for Cardiology have recommended 1 g/day EPA + DHA as a secondary prevention measure for cardiovascular disease (Smith and others 2006; von Schacky and Harris 2007). An intake of 1 g/day EPA + DHA shows no potential for adverse effects and is achievable in the diet (Kris-Etherton and others 2002). In 2000, the US Food and Drug Administration (FDA) recommended that consumers ingest 3 g of n-3 FA daily, with no more than 2 g/day from a dietary supplement (Kris-Etherton and others 2002; Kolanowski and Laufenberg 2006; Packaged Facts 2007). Whereas there is little evidence that consumption of <3 g n-3 FA/day has led to any significant bleeding in patients (Kris-Etherton and others 2002), consumption of >3 g n-3 FA may lead to excessive bleeding and should only be done under the care of a physician (Harris 1996; Kris-Etherton and others 2002; Whelan and Rust 2006).

In September 2004, a qualified health claim for n-3 FA was issued by the FDA. This claim stated,

“Supportive but not conclusive research shows that consumption of EPA and DHA omega-3 fatty acids may reduce the risk of coronary heart disease. One serving of [name of food] provides [x] grams of EPA and DHA omega-3 fatty acids.”
acids. [See nutrition information for total fat, saturated fat and cholesterol content.]” (Harris 2007; Packaged Facts 2007).

This health claim was not extended to include ALA due to the lack of supportive evidence that this 18-carbon FA provide the same cardioprotective effects as the longer-chain n-3 FA (i.e., DHA, EPA) (Albert 2007).

The AHA Scientific Statement notes that prospective secondary prevention studies of cardiac events suggest that supplementation of 0.5-1.8 g/day EPA+DHA and 1.5-3.0 g/day ALA reduces subsequent acute cardiac mortality (Kris-Etherton and others 2002). A more firm recommendation as to specific food sources of n-3 FA cannot be given at the current time and are not included in the guidelines provided by these professional societies (von Schacky and Harris 2007).

**Ratio of essential fatty acids**

Major dietary shifts have occurred over the past two centuries. Based on estimations of Paleolithic nutrition (200,000 to 12,000 years ago) and modern day nutrition, there has been a significant change in the fat content of the human diet, including a notable shift in the dietary intakes of n-6 and n-3 FA. Eaton and Konner (1985) previously developed a model to analyze the nutritional properties of a diet composed of wild game and uncultivated plants as well as evaluating archeological remains. This enabled these researchers to estimate the nutritional properties of an “average” Paleolithic diet (Eaton and others 1997). The Paleolithic diet was substantially lower in total and saturated fat in comparison with the modern day diet which is comprised of relatively high amounts of total and saturated fat (Simopoulos 1999; Kris-Etherton and others 2000). Eaton (1992) estimated that saturated fat in the Paleolithic diet provided approximately 6% and the overall total fat intake provided 20-25% of the total energy intake. The 2005 Dietary Guidelines for Americans recommend a saturated fat intake of ≤10% and total fat intake between 20-35% of the total daily energy intake (US Department of Health and Human Services and USDA, 2005). The total fat intake of the average American diet currently provides 36-38.5% of total caloric intake (Eaton 1992; Chanmugam and others 2003). Although the percentage of fat is not excessively greater in the modern day diet compared to the Paleolithic diet, a
significant increase in total caloric intake has taken place, including an increase in the absolute fat gram intake.

In addition, the profile of dietary fat has changed considerably between these two diets. Major changes occurred in the human diet over the past 10,000 years with the dawn of the Agricultural Revolution and the introduction of cereal grains into the food supply (Simopoulos 1999). The pre-agricultural food supply mainly consisted of wild plants, wild game animals and fish, all rich in n-3 FA, specifically ALA. Within the past 10,000 years, cereal grains have become a dietary staple and have created a dramatic departure from the foods with which humans evolved. Cereal grains are a rich source of n-6 FA, but are low in n-3 FA and antioxidants. In modern day nutrition, humans are fully dependent on cereal grains for the majority of their energy source, as well as carbohydrate and protein, leading to a significant increase in the dietary n-6:n-3 FA ratio (Simopoulos 1999).

The advent of the modern food oil industry within the past century further escalated the n-6:n-3 FA ratio. The process of solvent extraction of oils became more sophisticated, enabling more efficient and economical large-scale production of vegetable oils (Simopoulos 2002a). The most common vegetable oils consumed were from corn, sunflower and soybean sources, all rich in n-6 FA (James and others 2000). In the 1950s, studies showed strong evidence that n-6 FA reduced serum cholesterol concentration by 20-35%, lowering the risk of certain cardiovascular events (Simopoulos 2002a; Sacks and Campos 2006). With this in mind, vegetable oil production focused to increase the n-6 FA content in these oils, to help promote intake of n-6 FA, further increasing the essential FA ratio.

Another possible explanation for the significantly increased n-6:n-3 FA dietary ratio, due to advances in modern agriculture, is the emphasis on production over nutritional quality (Simopoulos 2002b). For example, grain feed is commonly used for domesticated livestock which subsequently decreases the n-3 FA content in animal carcasses. Domesticated livestock is estimated to contain four times the amount of polyunsaturated FA compared to wild animals, such as deer (Simopoulos 2002b). However, the FA profiles of domesticated livestock are primarily composed of FA of the n-6 family, with a very small, even undetectable amount of ALA. Another example is
found in modern aquaculture, where wild trout and salmon have higher amounts of \( n\)-3 FA (EPA and DHA) compared to cultured trout and salmon (van Vliet and Katan 1990). Levels of \( 18:2n-6 \) were higher in cultured fish affecting the \( n-6:n-3 \) FA dietary ratio, which was estimated to be at least 2-3 fold greater in cultured fish compared to wild fish (van Vliet and Katan 1990). The authors conclude that wild fish are a better source of \( n\)-3 FA than their cultured counterparts.

With these suspected dramatic differences between the modern day and Paleolithic diets, the dietary ratio of essential FA has significantly increased. In addition to the large consumption of cereal grain products, vegetable oils and farm-raised meat products, regular consumption of fish and fish products has diminished leading to an 80% reduction in dietary intake of \( n\)-3 FA in the last century (Saldeen and Saldeen 2004). The current \( n\)-3 FA consumption in the US is approximately 1.5-1.6 g/day, where ALA accounts for 1.4 g/day and 0.1-0.2 g/day is from EPA+DHA (Kris-Etherton and others 2000; Burdge 2004). This is estimated to be at least 10-fold lower than the current \( n\)-6 FA intake (Burdge 2004). Our Paleolithic ancestors are estimated to have consumed a diet composed of a 1:1 \( n-6:n-3 \) FA ratio. Observational studies have reported that the modern day diet consists of greater amounts of \( n\)-6 FA compared to \( n\)-3 FA intake (Leaf and Weber 1988), with the average \( n-6:n-3 \) FA ratio to be between 10-20:1 (Kris-Etherton and others 2000; Burdge 2004; Wahrburg 2004; Rakel and Rindfleisch 2005).

Diet plays an important role in the risk of development of chronic diseases. The body is able to desaturate and elongate ALA to form EPA and DHA. Unfortunately, the conversion rate of this series of reactions is inefficient in humans due to dietary factors and has been estimated to be between 0.2-6% for EPA and <0.05% for DHA in men (Burdge and Calder 2005; Burdge 2006). A high dietary \( n-6:n-3 \) FA ratio results from an elevated intake of \( n\)-6 FA, which creates a pro-inflammatory environment, increasing a person’s vulnerability of developing a chronic inflammatory disease. Simopoulos (2002a), among others, recommends altering the current diet to achieve a 4-6:1 \( n-6:n-3 \) FA ratio (Simopoulos 2002a; Meyer and others 2003) to attenuate inflammation in the body. Therefore, in order to improve the current imbalanced \( n-6:n-3 \) FA ratio, it is necessary to not only increase the \( n\)-3 FA intake but to also decrease the \( n\)-6 FA intake.
The inflammatory response

The human body has a natural defense system known as the immune system. Inflammation is the human body's natural reaction to infection, irritation or injury. It is the body's first response by the immune system to begin the elimination of foreign pathogens and toxins and repair damaged tissue. Inflammation and the immunological response are components of the normal, innate immune response and it is vital that these inflammatory responses are ordered and controlled. However, when inflammation occurs in an uncontrolled manner, excessive damage to body tissue and/or disease may result (Calder 2003, 2006a). Some diseases can trigger an inappropriate inflammatory response in the body, despite the absence of foreign substances. When this happens, the same immune response may now cause damage in the body.

There are two types of inflammation involved in the inflammatory response: acute and chronic. Acute inflammation is short-lived, lasting only a few days. The inflammation may be in response to physical damage, chemical substances, microorganisms or other foreign antigens to the body. Acute inflammation is not dependent on which body region is infected, and the same inflammatory response occurs everywhere. Inflammation promotes white blood cells and chemicals to be released into the blood or the tissue to destroy the foreign substance. The presence of the chemicals causes an upsurge of blood flow to the affected area, often resulting in redness and warmth. The blood capillaries become more permeable allowing large molecules including cytokines, to leave the blood stream and pass through the endothelial wall as exudate. This also increases leukocyte movement to enter the surrounding tissue (Calder 2006a). The affected area is filled with an exudate that contains proteins, fluid and cells to help mediate the defense mechanism and fight the infective agent in the damaged area. Swelling may occur when the chemicals induce leakage of fluid into the affected tissues. The inflammatory process may also stimulate nerves, creating pain stimuli (Sears 2005).

When the inflammation lasts longer than a few days, it is considered chronic (Movat 1985; Sears 2005). Chronic inflammation occurs when the inflammatory process persists for a prolonged time period - weeks, months or indefinitely. This
extended time allows inflammation to occur in the tissue, leading to tissue damage. Although the body simultaneously attempts to heal and repair the damaged tissue, it is not guaranteed for the body to completely destroy the causative agent, which is most often pro-inflammatory eicosanoids (Sears 2005).

**Pro-inflammatory cytokines**

Cytokines are small secreted proteins that mediate and regulate the inflammatory response (Kettler 2001). The cytokines communicate between the immune cells and the injured region of the body by activating lymphocytes, monocytes and macrophages, which form cytokines to provide protection against foreign antigens and/or tissue damage. Several cytokines play key roles in mediation of inflammation; however, this discussion is limited to the pro-inflammatory cytokines: interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). Both IL-1 and TNF-α are produced at sites of local inflammation. They stimulate their own and each other’s synthesis in an autocrine and synergistic manner (Jilka 1998; Kettler 2001). IL-1 and TNF-α often act synergistically on the synthesis of AA metabolites as well as stimulate the production of IL-6 (Endres and others 1989; Kettler 2001). Only small amounts of IL-1 or TNF-α are necessary to significantly increase levels of the three pro-inflammatory cytokines. However, a deficit of any of the pro-inflammatory cytokines will lead to a decrease in all three.

Studies examining the association between dietary n-3 FA intake and pro-inflammatory cytokine concentrations in plasma have shown concordant results in which n-3 FA have beneficial effects on active inflammation. Lopez-Garcia and colleagues (2004) conducted a cross-sectional study in a cohort of 727 healthy women aged 43-69 years from the Nurses’ Health Study I. Each participant completed a food frequency questionnaire (FFQ) in 1986 and 1990, which included consumption of fish and seafood products. These FFQs were analyzed to provide estimated total n-3 FA intake, as well as ALA, EPA and DHA intakes. Blood collected between 1989 and 1990 were analyzed for plasma IL-6 concentration. Data were divided into quintiles based on consumption of n-3 FA. Interleukin-6 was 23% lower in the highest quintile of total n-3 FA intake compared to the lowest intake. A trend for decreasing plasma IL-6 concentration was
seen with increasing ALA intake quintiles. However, EPA and DHA did not show a significant association with IL-6.

Pischon and colleagues (2003) performed a similar cross-sectional study in a cohort of 405 men, aged 40-75 years (at baseline in 1986) from the Health Professionals Follow Up Study and 454 women, aged 25-42 years (at baseline in 1989) from the Nurses’ Health Study II. In 1994 and 1995, FFQs were administered and completed by each participant. From completed FFQs, the estimated dietary ALA and EPA + DHA intakes were computed. Blood collected between 1993 and 1995 from males and between 1996 and 1998 for females was analyzed for plasma IL-6 concentration and TNF-α receptor numbers. TNF-α receptors are biomarkers derived from the TNF cell surface after induction by pro-inflammatory cytokines. TNF-α receptors have a longer half-life and are more easily detected with a higher sensitivity than TNF-α concentration and are, therefore, excellent biomarkers of inflammatory processes (Aderka 1996). In both males and females, those with the highest EPA+DHA intakes had the lowest TNF-α receptor numbers, whereas ALA intake levels was not significantly associated with plasma concentration of IL-6 or TNF-α receptor number.

The InCHIANTI Study examined the association of dietary FA levels and plasma inflammatory markers of 1123 Italian subjects, aged 20-98 years (Ferrucci and others 2006). Average daily intakes of polyunsaturated FA were estimated using a FFQ, previously validated for the Italian population. After controlling for major confounding variables, inverse relationships were found between intakes of AA and DHA with both plasma IL-6 and IL-1 concentrations. Low ALA intake was associated with high IL-1 concentration, and low EPA intake was related to high IL-6 concentration.

These aforementioned studies were all cross-sectional cohort studies, which were unable to demonstrate a causal effect of n-3 FA on the reduction of pro-inflammatory cytokines. These epidemiologic studies were also unable to collectively demonstrate an inverse correlation between high ALA intake and low pro-inflammatory cytokine production. Clinical trials involving dietary interventions are necessary to explore whether there is a causal effect of n-3 FA on inflammatory biomarkers and whether ALA is as effective in attenuating inflammatory responses as EPA and DHA.
It has been proposed that the increase of n-3 FA intake in the diet may attenuate chronic inflammation and help prevent the onset of inflammatory diseases in healthy individuals. Several clinical studies have been conducted with healthy subjects exploring the possible causal effect of n-3 FA intake on pro-inflammatory cytokines production. A few key studies are reviewed here.

Endres and colleagues (1989) had nine healthy subjects consume 18 g marine oil (2754 mg EPA and 1854 mg DHA) daily with their normal diets for six weeks. Peripheral blood mononuclear cells (PBMCs) and plasma phospholipids were analyzed for their FA profiles, as well as pro-inflammatory cytokine production activity. After six weeks, all subjects showed a significant decrease in plasma AA and significant increase in plasma EPA and DHA. In addition, IL-1β and TNF-α production decreased significantly. Ten weeks after the intervention, PBMCs exposed to heat-killed *Staphylococcus epidermis* maintained the decreased level of cytokine production. At week 26 (after baseline) cytokine production returned to pre-supplementation levels. These results suggest that dietary supplementation of EPA and DHA suppress cytokine production but not the release of cytokines in cells.

Meydani and colleagues (1993) conducted a dietary intervention where 22 healthy normotensive and normolipidemic subjects consumed similar low-fat, low-cholesterol diets that only varied in the EPA and DHA content. All subjects consumed a typical American diet for six weeks before the low-fat diet treatment for 24 weeks. The high-fish diet (n=11) was composed of 1.23 g/day EPA+DHA (from 8 servings of fish per week), while the low-fish diet (n=11) was composed of 0.27 g/day EPA+DHA (from 2 servings of fish per week). Fish served as the primary source of EPA and DHA, while the remainder of the n-3 FA content was from plant-derived sources (soybean oil, rapeseed oil and walnuts). Blood samples were collected three times during the last three weeks of the baseline and low-fat diets. Plasma concentrations of fatty acids were analyzed. Linolenic acid, ALA, EPA, DHA and total n-3 FA content increased and AA level decreased in subjects consuming the high-fish diet for 24 weeks. *Ex-vivo* cytokine production was measured by the production of cytokines when PBMCs were exposed to heat-killed *Staphylococcus epidermis* or *Escherichia coli* 0111:B4. After the 30-week study, blood samples of subjects who consumed the high-fish diet had
significantly lower concentrations of plasma IL-6, IL-1β and TNF-α compared to subjects in the low-fish diet group who showed increases in plasma IL-1β and TNF-α concentrations. One limitation of this study included a non-uniform timing of diets. Subjects began diets at varying times; this may have introduced confounding factors. In addition, three of the 11 subjects in the low-fat, low-fish diet group were previously subjects in the low-fat, high-fish diet. Despite the 6-month washout period, this may have introduced bias. Lastly, each serving of fish was 121-188 g, quite a large serving of fish. Therefore, the low-fish diet group consumed more fish than a typical American consumer; this was not a well-chosen control group for this study. Two servings of fish per week is recommended by the AHA for prevention of cardiovascular disease (CVD). It would have been more valuable to have a no-fish diet group in addition to the high-fish and low-fish diet groups, to demonstrate the effects of these fish-containing diets compared to a more typical American diet containing low EPA and DHA content.

Caughey and colleagues (1996) examined the effect of fish oil (FO) supplementation in healthy male subjects (n=13) who were consuming a flaxseed oil-based diet compared to a control sunflower oil-based diet (n=15). All subjects followed their respective diets for four weeks and then began supplementation with nine FO pills to provide 1.62 g/day EPA and 1.08 g/day DHA for four more weeks. Fish intake was limited during the study period. These investigators did not indicate the quantity of flaxseed oil that was mandatory, but the replacement of cooking oils and spreads with flaxseed oil and an enriched butter-like spread was required. The treatment group had an average intake of 13.7 g/day ALA, while control subjects averaged 1.1 g/day ALA. In addition, the average LA intake was approximately 8.4 g/day and 20.3 g/day for the flaxseed oil based and sunflower-oil based diet groups, respectively. After the initial four weeks, the flaxseed oil-based diet group had significant increases in cellular concentrations of ALA (3.0-fold) and EPA (2.3-fold), while these increases were not observed in the control group. There were also significant decreases in IL-1β and TNF-α in the flaxseed oil diet group after the initial four weeks. After FO pills were added the treatment group showed further significant increases in cellular EPA concentration, but not ALA, while the control group also showed similar increases in cellular EPA concentration. Markedly lower concentrations of IL-1β and TNF-α were
seen in both groups after the addition of FO capsules. These lowered cytokine concentrations suggest that the different sources of n-3 FA were incorporated into plasma and tissue and inhibited production of pro-inflammatory cytokines. Limitations to this study included the inconsistency of diets in subjects due to the lack of a controlled feeding trial. It was unclear whether it was ideal for subjects to substitute cooking oils with flaxseed oil, because of the high degree of unsaturation in flaxseed oil may have altered food flavor (due to oxidation), or had other detrimental effects in the body. These investigators did not control for these factors.

It is difficult to relate these results to the average American diet, as most research studies used greatly elevated dietary intakes of n-3 FA, especially of EPA and DHA, as treatments. For example, most human studies used at least 1230 mg and up to 9600 mg EPA + DHA daily, while habitual intake is <150 mg/day EPA+DHA (Meydani and others 1993; Thies and others 2001). Thus, studies use an 8-64-fold higher level. Similarly, subjects consumed approximately 14 g/day of ALA in order to induce a decreased production of pro-inflammatory cytokines (Caughey and others 1996), whereas the current intake of ALA is estimated to be much lower.

Little is known about effects of more moderate levels of n-3 FA on the inflammatory response. A group of researchers was unable to demonstrate that an intake of 2 g/day of ALA had an effect on human inflammatory cytokine production (Thies and others 2001). Forty-six subjects (mean age = 63 years) consumed nine oil capsules daily for 12 weeks. All capsules contained varying amounts of palm and sunflower oils. The six treatments were placebo (palm and sunflower oil only), ALA (flaxseed oil added), gamma-linolenic acid (GLA; GLA-rich oil added), AA (AA oil added), DHA (DHA oil added) and FO (EPA- and DHA-rich FO added). Fatty acid content in plasma and PBMCs phospholipids and pro-inflammatory cytokine concentrations were measured at baseline and week 12. Despite having a higher percentage of the respective oil in the ALA capsule (53.5% ALA of the total FA), compared to the DHA (19.1% of the total FA) and FO capsules (18.8% EPA and 7.4% DHA); the ALA treatment did not demonstrate any significant increases in the specified oils in the plasma and PBMCs phospholipid levels after the treatment period. It is likely that an even larger amount of ALA is necessary to show any significant increase of the
incorporation into the phospholipids, compared to the other oils. There were no significant effects on cytokine production, despite increases of FA in phospholipids for any of the treatments. Yet, the ALA group had the largest decreases in TNF-α and IL-6, even compared to FO subjects, as well as the smallest increase in IL-1β compared to the other oils. This may suggest promising results for attenuation of cytokine production. However, a larger scale trial is needed. Each treatment group had eight or fewer subjects, resulting in insufficient statistical power.

Most research has focused on prevention of inflammation and has included healthy subjects. With the idea that n-3 FA can be used as a therapeutic supplement to attenuate inflammation, some research has included subjects with inflammatory conditions. Kew and colleagues (2003) conducted a placebo-controlled study adding modest amounts of n-3 FA to the diet of 150 moderately hyperlipidemic, but otherwise healthy, adults. Subjects were randomly assigned to incorporate 25 g of a fat spread and oil capsules (five treatments: containing 4.5 or 9.5 g ALA, 0.77 or 1.7 g EPA+DHA or an n-6-rich placebo) into their daily diet for six months. Changes in the fatty composition of PBMCs were observed in subjects consuming 9.5 g/day ALA or 1.7 g/day EPA+DHA. The n-6:n-3 FA ratio of the placebo group was approximately 11:1, whereas the ratios of the treatment groups ranged from 3.3 to 10.1:1, depending on treatment. Despite the increasing amounts of n-3 FA, no changes in plasma pro-inflammatory cytokine production (IL-1β, IL-6 and TNF-α) or immune function were seen in these subjects. It is possible that the increase in n-3 FA consumption of these patients with moderate hyperlipidemia would require higher amounts of ALA, EPA or DHA in order to show any beneficial effects on cytokine production. A similar study conducted with normolipidemic subjects would produce a better understanding of whether these modest amounts of n-3 FA would be sufficient in producing a beneficial protective effect on chronic inflammation in the body.

The increase of pro-inflammatory cytokines may lead to or be a direct effect of inflammatory diseases. The next step is to investigate the effect of increased intake of dietary n-3 FA on subjects with chronic inflammation, as seen in inflammatory diseases.
Essential fatty acids and inflammatory diseases

When inflammation is left uncontrolled, disease and tissue damage occurs, resulting in high levels of the destructive pro-inflammatory cytokines TNF-α, IL-1 and IL-6. An imbalance of greater production of pro-inflammatory cytokines and less anti-inflammatory cytokines leads to inflammation in the body. Chronic overproduction of pro-inflammatory cytokines may lead to muscle wasting, decreased bone mass, endotoxin shock, body composition changes and tissue loss, as well as chronic inflammatory diseases (Calder 2002, 2006a, 2006b).

The main link between FA and inflammation is the eicosanoids that are responsible for mediating and regulating inflammation in the body. The eicosanoids of the n-6 and n-3 FA have opposing properties. Due to the elevated amount of n-6 FA found in the Western diet, the AA-derived eicosanoids are formed in larger quantities compared to those generated from n-3 FA, thereby promoting inflammation. Arachidonic acid is the main precursor to n-6 eicosanoids which are responsible for the secretion of the pro-inflammatory cytokines (von Schacky and Harris 2007). Alpha-linolenic acid, EPA and DHA reduce the secretion of the same pro-inflammatory cytokines to achieve a healthy balance in the body. It is proposed that an adequate amount of ALA present would provide an inhibitory action of the elongation and desaturation of LA, thereby shifting the metabolic pathway to favor the production of longer-chain FA and consequently decrease the amount of AA produced (Nannicini and others 2006). The longer-chain FA can directly replace AA as an eicosanoid substrate, inhibiting AA metabolism (Calder 2006a). These longer-chain FA can also act indirectly by altering activation of transcription factors, which would alter the expression of the inflammatory genes (Calder 2006a).

The fatty acid compositions of typical Western diets generally contain 20% AA. These n-6 FA are stored in the cell membrane phospholipid bilayer. When released, the FA are mobilized by phospholipase A₂ such that the free fatty acid becomes substrate for the generation of eicosanoids. Metabolism by cyclooxygenase-2 (COX-2) enzyme is responsible for the high production of prostaglandin-2 (PGE2), which has a number of pro-inflammatory effects (von Schacky and Harris 2007). These pro-inflammatory eicosanoids then induce increased levels of the pro-inflammatory
cytokines. Conversely, the n-3 FA replace AA and LA in the phospholipids and force a redistribution of the n-6 FA from the phospholipids into cholesterol esters and triglycerides (Weber and Leaf 1991). In addition, the n-3 FA may shift the delta-6 desaturase activity to act on ALA and suppress the production of these pro-inflammatory eicosanoids and subsequently pro-inflammatory cytokines. This may lead to a physiologic state containing anti-inflammatory properties (Smith and others 2006).

Previous research regarding the therapeutic use of n-3 FA for acute and chronic inflammation has focused primarily on the longer-chain n-3 FA: EPA and DHA. Studies exploring the potential benefits of ALA are few in number. Chronic inflammatory diseases affect millions of people across the US and include but are not limited to rheumatoid arthritis (RA), CVD and osteoporosis.

*Rheumatoid arthritis*

Rheumatoid arthritis is a chronic inflammatory disease depicted by inflammation of synovial tissue in the joints, where pro-inflammatory cytokines are detected in synovial fluids and serum of RA patients (Rindfleisch and Muller 2005). Approximately 0.8% of the adult population worldwide is affected. Without treatment, severe joint damage may occur. Researchers explored whether supplementing diets of patients with RA with n-3 FA would alleviate inflammation in affected joints.

Sundrarjun and colleagues (2004) had three groups of subjects diagnosed with RA, where two groups (FO and placebo) were provided dietary advice to consume diets low in n-6 FA. The FO group consumed four FO capsules daily, containing 3.36 g/day n-3 FA (1880 mg EPA and 1480 mg DHA), in addition to their background medication. The EPA+DHA content of the placebo capsules was not reported. The last group (control) was not given any dietary advice or capsules. Subjects in the FO and placebo groups were given dietary advice for the first six weeks, followed by the treatment period for 12 weeks, and concluded with a 6-week follow-up period. Starting with 60 subjects, the study population diminished to 35 (13 FO, 13 placebo and 9 control) by the end of the treatment period. Serum cytokine concentrations of IL-6 and TNF-α were measured at baseline and weeks 6, 18 and 24. Serum TNF-α concentration at week 24 in the FO group was 40.7% lower than TNF-α in the control group; however, this was not significantly different. Of the remaining FO and placebo subjects, significant
decreases in serum concentrations of IL-6 were seen, suggesting that a diet low in n-6 FA was equally effective as FO supplementation.

Cleland and colleagues (2006) conducted a long-term study lasting three years, with subjects who had recently been diagnosed with RA. There were 18 subjects in the FO group, who were advised to add FO to juice daily to consume 4.5-5 g EPA + DHA for the three years. Cellular concentrations of AA and PGE2 were measured every three or six weeks in FO and control (no supplement therapy) subjects. After the three years, AA synthesis in platelets and PBMCs were significantly lower in the FO group (-30% and -40%, respectively) compared to control group, in addition to a significant decrease (-41%) in PGE2 production.

There is evidence of clinical efficacy of the use of FO as adjunctive therapy in patients with RA, as seen with a reduced number of tender joints and amount of morning stiffness (Kremer 2000). It is predicted that dietary FA alter the metabolism of the pro-inflammatory eicosanoids to aid in the amelioration of inflammation. However, ALA has not been shown to be similarly effective in RA patients.

Nordstrom and colleagues (1995) conducted a placebo-controlled trial with ALA, as it is a cheaper source of EPA and DHA, in hopes of observing similar beneficial clinical effects as previously observed with EPA and DHA. Patients with RA consumed ALA (treatment group, n=11) or LA (placebo group, n=11). Treatment group subjects ingested 30 g flaxseed oil (32% ALA) while placebo group subjects consumed 30 g safflower seed oil (33% LA) daily for three months. As expected, after the 3-month supplementation period, the treatment group had a significant increase in serum ALA concentration while the placebo group had a significant increase in serum LA concentration. Blood coagulation time increased in the treatment group, supporting the possible use of ALA as a form of therapy for RA and other inflammatory diseases. However, clinical benefits such as global assessment, classification of functional status, joint score index, visual analogue scale and pain tenderness score did not show any statistical differences. Cellular concentrations of AA, EPA and DHA did not change, despite the significant increase in ALA in the treatment group, thus, confirming the low conversion rate of ALA to its longer-chain derivatives. These authors concluded that supplementation of ALA for three months was not beneficial for RA therapy.
It can be concluded from the aforementioned studies that FO (rich in EPA and DHA), but not their precursor, ALA, aids in the attenuation of pro-inflammatory cytokines and shows clinical benefits in RA patients who are currently taking medication for RA. Therefore, EPA and DHA appear to be an effective supplemental therapy for the treatment of RA. In addition, FO has been found to have protective effects on the cardiovascular system as well.

Cardiovascular disease

Cardiovascular disease is an umbrella term to describe diseases that affect the heart and blood vessels (veins and arteries), which is the leading cause of mortality and morbidity in Western countries. Commonly known types of CVD include coronary heart disease (CHD), stroke, coronary artery disease, angina, arrhythmia, heart failure/attacks and myocardial infarction. Inflammation is recognized to play a key role in the pathology of CVD (Calder 2006b). Atherosclerosis, a main cause of CVD, is the accumulation of lipids within artery walls. The formation of this plaque limits the artery’s flexibility or reactivity and eventually becomes hardened. The thickened and stiff artery decreases blood flow and reduces the oxygen supply to vital organs of the body, possibly leading to organ dysfunction. Lesions of atherosclerosis are recognized as an inflammatory disease (Ross 1999). For example, specific to coronary artery disease, these lesions cause endothelial dysfunction that alters the homeostatic properties of the artery’s endothelium, producing pro-coagulant properties forming vasoactive molecules, cytokines and growth factors (Ross 1999). The inflammatory response stimulates migration and proliferation of smooth muscle cells which can thicken artery walls. The pro-inflammatory cytokines increase binding of lipids to the endothelium and smooth muscle, further upregulating the inflammatory response (Patrick and Uzick 2001).

The importance of dietary \( n-3 \) FA in the role of anti-inflammation was first noted in epidemiologic observations of the low occurrence of autoimmune and inflammatory diseases in Greenland Eskimos compared to gender- and age-matched populations in Denmark (Kromann and Green 1980). It was reported that in 1976, only 3.5% of all deaths in native Greenland Eskimos were heart disease related. When their blood samples were compared to Danish controls, the Greenland Eskimos had lower concentrations of plasma cholesterol, triglycerides, low-density lipoproteins (LDL), and
very-low-density lipoproteins (VLDL) and a higher concentration of high density lipoproteins (HDL) (Dyerberg and others 1975).

The dietary composition of Greenland Eskimos was later compared to that of Danes (Bang and others 1980). There was a significantly lower level of saturated fat in the Greenland Eskimo diet along with a high level of polyunsaturated FA compared to the Danish diet. A closer examination of the dietary fat composition of Greenland Eskimos revealed that the diet was composed of 13.1% EPA and DHA, whereas the Danish diet contained a mere 0.8%. This high dietary intake of n-3 FA was due to the high availability of seafood in the environment for Greenland Eskimos. Compared to their Western ethnic counterparts, Greenland Eskimos had a lower incidence of chronic inflammatory diseases and myocardial infarction (Simopoulos 2002b).

In a multivariate analysis of diets and CHD in various countries, it was reported that these countries with the lowest mortality rates for CHD were associated with the highest intake of n-3 FA intakes (Keys 1980). Numerous studies have demonstrated the benefits of the longer-chain n-3 FA on CVD (Breslow 2006; Wang and others 2006; von Schacky and Harris 2007). The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico (GISSI)-Prevenzione trial was a multi-center randomized, intervention study conducted from October 1993 to September 1995 (GISSI-Prevenzione Investigators 1999). Patients (n = 11324) with recent myocardial infarction were enrolled in the study and randomly assigned to one of four treatment groups [n=2836, 850-882 mg/day EPA+DHA; n=2830, 300 mg/day vitamin E; n=2830, daily combination of n-3 FA and vitamin E; and n=2828, no supplement (control)]. The patients resided in Italy and regularly followed Mediterranean dietary habits. The trial procedures mimicked the routine care after myocardial infarction, including follow-up visits at 6, 12, 18, 30 and 42 months. Primary combined efficacy endpoints were death, non-fatal myocardial infarction, and stroke. After the trial period, investigators found that patients who had any EPA+DHA treatment had significantly lower risk of these primary endpoints, whereas the vitamin E had no benefit. These investigators concluded that long-term consumption of 1 g n-3 FA daily was beneficial for decreasing risk for overall and cardiovascular death.
In a randomized, placebo-controlled study based in India, Singh and colleagues (1997) had 360 patients (mean age = 48-49 years) with acute myocardial infarction consume either FO (n=122, 1.08 g EPA, 0.72 g DHA), mustard seed oil (n=120, 2.9 g ALA) or a placebo (n=118, 100 mg aluminum hydroxide) daily. All patients were advised to follow a low-fat diet. Cardiac events, such as cardiac deaths and nonfatal infarctions, were followed and reported in the three groups during one year of supplementation. The FO and mustard seed oil groups both had significantly fewer cardiac events at week 28 and the 1-year intervals compared to the control group. In addition, a significant decrease in cardiac arrhythmias, angina pectoris and left ventricular enlargement events were seen in the intervention groups. The authors concluded that both FO and mustard seed oil prevented cardiac events.

Very few studies have found beneficial effects of ALA, although most of these studies had small sample sizes or poorly designed methods (Harper and Jacobson 2005). In an epidemiologic study, the relation of reported intake of ALA and prevalent coronary artery disease in 4406 participants (mean ages = 51.8 years for males, 52.4 years for females) of the National Heart, Lung and Blood Institute Family Heart Study was analyzed (Djoussé and others 2001). Mean intake of ALA was 0.81 g/day for men and 0.68 g/day for women. Alpha-linolenic acid intake was inversely associated with the prevalence of coronary artery disease, when subjects were partitioned into quintiles of ALA intake. Although there is no proof of a causal relationship in this study, these results were interesting.

A recent meta-analysis of 14 research studies examined ALA and the cardiovascular risk markers of fibrinogen and fasting plasma glucose (Wendland and others 2006). All studies included at least four weeks of treatment using some form of ALA supplementation. Small reductions in fibrinogen concentration (-0.17 μmol/L) and fasting plasma glucose (-0.20 mmol/L) were seen in subjects with ALA supplementation. No other significant clinical findings were observed. Most trials had small sample sizes, and not all methods included blinding or randomization of treatments, suggesting a limited quality of these existing studies. At least one large randomized controlled trial is necessary before any recommendation for ALA can be made for patients with CVD. It
is likely that the doses of ALA in the studies were not large enough to be converted to EPA and/or DHA to play significant roles in lowering biomarkers that lead to CVD.

**Osteoporosis**

Osteoporosis is a problematic skeletal condition characterized by low bone mass and microarchitectural deterioration of bone tissue in which calcium and other minerals are depleted. This disorder of the skeleton can lead to an increased risk of bone fracture, more commonly seen among older adults. Previous clinical observations have shown that osteoporosis occurs simultaneously with inflammation in the same body regions (Ginaldi and others 2005). Recently, proinflammatory cytokines have been linked to different stages in the bone remodeling cycle.

An epidemiologic study with older Rancho Bernardo residents (mean ages: male = 72.9 years and female = 74.0 years) was conducted to examine relationships between the dietary \( n-6:n-3 \) FA ratio and bone mineral density (BMD) at the hip and lumbar spine. Subjects completed a FFQ, and BMD was measured using dual energy X-ray absorptiometry (DXA). A significant inverse association between the \( n-6:n-3 \) FA ratio and BMD at the hip was reported in both men and women. These results were independent of age, body mass index (BMI), and lifestyle factors (Weiss and others 2005). The average \( n-6:n-3 \) FA ratio was 8.4:1 in men and 7.9:1 in women.

Van Papendorp and others (1995) conducted a study with osteoporotic women (mean age=80 years) residing in an extended care facility. Subjects were divided into four treatment groups and fed 4 g of: (1) evening primrose oil (\( n-6 \) FA); (2) FO (\( n-3 \) FA); (3) mixture of evening primrose oil and FO; or (4) olive oil, for 16 weeks. An increase in osteocalcin, a bone formation biomarker, was found in both FO groups compared to the evening primrose oil and olive oil groups. In a separate study, Kruger and others (1998) supplemented postmenopausal women with low BMD with 6 g of an evening primrose and FO mixture, while a control group of women received 6 g of coconut oil. In addition, all women were supplemented with 600 mg calcium carbonate daily for 18 months. Calcium intake remained constant between treatment groups. Lumbar spine and femoral neck BMD decreased in the coconut oil group, while lumbar spine BMD remained the same and femoral neck BMD increased in the oil mixture group (Kruger
and others 1998). These clinical studies in older adults suggest that the ratio of dietary $n$-6:$n$-3 FA may affect bone health.

Few published studies have examined the role of flaxseed (rich plant source of $n$-3 FA) on bone health. Lucas and colleagues (2002) provided 36 women with 40 g ground flaxseed or wheat germ daily for three months. The $n$-3 FA content from flaxseed was not published. Each subject was also provided with a supplement of 1000 mg calcium and 400 IU vitamin D. There were no significant differences in bone metabolism biomarkers between the treatments. Dodin and colleagues (2005) conducted a 12-month study in which 179 women were supplemented daily with 40 g flaxseed (9.12 g $n$-3 FA) or wheat germ in ground form as well as incorporated into baked products. Serum total and HDL cholesterol concentrations were lower in the flaxseed group; however there were no differences in BMD (at L2-L4 lumbar vertebrae and femoral neck) between groups. These two flaxseed supplementation studies were unable to demonstrate any appreciable improvements in BMD or bone turnover biomarkers.

In contrast, in a double-blind crossover study, 38 women consumed either 38 g of flaxseed (8.5 g $n$-3 FA) or sunflower seed in the form of muffins and bread, daily for six weeks. During the flaxseed treatment, subjects experienced lower serum tartrate-resistant acid phosphate activity (bone resorption biomarker), but not bone-specific or total alkaline phosphatase activities (bone formation biomarkers) in comparison to the sunflower seed treatment (Arjmandi and others 1998).

A randomized, double-blind, balanced order, three-period crossover study with 23 overweight or obese subjects (mean age = 49.3 years) who were diagnosed as moderately hypercholesterolemic, was conducted by Griel and others (2007). Most subjects were males, and the females were postmenopausal. Each subject consumed the specific diets for six weeks, with a 3-week washout period in between diets. The three diets were: 1) an average American diet (control), providing a 9:1 $n$-6:$n$-3 FA ratio; 2) high LA diet, providing a 3.5:1 $n$-6:$n$-3 FA ratio; and 3) high ALA diet, providing 1.6:1 $n$-6:$n$-3 FA ratio. Walnuts were the main source for the essential FA, and flaxseed was added to provide additional $n$-3 FA into the high ALA diet. Compliance with diets was recorded by staff dietitians. Serum samples were collected and analyzed for bone-
specific alkaline phosphatase and N-telopeptide of type I collagen (bone formation and resorption biomarkers, respectively). TNF-α concentration was also measured after each diet period. The high ALA diet significantly lowered N-telopeptide of type I collagen concentration and TNF-α concentration in comparison to the control diet, whereas the bone-specific alkaline phosphatase concentration was unaffected by diet. These findings confirm a beneficial effect of n-3 FA and a reduced n-6:n-3 FA ratio on bone health, and suggest that the incorporation of n-3 FA, including those from plant sources, may provide health benefits to the skeletal system.

Although previous studies are equivocal, attenuation of bone loss with the addition of flaxseed and other plant sources of ALA, has unexplored potential. The current imbalanced n-6:n-3 FA ratio, can be improved by not only increasing the n-3 FA intake but also decreasing the n-6 FA intake. This, in turn, will aid in the attenuation of the risk of the onset of inflammatory diseases, such as CVD, RA and osteoporosis. It is still unclear whether ALA itself has a suppressive effect on pro-inflammatory cytokines, by modulation of AA activity or from its conversion of ALA to EPA. In order to fill the void of n-3 FA in the diet, many food manufacturers are researching and developing new food products to increase the dietary n-3 FA intake, if not decrease n-6 FA intake as well.

**Functional Foods**

As more consumers become aware of the potential therapeutic benefits of n-3 FA on the attenuation of chronic inflammation, an increased interest and demand for food and beverage products containing n-3 FA will occur. A growing trend towards self-medication with natural products, such as foods, has been observed, in response to increasing costs of prescription drugs and health care (Brouns and Vermeer 2000). These food and beverage products with potential health benefits are referred to as functional foods. The term *functional food* has many definitions ranging from simple to elaborate, without an established consensus in the US. The Institute of Food Technologists’ Expert Panel defines functional foods as “foods and food components that provide a health benefit beyond basic nutrition (for the intended population)” (Institute of Food Technologists 2005). The market for functional foods is dramatically growing, and the food industry is actively and intensely searching for natural food
ingredients that may provide positive effects on health, disease prevention or management (Brouns and Vermeer 2000).

During the late 20th century, functional foods and beverages found on the market were based on general discoveries in nutritional scientific research rather than deliberate research on specific functional food products. The most common products available were sport drinks, probiotic dairy products, heart-healthy spreads and ready-to-eat cereals (Weststrate and others 2002). The functionality claims of these products include improved gut and heart health, better immunity and higher energy levels. Interest in developing functional foods targeted at chronic diseases (based on more deliberate nutritional research) is currently growing and is predicted to emerge into the market soon enough. There is already a developed market for health-driven foods in the form of vitamin and mineral fortification of popular foods for the general public (Weststrate and others 2002). The consumer drive for buying healthful foods and beverages coupled with nutritional scientific evidence will aid in the development of an active functional foods and beverage market (Milner 2002). In 2000, the total market for functional ingredients, foods, beverages and dietary supplements was estimated at $50 billion. This was a 7% increase over the previous year. An overall growth rate of 10% per year for the next five years is possible, potentially outperforming the current foods and beverage market growth rate of 2% per year (Weststrate and others 2002). Weststrate and colleagues (2002) suggest that the area of functional foods is beginning to come of age and its development will be driven by exciting nutritional scientific discoveries which are currently taking place.

In response to the qualified health claim issued for EPA and DHA in 2004 by the FDA (Harris 2007; Packaged Facts 2007), food manufacturers began formulating various enriched functional food and beverages with marine derived FA to fill the void of sufficient n-3 FA intake in the American diet. In early 2006, it was reported that EPA- and DHA-enriched foods and beverages have entered mainstream US supermarkets (Packaged Facts 2007). In a recent newspaper survey, n-3 FA were identified as one of the “hottest food additives” of 2007 (Horovitz, 2007). According to a HealthFocus USA Trend Survey, four out of 10 adults are seeking to add more n-3 FA into their diets (Horovitz, 2007).
Omega-3 fatty acid enriched animal products

There has been much effort to increase the n-3 FA content of animal products (i.e. milk, meat and eggs) by the enrichment of animal feed. Animal feed enrichment has successfully increased the concentrations of ALA, EPA and/or DHA in the tissues and products of the animals such as muscles or the egg yolk (Kolanowski and Laufenberg 2006). In addition, the fatty acid profile of fluid milk has been modified to include greater amounts of unsaturated FA compared to normal bovine milk. However, the enrichment of animal products with n-3 FA raises concerns of whether significant organoleptic changes occur.

Fluid milk

Attempts to modify the FA profile of cow’s milk have been carried out. Milk fat is typically composed of 5% polyunsaturated FA, 25% monounsaturated FA and 70% saturated FA. The Wisconsin Milk Board indicated at their 1988 Milk Fat Roundtable that the ideal profile for milk fat should be composed of 10% polyunsaturated FA, 82% monounsaturated FA and only 8% saturated FA (Grummer 1991). Unfortunately, this cannot be accomplished solely with the alteration of cattle feed. Unsaturated FA are converted to saturated FA from rumen biohydration, resulting in a high content of saturated FA in milk fat despite the cattle’s consumption of a diet rich in unsaturated FA. There has been previous success in lowering the saturated FA content of milk fat by various methods. The infusion of fatty acids post-ruminally has been shown to decrease short and medium chain FA with an increase in oleic acid (monounsaturated FA) (Mansbridge and Drake 1997). Another option is to encase and protect the lipid present in the feed. For example, the addition of a protective whey protein coating around a lipid core preserves the lipid content within, while present in the rumen. The lipid core is released post-ruminally, and the unsaturated FA is available for absorption by the small intestine (Heguy 2006). More innovative approaches to modify the fat composition of milk are still underway.

Eggs

Eggs have been described as the ideal model for the transfer of dietary lipids (Bourre 2005). The intestinal physiology of hens allow for preservation of polyunsaturated FA content of their feed, producing a consistent enrichment of n-3 FA
into the egg yolk (Bourre 2005). Researchers have previously supplemented hen diets with EPA-rich marine algae (Nitsan and others 1999), DHA-rich marine algae (Herber-McNeill and Van Elswyk 1998), FO or flaxseed oil (Ferrier and others 1995). All were able to successfully deposit the specific n-3 FA into the egg yolk.

Consumer acceptance panels were conducted with these nutritionally enriched eggs, without a unanimous conclusion. Some consumers indicated a detection of a significant fishy off-flavor (Jiang and others 1992), where others found little to no difference in the flavor of the enriched eggs compared to control eggs (Van Elswyk 1997; Farrell 1998; Lewis and others 2000a). Eggs were scrambled, hard-boiled or poached, without additional seasonings. Consumers used various hedonic scales to indicate the palatability of egg dishes. The differences in palatability of the eggs may depend on source of n-3 FA as well as the amount added into the hens’ diets.

The consumption of enriched eggs laid by hens on a flaxseed feed produced significant increases in plasma concentrations of ALA, DHA and total n-3 FA of the consumers of these enriched eggs compared to control eggs. Twenty-eight normolipidemic subjects consumed either four n-3 FA enriched or four standard eggs per day for two weeks. No significant changes were observed in concentrations of total cholesterol, HDL or plasma triglycerides. Lewis and colleagues (2000a) had 23 hypercholesterolemic patients incorporate either 12 n-3 FA enriched eggs or 12 standard eggs per week into a low-fat diet for six weeks. Similar to results of Ferrier and others (1995), these 23 subjects showed no changes in serum total cholesterol or LDL concentrations but a significant decrease in serum triglyceride concentration (Lewis and others 2000a).

There is general concern that high egg intake results in high blood cholesterol and the onset of coronary artery disease. Although previous experimental studies have shown slight increases in serum cholesterol (~ 1-3%) with high egg intake (Kritchevsky 2004), more current clinical studies indicate no significant changes in plasma triglycerides, cholesterol or HDL concentrations (Ginsberg and others 1994; Ferrier and others 1995; Lewis and others 2000a). Interestingly, analysis of National Health and Nutrition Examination Survey (NHANES III, 1988-1994) data has shown that consumers who ate ≥4 eggs per week had lower average serum cholesterol concentration...
compared to consumers who ate ≤1 egg per week (Song and others 2000). Previous AHA guidelines instructed consumers to limit egg intake due to the misconception that the dietary cholesterol found in eggs appreciably increased serum cholesterol concentration (Kritchevsky 2004). The AHA revised their guidelines to focus on dietary patterns rather than specific foods such as eggs.

**Coldwater fatty fish**

Coldwater fatty fish are the primary dietary source for the longer-chain n-3 FA: EPA and DHA. Due to a limited resource of wild fish, aquaculturers resort to fish farming to increase the availability of fish for consumption. Although the fish supply for consumption increases, it has been noted that cultured fish contain less n-3 FA than fish naturally grown in the wild (Hamilton and others 2005). Farm-raised salmon has been reported to have more than 3-fold the n-6:n-3 FA ratio than wild salmon. Analysis of wild yellow perch also demonstrated a significant lower n-6:n-3 FA ratio compared to the farm-raised yellow perch. Additionally, the wild yellow perch had greater amounts of ALA and EPA, although total n-3 FA content did not differ from the farm-raised yellow perch (Gonzalez and others 2006).

It is proposed that n-3 FA enriched feed can lower the n-6:n-3 FA ratio and match the lower ratio naturally present in wild fish. It is widely recognized that the FA composition of fish changes easily to reflect the feed composition (Pickova and Morkore 2007). Seierstad and colleagues (2005) cultivated Atlantic salmon with varying levels of FO and/or rapeseed oil. Fifty-eight patients with CHD consumed 700 g of the tailored salmon weekly for six weeks, while maintaining their cardiovascular medication regimens throughout the study period. After the dietary intervention period, all subjects showed significant decreases in serum concentrations of total n-6 FA, AA and total cholesterol. Subjects who consumed the fish fed the greatest amount of FO had significant decreases in concentrations of serum EPA, total n-3 FA and the n-6:n-3 FA ratio. These subjects also had significant decreases in vascular inflammatory biomarkers such as IL-6 and TNF-α. The subjects who consumed the fish fed the greatest amount of rapeseed oil did not share these results, suggesting the FO had more beneficial effects on vascular inflammation than rapeseed oil. A major strength of this study was the ability to follow the FA composition of the fish feed, to the lipid profile
of the fish fillet and further observe the effect on serum lipids in these consumers. However, all subjects were instructed to continue their medication regimen, which may have had a confounding effect on serum lipid measurements.

Sensory evaluation of salmon fillets has shown that salmon feed supplemented with FO, produced an undesirable fishy odor as well as less acceptable color and taste compared to salmon feed that contained soybean oil or erucic acid with rapeseed oil (Thomassen and Rosjo 1989). However, consumers who evaluated baked filets of yellow perch for overall flavor were unable to detect a difference between wild and farm-raised yellow perch (Gonzalez and others 2006).

Fortunately, farm-raised fish still contains high levels of $n$-3 FA and can be consumed to help lower the dietary $n$-6:$n$-3 FA ratio. However, tailored or wild fish would be more efficient in decreasing the $n$-6:$n$-3 FA ratio.

*Chicken broiler meat*

Aside from eggs, $n$-3 FA-enriched animal feed has also been used to manipulate the white and dark meats of broiler chickens. The diets of broiler chickens were supplemented with varying amounts of flaxseed oil and/or FO (Lopez-Ferrer and others 2001a, 2001b; Rymer and Givens 2006). Rymer and Givens (2006) compared the $n$-3 FA rich diets to a control diet high in vegetable oil (low in $n$-3 FA). Meat quality was analyzed for FA composition in thigh samples. The supplemented diets lowered the LA concentration in poultry meat compared to the control diet (Rymer and Givens 2006). Alpha-linolenic acid concentration significantly increased in all of the supplemented diets, with the greatest increase seen in the higher concentrated ALA diet. The concentrations of the longer-chain FA were also significantly increased; however, the FO diets deposited more than 3-times the EPA and DHA than the flaxseed diets. This demonstrated the inefficiency of conversion of ALA to its longer-chain derivatives in poultry.

Sensory evaluation was performed only on thigh samples from chickens fed the FO enriched diets (Lopez-Ferrer and others 2001a). The highest dose of FO (4% by weight) produced an unacceptable odor and those samples were not evaluated. The thigh samples of chickens fed a combination of fish and linseed oils were most palatable, and consumers found it similar to the control (Pawlosky and others 2001).
However, sufficient details describing the sensory evaluation portion of the study were lacking. It is unknown whether the thigh samples of the chickens solely fed flaxseed oil had defects in its flavor, or how they compared to the evaluated thigh samples.

Lewis and colleagues (2000b) estimated that an intake of three longer-chain $n$-3 FA-enriched eggs provided the equivalent amount of $n$-3 FA as one serving of fish. The replacement of standard eggs with $n$-3 FA enhanced eggs may be a feasible option to increase the $n$-3 FA intake in the diet, especially for low fish-consuming populations.

**Livestock meat products**

Fish oil is not generally used in the supplementation of livestock animal feed, due to the potential production of a fishy off-flavor derived from lipid oxidation of the longer-chain $n$-3 FA. Instead, research has focused on the addition of flaxseed into the animal feed to increase $n$-3 FA content in the animal carcasses. In previous zootechnical studies, 5-6% flaxseed diets were fed to lamb, pig and cattle feeds and growth and carcass characteristics were observed (Weill and others 2002; Kouba and others 2003). The $n$-6:$n$-3 FA ratio was reduced in animals fed experimental diets compared to animals fed a standard livestock diets (no ALA added). Kouba and colleagues (2003) demonstrated increased levels of ALA and EPA in plasma, muscle and adipose tissue, while DHA remained stable, as confirmed by Romans and colleagues (1995) who found increases in ALA and EPA concentrations in the raw belly, longissimus thoracis and liver of pigs fed a flaxseed-enriched diet. Weill and colleagues (2002) conducted a double-blind, randomized, cross-over clinical trial with 75 healthy subjects who consumed livestock products (flaxseed vs. standard livestock diet). An increase in concentrations of ALA and its $n$-3 FA derivatives were found in the consumers’ plasma after consumption of the enriched meats. The consumers of the flaxseed-fed livestock products also had a reduction in their $n$-6:$n$-3 FA ratio compared to those who consumed the standard diet-fed livestock products. Hedonic and consumer preference was evaluated on the lamb, beef and pork meats for the color, taste, flavor, softness and juiciness attributes. For the lamb and beef products, no significant differences in consumer preference were noted; whereas a preference towards the control sample was seen for some pork products. Interestingly, there was a preference towards ham products from flaxseed-fed swine (Weill and others 2002). In higher-fat livestock
products, trained panelists were able to identify those derived from flaxseed-fed animals, as seen in bacon compared to pork loin products (Romans and others 1995). It may be concluded that the inclusion of flaxseed in livestock diets is a feasible method to improve the nutritional value without significant sensory losses, especially in the lower-fat livestock meat products (Romans and others 1995; Weill and others 2002; Maddock et al 2006).

**Health concerns**

There is potential to replace animal products low in \(n\)-3 FA, with its nutritionally enhanced alternatives to help increase dietary \(n\)-3 FA intake. However it is important to keep in mind that when increasing the amount of animal products in the diet, an accompaniment of an increase of animal fat intake occurs. Although the fats are not all composed of saturated FA (as in normal non-enriched meat products), consumption of such high amounts of animal products may raise health concerns of excessive energy intake, weight gain and obesity.

Seafood, especially fatty fish are the best inherent food source of the long chain \(n\)-3 FA, EPA and DHA. There is concern that increased consumption of seafood may lead to a higher intake of impure marine oils. The uncertainty of the purity of the marine oils has raised apprehension about toxins such as parachlorobenzoic acid, Dichloro-Diphenyl-Trichloroethane, dioxin and mercury, which may be present in the marine origin foods (Mahaffey 2004; Melanson and others 2005; Garg and others 2006). It has not been determined whether the costs (monetary, natural resources, etc) of animal feed enrichment are greater than fortifying food products to deliver \(n\)-3 FA. Even though \(n\)-3 FA enriched animal sources have been successful in increasing dietary intakes of \(n\)-3 FA, plant sources may provide a more optimal avenue to decrease the \(n\)-6:\(n\)-3 FA ratio. The use of plant derived \(n\)-3 FA products into the diet is growing, as more formulations of novel food products are available.

**Formulations of functional foods using omega-3 fatty acids**

It has been strongly suggested by the AHA and the United Kingdom Scientific Advisory Committee on Nutrition to consume fish and fish products (including FO capsules) at least twice a week to achieve a “healthy intake” of \(n\)-3 FA in the diet (Whelan and Rust 2006). However modern Western societies seldom consume
sufficient fish or fish products to fulfill this recommendation and FO capsules are not suitable for daily use (Mantzioris and others 2000). Due to the small amount of $n$-3 FA in each FO capsule, a consumer would need to take at least 4-6 FO capsules in order to attain the large dose necessary for any beneficial effect. Consequently, for many consumers, it is unlikely that they will incorporate fish products and/or FO capsules into their diets due to high cost, aversion to fishy flavors and inconvenience (Davidson and others 1991). Formulation of $n$-3 FA enriched food products is a practical alternative to fill the void by increasing the dietary intake of $n$-3 FA and lowering the dietary $n$-6:$n$-3 FA ratio. Without dramatically altering a consumers’ dietary habits, it may be feasible to increase the intake of $n$-3 FA and benefit from its anti-inflammatory properties with the incorporation of $n$-3 FA enriched products.

As implied earlier, the $n$-3 FA enriched functional food and beverage market is rapidly growing. In 2006, an estimated $2$ billion was spent on retail sale of these enhanced products (Packaged Facts 2007). This was a 111.5% growth from retail sales in 2005. An annual growth of 60.3% is predicted to occur over the next few years, with $7$ billion in retail sales in 2007 (Packaged Facts 2007).

Previous efforts to incorporate $n$-3 FA into foods have been performed with various products. Lovegrove and colleagues (1997) recruited nine middle-aged (mean age = 50 years) normotriglycerolaemic British males to participate in a randomized, single-blind, controlled crossover study. Each treatment period lasted 22 days with a 5-month washout period in between treatments. Men used either food products formulated with higher levels of EPA and DHA or their non-enriched counterparts for the treatment periods. The researchers had originally hoped for the men to consume an average daily intake of 1.8 g EPA + DHA. Enriched products included sweet products such as baked goods, ice cream and milkshakes, pasta, mayonnaise, vinaigrette and table spread. Most of these enriched products were not regularly consumed in the subjects’ diets; therefore, these men were only capable of consuming 1.4 g EPA+DHA per day. It is also likely the amounts of longer-chain FA present in the enriched food products may have been too small and the subjects were unable to consume enough of the products to attain the targeted 1.8 g EPA+DHA without overeating and gaining weight.
Plasma and phospholipid fatty acid concentrations were measured, and significant increases in EPA + DHA were seen after consumption of the enriched products (Lovegrove and others 1997). This demonstrates that the longer-chain FA of the enriched products were incorporated into plasma and phospholipids in the body.

Mantzioris and colleagues (2000) recruited 15 healthy Australian males (mean age = 37.7 years) to use n-3 FA rich foods at home for four weeks. These food products included ALA-rich cooking oil, margarine, salad dressing, mayonnaise and muffin mix and EPA + DHA enriched sausages and French onion dip. The selection of products used was composed of mostly savory products. The subjects were also provided with canned fish products and ground flaxseed. Food products were commercially manufactured by the Australian company, Meadow Lea Foods and were chosen because they were core items in the Australian diet.

These male subjects had an average intake of 9 g/day of ALA and 1.83 g/day of DHA+EPA, equivalent to consuming six FO pills daily (Mantzioris and others 2000). The n-3 FA were efficiently incorporated into plasma, demonstrated by significant increases in plasma concentration of the n-3 FA. Alpha-linolenic acid significantly increased 3-4-fold in the first two weeks, but little additional change occurred after two weeks. Eicosapentaenoic acid significantly increased 2.5-fold in the first two weeks, and further increased afterwards, but not significantly so. Docosahexanenoic acid significantly increased 1.5-fold in the first two weeks, but no change was seen afterwards. It is likely that ALA and DHA capacity levels were reached within two weeks while the body continued to incorporate more EPA into tissues. Conversely, both LA and AA significantly decreased due to the higher availability of ALA in the system to react with delta-6-desaturase.

Concentrations of pro-inflammatory cytokines (IL-1β and TNF-α) both decreased in response to the diet in the first two weeks. However, TNF-α increased after two weeks, suggesting that the decrease may not have been associated to the n-3 FA enriched diet. Instead, increases in TNF-α concentrations may be due to non-inflammatory macrophages in response to other bodily ailments.

Metcalf and colleagues (2003) conducted a similar study, where 16 healthy Australian men (mean age = 39.2 years) incorporated n-3 FA enriched food products
into their diets for four weeks. Similar food products were used as in the study by Mantzioris and others (2000). Subjects significantly increased their \( n \)-3 FA intake and coincidently decreased their \( n \)-6 FA intake. Their BMIs also increased significantly in the first two weeks of the study. After two weeks of the dietary intervention period, subjects had significant increases in their average concentrations of plasma ALA (56%), EPA (174%), DHA (80%) and total \( n \)-3 FA (79%), as well as notable decreases in plasma LA (-10%), AA (-3%) and total \( n \)-6 FA (-6%). The only notable change in plasma fatty acid concentration between week two and four was a -19% decrease in ALA. Cytokine concentrations were not measured in this study.

Patch and colleagues (2005) recruited 75 overweight males and females to participate in a double blind, randomized, controlled 6-month study. Thirty-eight subjects incorporated longer-chain \( n \)-3 FA enriched foods, while remaining subjects consumed control counterparts of the food products. Specific dietary \( n \)-3 FA intake was determined, and ALA intake increased by 75%, EPA by 490% and DHA by 370%. Additionally, the \( n \)-6:\( n \)-3 FA ratio decreased by 5%. Since the food products were enriched with the longer-chain \( n \)-3 FA, these dietary increases were expected. It was estimated that subjects consumed an average of 1 g EPA+DHA from the enriched foods over the study. Some limitations of this study were that it was not a crossover study, limiting the generalization of the results. In addition, neither plasma concentration of FA nor cytokines were determined.

Various food products have been fortified with \( n \)-3 FA, both in the sweet and savory markets. There are currently more food products fortified with \( n \)-3 FA than enriched beverages. However, as the functional food and beverage market grows, it is expected for enriched beverages to become more common, popular and available in the market.

**Previous omega-3 fatty acid enriched beverages**

A survey was conducted in the October 2005 issue of Food Technology providing subscribers a list of foods and asking which held “the greatest promise as a delivery vehicle for nutraceuticals” (Institute of Food Technologists 2006). Approximately 45% of the 327 respondents chose beverages and juices as the most promising delivery vehicle for health-promoting ingredients (Institute of Food
Many beverages are often consumed not only to help provide moisture to foods in the meal, but also for their nutritive value. It is not uncommon to drink a glass of orange juice or a bowl of chicken noodle soup with the belief that it helps prevent colds or nourish the body (Hazen 2003).

The functional beverage industry has been declared as one of the fastest growing segments of the food industry (Boland and others 2001; Hazen 2003; Sharma 2005). According to market research performed by a New York-based Beverage Marketing Corporation, the functional beverage industry is predicted to account for 85% of beverage sales by 2008, surpassing carbonated soft drinks as the largest non-alcoholic beverage category. Nutrient enriched beverages are projected to increase 84% in sales during the 2004-2008 period (Dairy Management Inc 2005). Market research performed by Mintel International Group reported the functional food market in the US to be worth over $10.4 billion in 2004, up from $8.9 billion in 1999 (McCoy 2005). Functional beverages constituted 96.3% of these functional food products in 2004, an increase from 81.7% in 1999 (McCoy 2005).

Sales data show that grain-based products are the most common $n$-3 FA enriched food products, constituting 86% of the market (Packaged Facts 2007). This is followed by enriched eggs at 6% and dairy products at 4%. By 2011, it is predicted that a shift towards $n$-3 enriched dairy products (including milk, cheese, yogurt and ice cream) will occur, earning a larger percentage of the market.

**Milk based functional beverages**

A report by the Fluid Milk Strategic Thinking Initiative proclaims that milk serves as an ideal base for functional beverages due to its inherent nutrient content (Boland and others 2001; Hazen 2003). Consumers of Western cultures often enjoy dairy products for their sensory properties while simultaneously recognizing their health promoting attributes, such as bioavailable calcium, protein and some B vitamins (Boland and others 2001).

The reported US per capita total fluid milk consumption in 2004 was 21.2 gallons (US Census Bureau 2006). In the early 1900s, whole milk and buttermilk were primarily consumed. Consumption of these milks took place mainly on farms. As methods of milk production improved, milk consumption became more widespread. A decline in fluid
milk consumption occurred throughout the century due to competition with other beverages (most commonly sweetened beverages) as well as the concern of milk fat as a culprit to CVD, obesity and a dietary source of saturated fat. In response, the consumption of low-fat milk and skim milk rose starting in 1958 and surpassing whole milk consumption in 1988 (Putnam and Allshouse 2003). The International Dairy Foods Association reported a steady decrease in the sale of regular whole, low-fat and skim milks between 1975-2004 (Miller and Blayney 2006). This coincided with an increase in flavored milk beverages, specifically chocolate milk (Dairy Market Trends 2002).

The use of milk as the base for a functional beverage may aid in increasing milk consumption in US. The NHANES, 1999-2002, reported a steady decline in milk consumption across increasing age groups (Storey and others 2006). In fact, only 30% of the population aged two years or older meets the recommended servings for milk and other dairy products. As the consumption of sweetened beverages increased, the per capita total fluid milk consumption decreased from 36.4 gal/person in the 1950s to 22.6 gal/person in 2000 (US Dept of Agriculture, Office of Communications 2003).

Low-fat dairy products have a strong advantage of serving as an effective base for bioactive ingredients (Sharma 2005). Milk is an efficient vehicle for fat absorption due to the dispersion of fat in very small micelles (Baró and others 2003). These small micelles increase the surface area for the absorption of fats and fat-soluble compounds. This allows FA to be successfully incorporated into a milk-base beverage, and produce significant increases of plasma concentrations of the fatty acids, especially the longer-chain FA, EPA and DHA (Visioli and others 2000). Functional beverages also have potential, because the beverage may be consumed along with a typical meal without altering the dietary habits of the consumer or may become an easy component of the consumer’s normal lifestyle (Temelli and others 2004).

Metcalf and colleagues (2003) examined the subjects’ food preferences for various n-3 FA enriched products in their study, which included a flaxseed-based energy drink and long-life milk fortified with FO. The higher the frequency of usage of a food product was, the greater its popularity. One of the most popular products was the shelf-stable EPA+DHA enriched long-life milk.
Visioli and colleagues (2000) had eight normolipidemic subjects who were habitual low-fat milk drinkers consume 500 mL/day partially skimmed milk, without any fish intake, for four weeks to establish baseline uniformity in the subjects. After the first month, subjects consumed 500 mL daily of a commercially produced EPA+DHA enriched long-life milk (Parmalat Plus Omega 3©). The milk provided 400 mg total n-3 FA (100 mg ALA, 120 mg EPA, and 180 mg DHA). Plasma lipid parameters were measured and marked increases of plasma EPA (44%, 31%) and DHA (13%, 31%) were observed at week 3 and 6, respectively. After the 6-week period, plasma triglyceride concentration significantly decreased (-19%) and HDL concentration increased approximately (19%). The enriched milk was prepared with an ultra-high temperature processing which may have affected taste and availability of FA. However, this could not be determined from results of this study.

In 2003, Baró and colleagues (2003) conducted a study with 30 healthy Spanish volunteers who consumed 500 mL/day of semi-skimmed milk for four weeks, with restricted fish intake. This was followed by 500 mL/day of n-3 FA enriched long-life milk (Puleva Omega3®), also containing oleic acid, vitamins A, D, E, B₆, and folic acid daily for eight weeks. The enriched milk provided 0.6% ALA, 1.4% EPA and 2.1% DHA as total percentage in milk fat. After the initial four weeks, plasma concentrations of EPA and DHA were moderately decreased, without a significant effect on plasma FA. The 8-week supplementation restored the deficit EPA and DHA due to the abstinence from fish consumption as well as further increasing plasma EPA by 33% and plasma DHA by 30%. Based on these previous studies, it is feasible to formulate an n-3 FA enriched long-life milk with successful incorporation of the n-3 FA (ALA, EPA and DHA) into the body.

Carrero and others (2004) conducted a study with 30 middle-aged mildly hyperlipidemic Spanish subjects (mean age = 51.3 years). Similar to previous studies (Visioli and others 2000; Baró and others 2003), subjects in the present study consumed 500 mL/day of long-life milk (Puleva Omega3®) for four weeks to establish a uniform baseline. After the first month, subjects switched to 500 mL/d enriched milk for eight weeks. The enriched milk was formulated from the addition of fish and vegetable oils into a skim milk foundation as well as the fortification of vitamins A, D, E, B₆ and
folic acid. The enriched milk contained a total fat content similar to the long-life milk, composed of a different FA profile. It was reported that the enriched milk had equivalent palatability of the long-life milk with a healthier fatty acid and vitamin profile (FO-derived EPA+DHA, oleic acid, folic acid, and vitamins A, D, E and B₆). The enriched milk (containing 0.20 g EPA and 0.13 g DHA per 500 mL) had eight times more polyunsaturated FA than the control milk (containing no detectable amounts of EPA or DHA). Fish consumption was halted throughout the study. Plasma concentrations of triglyceride, LDL, EPA and DHA were measured at baseline, and at week 4, 8 and 12. At week 12, significant increases in plasma EPA (33%) and DHA (20%) were measured, as well as a significant decrease in LDL (-13%). At week 4, after subjects were restricted from fish intake and consumed the control milk, triglyceride concentration increased from baseline. After four weeks of the enriched milk, triglyceride concentration resumed to normal and continued to decrease as enriched milk consumption continued. The authors concluded that replacing semi-skimmed long-life milk with enriched milk was an effective way to decrease CVD risk factors.

Overall, these studies suggest that the formulation and usage of n-3 FA enriched milk beverages are feasible tools to increase dietary n-3 FA intake. This, in turn, will help lower the n-6:n-3 FA ratio and possibly attenuate inflammation in the body. However more research is necessary and measurement of pro-inflammatory cytokines at various intervals after consumption of n-3 FA enriched products is needed. Currently, the first commercially available n-3 FA enriched dairy is produced by Omega Farms (Hayward, CA), providing 75 mg EPA+DHA per serving (Packaged Facts 2007). A milk beverage enriched with ALA has not been commercially produced.

Oxidative susceptibility of EPA and DHA

There is consensus that the longer-chain n-3 FA (EPA and DHA) are more effective in the attenuation of inflammation and CVD than the moderate length n-3 fatty acid ALA. However, the use of these marine and algae oils are limited by their oxidative susceptibility and fishy and metallic off-flavors. It is a challenge in the industry to inhibit lipid oxidation of n-3 FA oils during processing, shipping and storage (Djordjevic and others 2004). Fats and oils are extremely prone to autoxidation and may potentially
harm cells with the formation of free radicals. Autoxidation is a primary factor in the degradation of the quality of fats in foods (Sattar and deMan 1975).

The oxidative stability of unsaturated FA decreases as their degree of unsaturation increases. In other words, the greater number of carbon double bonds present in the polyunsaturated fatty acid, the more vulnerable it is to autoxidation (Kim and LaBella 1987). Therefore, DHA is more vulnerable than EPA and ALA, respectively. In the 18-carbon FA series, the relative oxidation rate of 18:0 is 1. The relative oxidation rates of 18:1, 18:2 and 18:3 is 100, 1200 and 2500 times, respectively, the oxidation rate of 18:0 (De Man 1999). It is evident that the more unsaturated a FA is, the more susceptible it is to oxidation.

It has been suggested that presence of the oil in an oil-in-water emulsion may increase the oxidative stability for a lipid. The emulsion would consist of the oil being dispersed into a water-based food including beverages and dairy products (Djordjevic and others 2004). However, the incorporation of these long chain n-3 FA, in the form of FO, into a milk base still has issues with oxidative stability (Let and others 2003; Venkateshwarlu and others 2004; Let and others 2005). The combined unsaturated FA found in milk lipids, particularly the phospholipids, and the long chain n-3 FA added to the milk, gives the enriched beverage a noted vulnerability to oxidation producing an oxidized flavor. Alternatively, it has also been suggested that the use of n-3 FA in dairy products retards the lipid oxidation process, because dairy products are generally refrigerated. The lower temperatures help protect against oxidation during storage. In addition, the milk proteins provide an antioxidant property, also potentially aiding in the protection against lipid oxidation of the enriched dairy beverage (Packaged Facts 2007).

**Advantages of ALA over EPA and DHA**

The recommendation to increase dietary EPA and DHA is limited to patterns of food choice and natural resources of fish to sustain a sufficient supply of fatty/oily fish (Burdge and Calder 2005). For populations who do not consume seafood, muscle or organ meat products, such as vegetarians, the longer-chained n-3 FA are only accessible from plant sources containing ALA (Garg and others 2006).

The main role of ALA is to moderate the metabolism of LA and its subsequent eicosanoids by competing for available delta-6-desaturase. The affinity of delta-6-
desaturase is greater for ALA; however, due to larger amounts of LA available in cellular pools, the metabolism of the \textit{n}-6 family is favored. The human body is capable of converting ALA into EPA and DHA through the series of desaturation and elongation steps. There is sound evidence of a modest conversion rate of ALA to EPA, varying from 0.2-15\% depending on various methods of measurement (Emken 1994; Pawlosky and others 2001; Burdge and others 2002). The Institute of Medicine has previously stated that ALA is not known to have any specific functions aside from being a precursor to EPA and DHA (Packaged Facts 2007). Most \textit{n}-3 FA research focuses on EPA and DHA, and due to a lack of research, it cannot be concluded that ALA does not serve more purpose than a precursor of longer-chain FA. Burdge and Calder (2005) reported that an increase in dietary ALA over a period of weeks to months can result in elevated levels of EPA in plasma lipids. However, increased dietary ALA did not enhance the levels of DHA, most likely due to the extra necessary elongation and desaturation steps. Overall, it is still important to increase the intake of ALA in the diet to compete with LA for the delta-6-desaturase enzyme, which will help attenuate the production of AA and its inflammatory effects.

\textbf{Flaxseed as a functional ingredient}

An alternative to using the long chain \textit{n}-3 FA in functional food products, is the use of ALA in the form of flaxseed. Flaxseed (\textit{Linum usitatissimum}) is a blue-flowered crop, where the seed can be used for food and feed uses. The seed itself is dark brown in color, flat, oval with a pointed tip and slightly larger than a sesame seed. It has a crisp and chewy texture with a nutty and earthy flavor. The use of flaxseed as a source of \textit{n}-3 FA may be more optimal than FO, as flaxseed does not have the fishy, metallic flavor found in marine and algae oils. Flaxseed is the richest source of ALA in the North American diet, containing 37\% total fat, where 57\% of the total fat is ALA. Although the fat content is high in flaxseed, the absence of two or three carbon double bonds in ALA (18:3) compared to EPA (20:5) and DHA (22:6) reduces its susceptibility to lipid oxidation.

Flaxseed is also a dietary source for small amounts of some fat-soluble vitamins, where the presence of vitamin E compounds provide antioxidant properties which may help promote oxidative stability (Kamal-Eldin and Yanishlieva 2002). The main
tocopherols in flaxseed are γ- and δ-tocopherols present at 8.5-39.5 mg/100 g and 0.2-1.1 mg/100 g, respectively. The tocotrienol, plastochromanol-8 is present at 11.9-13.9 mg/100 g, which has been reported to be more effective in the prevention of lipid oxidation (Daun and others 2003). Flaxseed is also the richest source of lignans, which is a class of phytoestrogens and a natural antioxidant.

The presence of these antioxidants resides in the shell of the flaxseed. There are three types of flax products available in the market: whole flaxseeds, flaxseed meal and flax oil. Whole flaxseeds have a tough shell to protect its content. The human body cannot efficiently break the shell and make use of the contents within. Whole flaxseeds are used mostly for visual hedonic value. It has been demonstrated that the crushing or milling of flaxseed (into flaxseed meal) significantly increases the bioavailability of the natural antioxidants present in the shell (Kuijsten and others 2005). Flax oil is pressed from flaxmeal and contains only the oil component. Flax oil must be handled carefully to ensure freshness and prevent oxidation from heat, light and air during its processing and storage phases. The bioavailability of ALA from flaxseed meal is comparable as that from flax oil, despite the presence of the ground shell (Cunnane and others 1993).

**Association of ALA and flaxseed with inflammatory diseases**

The association between ALA and inflammation and CVD has received dramatically less attention than that with EPA and/or DHA. Although, flaxseed has been linked to reducing the risk of CVD through a variety of possible mechanisms such as reducing inflammation, serum cholesterol, platelet aggregation and acting as an antioxidant (Bloedon and Szapary 2004), there has been insufficient evidence to support these suggested actions.

To date, there are no published epidemiological studies of flaxseed or flax-containing food products and inflammatory diseases. This is most likely due to the lack of a defined population who consume flaxseed in significant amounts as part of their normal habitual diet (Bloedon and Szapary 2004).

The effects of flax oil (containing 14 g ALA/day) on pro-inflammatory markers (TNF-α and IL-1β) were studied in healthy males over four weeks (Caughey and others 1996). The flax oil led to modest increases in cellular levels of EPA. As expected, this increase in EPA (derived from the increased ALA intake) was approximately only 0.4%,
whereas subjects, who consumed much smaller amounts of EPA (from FO), had significantly more EPA accumulation in their PBMCs. The production of pro-inflammatory markers both decreased by approximately 30%, which occurred simultaneously with the increase of cellular EPA content. This suggests a dose-dependent relationship between cellular EPA content and the inhibition of inflammatory cytokine production. A more recent study demonstrated that a smaller amount of flax oil (containing 3.5 g ALA/day) in healthy adults for 12 weeks did not have similar inhibitory effects of the production of pro-inflammatory cytokines (Wallace and others 2003). As illustrated earlier, the use of flaxseed for attenuation of bone loss is unclear (Arjmandi and others 1998; Lucas and others 2002; Dodin and others 2005). Further research is still necessary to explore the effect of ALA-rich plant sources such as flaxseed on inflammation and inflammatory diseases.

**Current gap in knowledge**

There is still a gap in the knowledge of the effect of increased flaxseed consumption into the diet on inflammation of the body. Based on previous research, larger amounts of ALA are necessary to perform the same benefits as EPA due to the modest conversion rate. Cunnane and colleges (1993) have reported that a daily dose of 50 g flaxseed meal is palatable, safe and nutritionally beneficial. The 50 g of flaxseed would provide approximately 10.5 g ALA, which has been shown to cause changes in the fatty composition of PBMCs, but no changes in pro-inflammatory cytokines (Kew and others 2003). However as previously stated, this is only one study, where there is a very limited amount of information available on the effects of ALA on inflammation and cardiovascular related markers. A concrete amount of ALA necessary to show any effect on inflammatory markers is still unknown.

**Novel product development**

Flaxseed is primarily incorporated into grain-based products as a replacement of some or all oil or shortening in the recipes. To date, a flaxseed-enriched milk-based beverage has not been formulated. The idea of the incorporation of flaxseed in a milk base can provide a combination of benefits of the two. Milk has been referred to as a nutrient punch, being a great source for calcium, protein, vitamin A, B_{12}, D, riboflavin, niacin, potassium and phosphorus. However milk is low in n-3 FA, lignans, fiber and
vitamin E, which are being added into the beverage system with the flaxseed meal. There are concerns of potential disadvantages of the formulation of the beverage, such as, the additional fiber from flaxseed which may hinder the absorption of calcium provided in the milk.

Summary

Inflammation can lead to numerous devastating health conditions including RA, CVD, and osteoporosis. One avenue of prevention is to decrease the dietary $n$-6:$n$-3 FA ratio, generally with an increase of $n$-3 FA rich food sources. There is sound beneficial evidence of the long chain $n$-3 FA (EPA and DHA), generally found in marine derived oils, on attenuation of inflammation. However, due to limited resources of fish and fish products, and particular eating patterns, $n$-3 FA from plant sources (in the form of ALA) may be an attractive alternative. There has been limited research performed on the effect of ALA and inflammation on BMD; however some data appear promising. As more evidence of the beneficial effects of $n$-3 FA on human health becomes available, a surge of novel $n$-3 FA enriched products will emerge into the market. Food manufacturers are constantly working on developing new formulations of functional food products using EPA, DHA and ALA as bioactive ingredients. It is still unknown whether ALA (especially in the form of flaxseed) can lead to additional benefits or detrimental effects when incorporated into a food or beverage system, as EPA- and/or DHA-enriched products have previously demonstrated. Therefore, the purpose of this research was to: 1) develop a palatable flaxseed-enriched milk-based beverage (“flaxmilk”); 2) evaluate sensory attributes and conduct product characterization of the novel flaxmilk beverage; and 3) estimate the $n$-6:$n$-3 FA ratio in diets of young-adult and postmenopausal women and explore associations between their estimated $n$-6:$n$-3 FA ratio and bone mineral measurements.
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Chapter III

Consumer acceptance of a novel flaxseed-enriched milk-based beverage
Abstract

Increased awareness of the cardioprotective effects of omega-3 fatty acid-rich products has resulted in the development of novel functional beverages. Marine sources of omega-3 fatty acids have primarily been used, hindering consumption of these functional products by some consumers. The formulation of a flaxseed-enriched milk-based beverage (i.e., “flaxmilk”) offers omega-3 fatty acids from a plant source, while providing nutrients present in both milk and flaxseed. Sixty-two volunteers evaluated flaxmilk using a 9-point hedonic scale. Each participant provided demographic information and indicated whether he/she would consume flaxmilk daily. A mean (±SD) hedonic score, equivalent to a “likes slightly” descriptor, of 6.35 (± 1.53) was obtained, representing consumer acceptance. Trust and familiarity to chocolate milk likely influenced consumer judgment of the beverage. There were no significant differences in hedonic scores based on gender, age, household income or education. The knowledge of positive health benefits increased consumer willingness for daily intake of flaxmilk.

Keywords: flaxmilk, hedonic sensory evaluation, novel functional beverage, omega-3 fatty acids
Introduction

In the late 1990s, the awareness of health benefits of dietary omega-3 fatty acids increased. Some of these health benefits include, but are not limited to, anti-thrombotic and anti-inflammatory effects, which are important to cardiovascular health (Kris-Etherton and others 2002; Calder 2006a, 2006b). Because of these potential health benefits, consumers are encouraged to incorporate more omega-3 fatty acids into their diets. A common approach to achieve this goal is through the use of nutritional supplements (i.e., capsules and tablets). However, many consumers prefer the use of whole foods rather than nutritional supplements.

Natural food sources of omega-3 fatty acids include fatty coldwater fish, walnuts and flaxseed. Yet, Western societies seldom consume sufficient amounts of fish or fish products to fulfill the adequate intake of 1.6 g/day for omega-3 fatty acids (Institute of Medicine 2005). The aim of increasing overall fish consumption in the population is not practical, due to the high cost, limited resources and displeasure of fish consumption for some consumers. Walnuts and flaxseed also are difficult to consume in adequate amounts to provide sufficient levels of dietary omega-3 fatty acids. Therefore, fish, walnuts and flaxseed are limited in their effectiveness as sole sources of omega-3 fatty acids (Mantzioris and others 2000).

The development of functional foods and beverages high in omega-3 fatty acids has aided in the greater availability of alternate food sources for these valued lipids. The term functional food has many definitions ranging from simple to elaborate, without an established consensus in the United States (U.S.). The Institute of Food Technologists’ Expert Panel defines functional foods as “foods and food components that provide a health benefit beyond basic nutrition (for the intended population)” (Institute of Food Technologists 2005). Verschuren (2002) stated that functional foods and beverages may enhance a physiological role or help reduce disease risk in consumers. In this case, omega-3 fatty acids may have cardioprotective effects via anti-thrombotic and anti-inflammatory roles.

Currently available omega-3 fatty acid-rich products include table spreads, salad dressings, pasta, eggs and milk (Mantzioris and others 2000; Metcalf and others 2003). These products have been formulated using marine-based omega-3 fatty acid sources.
Marine-derived long chain fatty acids tend to create fishy or metallic off-flavors in these products due to lipid oxidation. In addition, non-seafood consumers are likely to avoid these food products. A less explored alternative is the use of plant-derived omega-3 fatty acids such as flaxseed and walnuts in the formulation of novel functional food products. Flaxseed is the richest plant source of omega-3 fatty acids, where over half of its fat content is composed of alpha-linolenic acid. Flaxseed is also an excellent source of fiber and lignans (antioxidants).

Milk serves as an ideal base for functional beverages due to its inherent nutrient content (Boland and others 2001; Huth and others 2006). Research supports the associations between adequate consumption of dairy products and high bone mineral density, lowered blood pressure and adequate body weight regulation (Miller and others 2000; McCabe and others 2004; Lanou and others 2005; St-Onge 2005; Zemel and others 2005 ; Huth and others 2006). Consumers in Western cultures often enjoy dairy products for their sensory properties while simultaneously recognizing their health promoting attributes (Boland and others 2001). For example, milk is recognized as a component of a healthy diet and a source of bioavailable calcium and protein (Gueguen and Pointillart 2000; Boland and others 2001; Ranganathan and others 2005; Huth and others 2006).

Flavored milks are nutrient-dense providing similar amounts of nutrients such as calcium, protein and B vitamins, as unflavored milk. Chocolate milk contains slightly higher levels of fiber and iron than unflavored milk, due to the presence of the additional flavoring ingredients (Capps and others 2005). However, due to the added sweetener, flavored milks also contain more carbohydrates and calories.

Although flavored dairy beverages such as chocolate milk have been in the market for decades, they have become more popular in recent years. In 1997, sales of flavored dairy products in the U.S. were approximately $1.4 billion (Dairy and Food Communications, Inc 2002). By 2001, a 40.4% sales growth increased revenues to $2.0 billion. Economists predict a continued annual growth rate of 8.4% (Dairy and Food Communications, Inc 2002); thus, sales of flavored dairy beverages will reach $5 billion by 2010.
Characteristics of the typical functional food consumer in the U.S. include well-educated, of high income status, female and aged 35 to 55 years (Childs 1997; Verbeke 2005). Consumption of a functional milk-based beverage would be especially advantageous in a female population in mid-life, because calcium present in dairy may attenuate bone loss.

Functional beverages have the potential to provide additional nutrients without significantly altering dietary habits of the consumer, because the beverage may be consumed along with a typical meal (Hazen 2003; Temelli and others 2004). Using flaxseed as a functional food ingredient in a beverage for delivery of omega-3 fatty acids may also help avoid fishy and metallic off-flavors associated with marine-derived sources. The development of a flaxseed-enriched milk-based beverage provides the nutrients present in milk (i.e. calcium, protein, vitamins A, B_{12} and D, riboflavin, niacin, potassium and phosphorus) in combination with nutrients present in flaxseed (omega-3 fatty acids, lignans, iron, fiber and vitamin E). The objective of this study was to formulate an acceptable flaxseed-enriched milk-based beverage (“flaxmilk”). A secondary objective is to see whether there was a consumer group that would show more preference to the beverage than other groups and compare to the “typical functional food consumer” as described above.

**Materials and Method**

*Recipe and Processing*

Raw milk was obtained from the Virginia Tech dairy farm (Blacksburg, VA). Processing of flaxmilk was completed in the Dairy/Beverage Processing Pilot Plant in the Department of Food Science and Technology at Virginia Tech. Raw milk was heated to 54.4°C and separated into cream and skim phases using a pilot plant separator (Elecrem separator, model IG, 6400 rpm, Bonanza Industries, Inc., Calgary, Alberta). The non-fat phase (<0.5% milkfat) was subsequently used for the formulation of flaxmilk while the cream phase was discarded. Finely ground flaxseed (2.47% by weight; Pizzey’s Meadow Pure™ O3 BevGrad™, Gurnee, IL), malted milk mix (9.60% by weight; Nestlé Carnation, Switzerland), 100% pure vanilla extract (0.61% by weight), guar gum (0.08% by weight; TIC gums, Belcamp, MD) and κ-carrageenan (0.09% by weight; TIC gums, Belcamp, MD) were incorporated into the unpasteurized non-fat milk.
(87.17% by weight) and mixed in a 50-gallon, double wall vat-pasteurizer with a bottom-sweep agitator (The Creamery Package, Chicago, IL) to gently stir ingredients together until well mixed. (See Appendix A for recipe formulation trials). The milk-based mixture was heated to 54.4°C and immediately homogenized (APV Gaulin, Inc, Model 15MR, Everett, MA with Reliance Duty Master AC Motor, Cleveland, OH) at 10.3/3.5 MPa in the first and second stages, respectively. Homogenization is a mechanical treatment where the beverage is passed under high pressure through a small orifice in order to decrease the average diameter of fat globules, increase the number and surface amount of fat globules and assist in dispersing ingredients and creating a stabilized fluid system (Wehr and Frank 2004). The reduction of fat globule size removes visible cream or fat separation in the milk, providing a well-mixed emulsion of flaxseed and milk with a more uniform and smoother consistency. Flaxmilk then was vat-pasteurized at 73.9°C for 30 min. Pasteurization inactivates pathogenic vegetative cells and destroys organisms that cause spoilage (Wehr and Frank 2004). After pasteurization, the flaxmilk was cooled and stored at 4°C in stainless steel containers prior to sensory acceptability study.

Participants and Sensory Evaluation

Flaxmilk samples were prepared for consumer evaluation on day 2 of storage. Flaxmilk (~22 mL) was poured into 1-ounce soufflé cups, capped and refrigerated at 4°C and transported to the Student Union on the Virginia Tech campus. Samples were stored in a cooler with ice packs to maintain product temperature throughout the duration of the sensory evaluation. Sixty-two participants were recruited using the intercept method. Advertisement of study participation included promotion of taste-testing of a novel chocolate-flavored milk beverage. Participants signed informed consent forms to participate in the sensory panel (Appendix B) and completed a demographic survey (Appendix B). Any individual with dairy, flaxseed, cocoa and/or chocolate allergies was excluded from participation. Participants included Virginia Tech students and staff, as well as visitors to the campus. This study was approved by the Institutional Review Board for Human Subjects at Virginia Tech.

Each participant sampled the flaxmilk and rated its overall palatability on a 9-point hedonic scale (Figure 3.1). Subjects were given the opportunity to write
comments about the sample beverage. Water was provided to cleanse the palate before and after tasting of flaxmilk. Candy was offered as compensation for participating in the study.

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**Consumer Preference Test for Novel Milk-based beverage**

Please taste the sample thoroughly and rate its overall palatability on the scale below. You may expectorate the sample if you wish.

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Comments? ___________________________________________________________________

Figure 3.1. Scorecard with 9-point hedonic scale used to rate acceptability of flaxmilk

Participants had the option to ask the investigator any questions while completing the demographic survey and scorecard to rate the flaxmilk beverage. In addition, informal conversation occurred between investigator and participants; often relating to what flaxseed was. The investigator never informed the participant that flaxmilk may have potential health benefits, but solely stated that the beverage contained flaxseed.

After tasting the product, subjects completed an exit questionnaire. The questionnaire provided an estimate of consumer intent of consumption, frequency and amount of consumption (Figure 3.2). Participants were not informed that flaxmilk may have potential health benefits. Therefore, the first set of questions referred to flaxmilk being a new beverage in the market. However the second set of questions referred to flaxmilk being a new beverage in the market that may provide a positive health benefit when consumed.
Flaxmilk Beverage Responses

Would you consume this beverage on a daily basis?   Yes   No
If yes, how much of this product would you consume on a daily basis? ___________
If yes, how much would you consume at one time? ____________________________

Would you consume this beverage on a daily basis if you knew that it provided a
positive health benefit?   Yes   No
If yes, how much of this product would you consume on a daily basis? ___________
If yes, how much would you consume at one time? ____________________________

THANK YOU FOR YOUR PARTICIPATION!!

Figure 3.2. Exit questionnaire completed by participants after tasting flaxmilk

Statistical Analysis

Consumer responses were evaluated based on a 9-point hedonic scale [where
1 = “dislike extremely, 5 = “neither like nor dislike”, 9 = “like extremely”] (Meilgaard and
others 1999). Mean (± SD) score was calculated from participant responses for the
entire population (n=62). This mean hedonic score was compared to the a priori
targeted hedonic score of 6.0 (“like slightly”), which would indicate acceptability and
palatability of the novel flaxmilk beverage. Analysis of variance (ANOVA) was used to
explore significant differences in mean (± SD) hedonic scores among subpopulation of
participants based on gender, age, income and education level. The binomial type
questions in the exit questionnaire were analyzed relative to the hedonic scores.
Statistical significance was set at p<0.05. All statistical procedures were completed

Results

Demographics

Demographics characteristics of participants are presented in Table 3.2.
Table 3.1. Demographic characteristics of all participants (n=62) and by gender evaluating flaxmilk beverage

<table>
<thead>
<tr>
<th>Marital Status</th>
<th>Participants (n=62)</th>
<th>Male: 42% (n=26)</th>
<th>Female: 58% (n=36)</th>
<th>Overall Mean Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number of responses</td>
<td>percentage</td>
<td>number of responses</td>
<td>percentage</td>
</tr>
<tr>
<td>Married</td>
<td>35</td>
<td>57%</td>
<td>13</td>
<td>50%</td>
</tr>
<tr>
<td>Single</td>
<td>24</td>
<td>39%</td>
<td>13</td>
<td>50%</td>
</tr>
<tr>
<td>Divorced</td>
<td>2</td>
<td>3%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>1%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[18-25 y]</td>
<td>22</td>
<td>35.5%</td>
<td>11</td>
<td>42%</td>
</tr>
<tr>
<td>[26-35 y]</td>
<td>7</td>
<td>11%</td>
<td>1</td>
<td>4%</td>
</tr>
<tr>
<td>[36-45 y]</td>
<td>14</td>
<td>23%</td>
<td>7</td>
<td>27%</td>
</tr>
<tr>
<td>[46-55 y]</td>
<td>15</td>
<td>24%</td>
<td>5</td>
<td>19%</td>
</tr>
<tr>
<td>[over 55 y]</td>
<td>4</td>
<td>6.5%</td>
<td>2</td>
<td>8%</td>
</tr>
<tr>
<td>Household Income</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;$20,000</td>
<td>17</td>
<td>27%</td>
<td>7</td>
<td>28%</td>
</tr>
<tr>
<td>$21-40,000</td>
<td>4</td>
<td>6%</td>
<td>3</td>
<td>11.5%</td>
</tr>
<tr>
<td>$41-70,000</td>
<td>9</td>
<td>15%</td>
<td>3</td>
<td>11.5%</td>
</tr>
<tr>
<td>$71-100,000</td>
<td>14</td>
<td>23%</td>
<td>6</td>
<td>23%</td>
</tr>
<tr>
<td>&gt;$100,000</td>
<td>14</td>
<td>23%</td>
<td>5</td>
<td>19%</td>
</tr>
<tr>
<td>NR</td>
<td>4</td>
<td>6%</td>
<td>2</td>
<td>7%</td>
</tr>
<tr>
<td>Education completed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High School</td>
<td>16</td>
<td>26%</td>
<td>10</td>
<td>38%</td>
</tr>
<tr>
<td>College</td>
<td>23</td>
<td>37%</td>
<td>8</td>
<td>31%</td>
</tr>
<tr>
<td>Advanced Degree</td>
<td>22</td>
<td>36%</td>
<td>8</td>
<td>31%</td>
</tr>
<tr>
<td>NR</td>
<td>1</td>
<td>1%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Employment status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full time</td>
<td>31</td>
<td>50%</td>
<td>14</td>
<td>54%</td>
</tr>
<tr>
<td>Part time</td>
<td>14</td>
<td>23%</td>
<td>6</td>
<td>23%</td>
</tr>
<tr>
<td>Student</td>
<td>13</td>
<td>21%</td>
<td>3</td>
<td>11.5%</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>6%</td>
<td>3</td>
<td>11.5%</td>
</tr>
</tbody>
</table>

NR = no response; columns may not add to 100% due to rounding
Fifty-eight percent of the participants were female, 57% married, 27% earned an annual income of <$20,000 while 46% earned >$71,000 annually. Nearly 75% of participants attained a college or advanced degree. Fifty percent work full time and 21% were enrolled as students.

On the demographic questionnaire, participants were asked to indicate how many cups of white milk he/she consumed on a normal, daily basis (Table 3.3). Sixty percent of participants consumed 1 cup daily, and 32% consumed at least 2 cups daily. The type of milk consumed was also noted; 38% of participants drank non-fat milk, 18% drank 1% milk, 31% consumed 2% milk and 8% drank whole milk when consuming milk. A vast majority of participants reported that they did not consume chocolate milk (82%) or flaxseed products (94%) on a regular basis. The majority of participants indicated that they enjoyed malty flavored beverages (52%) or foods (47%) such as malted milkshakes, beer, grapenuts cereal and malted chocolate ball candies. When completing the demographic survey, many participants asked what flaxseed was, indicating that they were not familiar with this product.
Table 3.2. Consumption patterns of subjects, overall and categorized by gender

<table>
<thead>
<tr>
<th>Cups of milk consumed daily</th>
<th>Participants (n=62)</th>
<th>Males (n=26)</th>
<th>Females (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>0 cups</td>
<td>5 (8%)</td>
<td>1 (4%)</td>
<td>4 (11%)</td>
</tr>
<tr>
<td>1 cup</td>
<td>37 (60%)</td>
<td>16 (61.5%)</td>
<td>21 (58%)</td>
</tr>
<tr>
<td>2 cups</td>
<td>13 (21%)</td>
<td>4 (15%)</td>
<td>9 (25%)</td>
</tr>
<tr>
<td>3 cups</td>
<td>3 (5%)</td>
<td>2 (8%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>≥ 4 cups</td>
<td>4 (6%)</td>
<td>3 (11.5%)</td>
<td>1 (3%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of milk consumed</th>
<th>Non-fat</th>
<th>1%</th>
<th>2%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>0 cups</td>
<td>23 (38%)</td>
<td>8 (32%)</td>
<td>15 (42%)</td>
</tr>
<tr>
<td>1 cup</td>
<td>11 (18%)</td>
<td>6 (24%)</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>2 cups</td>
<td>19 (31%)</td>
<td>7 (28%)</td>
<td>12 (33%)</td>
</tr>
<tr>
<td>3 cups</td>
<td>5 (8%)</td>
<td>3 (12%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>n/a</td>
<td>3 (5%)</td>
<td>1 (4%)</td>
<td>2 (6%)</td>
</tr>
</tbody>
</table>

Do you regularly consume:

| Chocolate milk?            | Yes                 | 11 (18%)     | 2 (8%)        | 9 (25%)       |
|                            | No                  | 51 (82%)     | 24 (92%)      | 27 (75%)      |

| Flaxseed products?         | Yes                 | 4 (6%)       | 1 (4%)        | 3 (8%)        |
|                            | No                  | 58 (94%)     | 25 (96%)      | 33 (92%)      |

Do you enjoy:

| Malty flavored beverages?  | Yes                 | 32 (52%)     | 14 (54%)      | 18 (50%)      |
|                            | No                  | 21 (34%)     | 8 (31%)       | 13 (36%)      |

| Malty flavored foods?      | Yes                 | 29 (47%)     | 12 (46%)      | 17 (47%)      |
|                            | No                  | 23 (37%)     | 9 (35%)       | 14 (39%)      |

NR = no response; columns may not add up to 100% due to rounding

**Sensory Evaluation**

The 9-point hedonic scale was used as a tool for untrained sensory panelists to indicate the level of like or dislike of the flaxmilk sample. A minimum mean hedonic score of 6.0 was determined *a priori* to represent “like slightly”, suggesting acceptance or palatability of flaxmilk. Seventy-six percent of participants responded with a score of 6 or higher, resulting in a mean hedonic score of 6.35 ± 1.53. Of panelist responses, 60% were ≥7 (“like moderately”), and only 16% were ≤4 (“dislike slightly”). The overall
distribution of hedonic scores is presented in Figure 3.3a. Figure 3.3b displays distribution of hedonic scores for flaxmilk acceptability by gender groups.

Figure 3.3. a) Overall consumer acceptability (n=62) of flaxmilk. b) Separated by gender: female (n=36), male (n=26). A score of 1 = “dislike extremely”, 5 = “neither like nor dislike”, 9 = “like extremely”.

In the current study, a greater percentage of female participants rated the flaxmilk with a score of “6” or higher compared to male consumers (Figure 3.3b). Although more female subjects (n = 36) were recruited, 83% of them rated the beverage with a score of 6.0 or higher, whereas among the male subjects (n = 26), only 65%
rated the beverage with a score of 6.0 or higher. The mean hedonic score for female consumers was $6.63 \pm 1.38$, and for male consumers was $5.96 \pm 1.66$. There was a trend toward gender differences in acceptability, with the females rating the drink more acceptable than the males ($p=0.08$).

In the current study, the largest age bracket subpopulation was the 18 to 25 years old at 35.5%. The 36 to 45 years and 46 to 55 years old brackets followed with 23% and 24% of the overall sample, respectively. The participants aged >55 years provided the highest mean hedonic score with 7.00 (Table 3.4). However, the hedonic scores of each age bracket did not differ significantly ($p=0.51$). As mentioned above, the most common annual household income earned were <$20,000 (27%), $71,000-100,000 (23%) and >$100,000 (23%). The participants, who earned $41-70,000 annually, rated flaxmilk with the highest hedonic score of 7.22 (Table 3.4). Analysis of the household income subsamples with respective hedonic scores showed no significant differences ($p=0.14$). All participants earned at least a high school degree. Those who earned an advanced degree scored the flaxmilk slightly higher than participants who have not (Table 3.4). However all hedonic scores were not significantly different between education levels ($p=0.58$).

The daily consumption of milk did not have an effect on the hedonic scores in the current study’s participating population. The highest mean was reported by subjects who regularly do not consume milk, with a hedonic score of 7.6 (Table 3.4). There were no significant differences between daily milk consumption subsamples and their reported hedonic scores ($p=0.20$).
Table 3.3. Mean (± SD) hedonic scores of panelists categorized by age brackets, income levels, education and daily milk consumption (n = 62)

<table>
<thead>
<tr>
<th>Demographic characteristic</th>
<th>Mean hedonic score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age bracket (years old)</strong></td>
<td></td>
</tr>
<tr>
<td>18 – 25</td>
<td>5.91 ± 1.48</td>
</tr>
<tr>
<td>26 – 35</td>
<td>6.57 ± 1.51</td>
</tr>
<tr>
<td>36 – 45</td>
<td>6.43 ± 1.74</td>
</tr>
<tr>
<td>46 – 55</td>
<td>6.67 ± 1.29</td>
</tr>
<tr>
<td>&gt;55</td>
<td>7.00 ± 2.00</td>
</tr>
<tr>
<td><strong>Household Income</strong></td>
<td></td>
</tr>
<tr>
<td>&lt; $20,000</td>
<td>5.71 ± 1.40</td>
</tr>
<tr>
<td>$21-40,000</td>
<td>6.50 ± 1.73</td>
</tr>
<tr>
<td>$41-70,000</td>
<td>7.22 ± 1.30</td>
</tr>
<tr>
<td>$71-100,000</td>
<td>6.07 ± 1.86</td>
</tr>
<tr>
<td>&gt; $100,000</td>
<td>6.71 ± 1.38</td>
</tr>
<tr>
<td><strong>Highest Education Level Completed</strong></td>
<td></td>
</tr>
<tr>
<td>High School</td>
<td>6.00 ± 1.71</td>
</tr>
<tr>
<td>College</td>
<td>6.43 ± 1.67</td>
</tr>
<tr>
<td>Advanced Degree</td>
<td>6.50 ± 1.26</td>
</tr>
<tr>
<td><strong>Daily Milk Consumption</strong></td>
<td></td>
</tr>
<tr>
<td>0 cups</td>
<td></td>
</tr>
<tr>
<td>1 cup</td>
<td>6.17 ± 1.60</td>
</tr>
<tr>
<td>2 cups</td>
<td>6.00 ± 1.53</td>
</tr>
<tr>
<td>3 cups</td>
<td>7.33 ± 0.58</td>
</tr>
<tr>
<td>≥4 cups</td>
<td>6.75 ± 0.50</td>
</tr>
</tbody>
</table>

**Intent of consumption**

Each participant completed a short exit questionnaire with two binomial questions to estimate the participant’s intent of consumption, frequency and amount of consumption of flaxmilk. When asked whether the participant would consume the flaxmilk beverage on a daily basis, 58% chose “no”. For consumers who indicated “yes”, they were asked how much they would consume on a daily basis and at one time. Most of these participants (88%) indicated that they would drink 6-8 oz daily, consuming half to all at one time. The second question asked whether the participant would consume the beverage on a daily basis if it was known to provide a positive health benefit. Eighty-five percent responded “yes”. When asked how much he/she would consume on a daily basis and at one time, 71% responded with 1-2 cups daily, and drinking about 6-8 oz at one time.
Discussion

**Milk Consumption Patterns**

Sixty percent of participants in this study reported consumption of one cup (8 fl oz) of milk daily, with 92% consuming at least one cup daily (Table 3.3). This is slightly higher than the overall U.S. population, which is estimated at 7.4 fl oz (Economic Research Service/United States Department of Agriculture 2005a). Fluid milk products most commonly consumed by 69% of participants in the present study included non-fat (<0.5% fat) or 2% fat milk. This was similar to milk consumption patterns in the overall US population, where non-fat and 2% were also the two most consumed fluid milk varieties (Fredonia 2006). In the current study, 42% of women reported intake of non-fat milk, while 33% drank 2% milk. A similar pattern was reported by men where 32% drank non-fat milk and 28% consumed 2% milk. In the overall U.S. population, 2% milk was most popular for both genders (Fredonia 2006). Non-fat milk is more frequently consumed in more highly educated consumers (“more than high school” vs. “high school” and “less than high school”) and by consumers with greater income (≥ $75,000 vs. ≤ $20,000) (Fredonia 2006). Subjects in the current study were all high school graduates, representing a more educated group than the overall U.S. population and likely explaining the prevalence of non-fat milk intake over other varieties of fluid milk.

**Familiarity**

The novel flaxmilk product surpassed the targeted goal for acceptability with this untrained consumer panel. The mean hedonic score (6.35) for flaxmilk was likely related to the consumers’ familiarity and appreciation of the chocolate flavor. According to Thompson and colleagues (2004), chocolate is the most popular flavored milk among both adults and children. Flavored milk beverages provide nutrients and a desired flavor. The consumption of flavored milk beverages has steadily increased in the U.S. population. The U.S. consumption of 1%, 2% or non-fat flavored milks was approximately 3,554 million pounds in 2005, almost double the 1,719 million pounds consumed in 1990 (Economic Research Service/United States Department of Agriculture 2005b). As the consumption of flavored milks has markedly increased, consumption of low-fat (1% and 2%) or non-fat plain milk has decreased from 25,039 million pounds in 1990 to 23,913 million pounds in 2005 (Economic Research
Service/United States Department of Agriculture 2005b). In previous sensory studies on chocolate milk, overall acceptability and liking was strongly correlated with acceptance of overall flavor, chocolate flavor and sweetness (Thompson and others 2004).

The best indicator to predict willingness to try and enjoy a novel food product is familiarity with components of a novel food product (Backstrom and others 2004; Martins and Pliner 2005; Huotilainen and others 2006). For example, the chocolate milk base of the flaxmilk likely increased acceptability in this consumer population, because chocolate is a well-liked flavor. With familiar flavors, texture or smells, a consumer is more willing to try a novel food product and to find it palatable and acceptable. A certain degree of apprehension is present when a consumer is presented with a novel food product. This is due to an innate protective function in preparation of encountering dangerous components in the food environment. This natural reaction is referred to as food neophobia, where novel foods are considered “more dangerous” than foods that are familiar to the person (Pliner and others 1993).

This conditioned preference behavior can be altered through a series of small incremental steps to overcome the phobia of new foods (Stallberg-White and Pliner 1999). The popularity of chocolate milk has slowly increased in the U.S. over the last century. This demonstrates the growing acceptability of chocolate milk over time, even though the addition of cocoa powder and sugar were novel concepts at one time. In the formulation of flaxmilk, chocolate milk (a now familiar beverage) was chosen to serve as the foundation into which the flaxseed would be incorporated. Using a common beverage (chocolate milk) with a novel ingredient (flaxseed) was anticipated to garner acceptability of the flaxmilk.

It was predicted that daily milk drinkers would be more likely to find flaxmilk acceptable. Contrary to expectations, the daily consumption of milk was not a factor in the acceptance of flaxmilk. Eighty-two percent of these participants did not consume chocolate milk. Informal discussions with participants revealed that they were familiar with chocolate milk but did not consume it regularly, due to their perception that chocolate milk was a children’s drink. Less than 10% of participants indicated consumption of flaxseed and products containing flaxseed on a regular basis.
The malt flavor in flaxmilk was included to complement the nutty and malty flavors inherent to flaxseed. It was anticipated that malted barley would create a smoother, balanced flavoring system in the beverage. Consumer responses indicated that 60% enjoyed malty flavored beverages (i.e. beer, malted milkshakes) and that 56% enjoyed malty flavored foods (malted milk balls, grapenuts). Although consumers indicated their enjoyment of malty flavored foods and beverages, the variety of sources of the malt flavor was limited. It is unclear whether the added malted barley enhanced the flavor of flaxmilk or not.

Interestingly, 94% of consumers did not consume products containing flaxseed on a regular basis. Most participants indicated that they did not know what products contained flaxseed or what flaxseeds were. Therefore, it is highly likely that the unfamiliarity toward flaxseed or flaxseed containing products may have brought about a neophobic reaction, resulting in a lower hedonic score for some subjects.

Socio-demographic characteristics

Certain socio-demographic characteristics have been identified to predict whether a consumer would be more likely to find a novel functional food product acceptable and intend to purchase and use a new product. In the U.S., the typical functional food consumer is more likely to be female, a college graduate, of high income status and aged 35 to 55 years (Childs 1997; Verbeke 2005).

More females rated flaxmilk with a score of 6.0 or higher than the males. The mean hedonic score for females was slightly greater than those for the male consumers. Although not significant, there was a trend toward gender differences in acceptability. These results supported previous studies in which women were less likely to show neophobic tendencies for unfamiliar food products (Fagerli and Wandel 1999). Fagerli and Wandel (1999) describe women as innovators and mediators for causing a shift towards consuming healthier diets. Men are less likely to be interested in health aspects of food, particularly in lower socio-economic classes.

Research supports the notion that neophobia decreases with age; younger individuals are generally more hesitant to try new foods (McFarlane and Pliner 1997). Willingness to taste novel foods linearly increased with age, as knowledge and awareness of health and nutrition became a priority in older subjects (Pelchat and
Factors that may reduce neophobia include exposure to more novel foods, more awareness of foods outside a person’s comfort zone and being more adventurous with foods (Mcfarlane and Pliner 1997; Backstrom and others 2003). In contrast, Backstrom and colleagues (2003) showed that young and middle-aged men were the least food neophobic, while elderly women were most neophobic. Results from the current study are unable to support either of these previous findings as significant correlations were not found between age and hedonic scores. Hedonic scores did not differ significantly between age groups. However the consumers in the >55 years old age bracket (n=4) did have the highest mean hedonic score (mean hedonic score = 7.00) albeit not significant, due to the very small sample size available and large standard deviation.

Consumers belonging in higher income classes are more accepting of novel functional food products. Steptoe and colleagues (1995) posit that lower income consumers tend to purchase the same products when shopping, afraid to spend money on a food product he/she may not enjoy. Price is an important factor on driving food choices, especially among consumers with low compared to high income (Steptoe and others 1995). This pattern was not found in the current study, as mean hedonic scores for the household income brackets did not differ greatly. Also, as household income increased, the mean hedonic score did not increase as predicted by Childs’ (1997) characteristics of functional food consumers.

Level of education is positively associated with food choices, due to more lifestyle experiences (Eddy and others 1999). This trend was not found within the participants of the current study. Consumers in the present study were well-educated, and the level of education was higher compared to the overall U.S. population (United States Census Bureau 2007). Another difference in the current study’s participants compared to the overall U.S. population is the distribution of participants across income categories (29% earning <$20,000 and 48% earning >$71,000). Subjects were asked to indicate their own annual income, or if they were financially dependent to specify his/her household annual income. Many students were financially independent; thus, many of the participants were in the <$20,000 bracket. Older participants and financially dependent students were in the $71-100,000 and >$100,000 brackets.
These education and income variables were unique and, thus, were not significant correlates to acceptability of flaxmilk. If a similarly designed hedonic sensory panel was held with a more representative sample of the U.S. population, results might differ.

Value of perceived health benefit

When consumers were informed that flaxmilk had potential positive health benefits, they were more willing to add the beverage into their normal diets and consume larger amounts as compared to the flaxmilk being simply a beverage. These findings support those of Verbeke (2005), who found that the belief of health benefits was the main basis for acceptance of novel functional food products. Consumers are more likely to accept a novel food product when it is “good for you” (Pliner and others 1993). The perception of healthiness in food products plays a large role on their acceptance (Bech-Larsen and Grunert 2003).

Urala and Lakteenmaki (2004) explored consumer attitudes towards the use of functional food products. These researchers reported a perceived sense of reward when consumers utilized functional foods. The consumers' confidence in the effectiveness of the functional food product was a crucial factor in their willingness to make use of the product. Consumers generally make dietary choices based on health claims, health benefits, cost, convenience and taste of foods and beverages (Steptoe and others 1995). Compared to European counterparts in Denmark and Finland, Americans are reported to be more inclined to try novel functional foods (Bech-Larsen and Grunert 2003). The perception of functional foods being healthier than foods not enriched with additional nutrients may play a factor in the willingness of Americans to try novel products. Debate continues, however, around whether consumers are willing to trade sensory pleasure for health benefits. Some research suggests that consumers are willing to sacrifice taste for health benefits (Tuorila and Cardello 2002), while other studies do not support this idea. Rather, consumers are reluctant to change their eating habits and preferences for health benefits (Bech-Larsen and Grunert 2003; Verbeke 2006). It is critical for a novel functional food product to be acceptable by consumers to obtain commercial success.
Conclusion

This study shows promising results that flaxseed can be incorporated into a milk base to produce a beverage that provides both nutrients inherent in milk as well as in flaxseed. When participants were informed that flaxmilk had positive health benefits, the intent to consume flaxmilk almost doubled within the study population. The interest to consume flaxmilk may aid a subpopulation that does not normally drink milk or consume low-fat, calcium-rich dairy products, to drink flaxmilk as a source of dairy.

Willingness by these study participants to consume flaxmilk suggests that this novel beverage has great potential to serve as a vehicle to deliver valuable omega-3 fatty acids to the body, potentially providing health benefits.

Acknowledgements

The authors wish to thank all participants of this sensory panel as well as Walter Hartman for assistance in processing of flaxmilk.
References


Chapter IV
Flaxseed Functionality in an Omega-3 Fatty Acid Enriched Milk-Based Beverage

To be submitted to Journal of Food Quality
Abstract

The intake of foods rich in omega-3 fatty acids is insufficient in the average American diet. Flaxmilk was formulated to serve as a beverage vehicle to deliver omega-3 fatty acids (inherent in flaxseed) into consumer diets. Two chocolate milk-based beverages were processed, where one contained 0.41% (by weight) omega-3 fatty acid and the other, serving as a control, had minimal amounts. These beverages were evaluated for quality attributes, including physical, chemical and sensory characteristics, over a 14-day storage (4°C) period. The objective was to determine how flaxseed addition altered product quality compared to a well-known and well-accepted traditional dairy product. Sensory evaluation by untrained panelists demonstrated that flaxmilk was thicker, possessed a maltier flavor and more aftertaste, and was less sweet and had less chocolate flavor compared to chocolate milk. No significant differences in color were identified. Instrumental analysis showed significant greater L, a and b values, viscosity and total solids in the flaxmilk compared to control. Beverages compositions remained stable throughout the 14-day storage period; however, flaxmilk had a significantly higher proportion of total fat and unsaturated fats (including omega-3 fatty acids) than the control. Flaxmilk had a 1:2.3 omega-6:omega-3 fatty acid ratio, whereas the control had a ratio of 5.7:1. The elevated content of omega-3 fatty acids in flaxmilk can be added into the diet to increase intake of the valuable lipids. Although there were significant changes in the beverages during the storage period, the nutritive properties of flaxmilk did not decline. This indicates flaxmilk is a stable beverage that may serve as a beverage to help attain suggested intakes of omega-3 fatty acids in the diet.

Keywords: functional beverage, sensory evaluation, product characterization, flaxseed, flaxmilk, chocolate milk
Introduction

Many Americans, regardless of age or gender, select foods based on the potential health benefits (Milner 2002). The desire to stay healthy in a fast-paced environment is becoming more prevalent in the United States (U.S.). Consumers are attracted to conveniences in food preparation that support health maintenance and disease prevention (Niness 1999; Brouns and Vermeer 2000). Three key factors in the successful formulation of a novel food product are familiarity of potential health benefits from use of the product, easy incorporation into an active lifestyle and acceptability of product by consumers (Weststrate and others 2002).

Development of foods and beverages enriched with omega-3 lipids have contributed to an increased intake of these essential fatty acids by Americans. The additional omega-3 fatty acids in the diet provides health-promoting benefits while still maintaining normal dietary nutrient properties (Temelli and others 2004; Devcich and others 2007). In this case, omega-3 fatty acids may have cardioprotective effects via anti-thrombotic and anti-inflammatory mechanisms. Products such as milk, eggs, pasta, salad dressings and table spreads have been enriched with omega-3 fatty acids (Mantzioris and others 2000; Metcalf and others 2003). Most of these products are enriched with long-chain fatty acids originating from marine sources, leading to adverse fishy or metallic flavors. An alternative that has unexplored potential is the use of omega-3 fatty acid-rich plant sources for the enrichment of functional food products. The richest plant source for omega-3 fatty acids is flaxseed, with alpha-linolenic acid comprising over half of the total fat content. Flaxseed is also an excellent source for fiber and lignans (an antioxidant).

Milk serves as an excellent foundation for functional beverages, especially for consumers of Western cultures, due to its naturally present nutrient content and characteristic sensory properties (Boland and others 2001). Functional beverages may provide additional nutrients without significantly altering dietary habits of the consumer, as beverages may be typically consumed along with a meal (Hazen 2003; Temelli and others 2004).

A flaxseed-enriched milk-based beverage (“flaxmilk”) was formulated to deliver the nutrients present in both milk and flaxseed (Lau and others 2007). It is unknown
how the components within the colloidal system of flaxmilk will interact over a period of time (i.e. storage period between processing to usage by consumer). The overall objective of this study was to determine whether the addition of flaxseed will disrupt or alter the dairy colloidal system or if “flaxmilk” will behave similarly to that of a commonly accepted product found on the market (chocolate milk) over a 14-day storage period. The physical, chemical and sensory characteristics of the beverages were analyzed and compared to explore the effect of the addition of flaxseed into a chocolate milk-based colloidal dispersion.

**Materials and Methods**

*Preparation*

A flaxseed-enriched milk-based beverage (“flaxmilk”) was formulated for this study. After a number of preliminary recipes were tested (see Appendix A), a finalized recipe for flaxmilk was developed and achieved consumer acceptance (Lau and others 2007). Finely ground flaxseed (Pizzey’s Meadow Pure™ O3 BevGrad™, Gurnee, IL), malt milk mix (Chocolate Malted Milk, Nestlé Carnation, Switzerland), 100% pure vanilla extract (Rodelle Pure Vanilla Extract, Custom Blending, Fort Collins, CO), guar gum (GuarNT Bland 200 HV Powder, TIC gums, Belcamp, MD) and κ-carrageenan (Colloid 517 “T” Powder, TIC gums, Belcamp, MD) were incorporated into unpasteurized skim milk. The formulation was mixed, homogenized (54.4°C), pasteurized (73.9°C) and stored (4°C) in stainless steel containers to avoid excess exposure to light and air. Details of the processing procedure are reported elsewhere (Lau and others 2007). A control chocolate beverage was formulated with equivalent amounts of the ingredients in flaxmilk, except the flaxseed. The control beverage served as a familiar and commonly accepted product on the market to which to compare flaxmilk. This allowed investigators to identify any significant alterations in the colloidal chemistry from the presence of flaxseed in flaxmilk. Beverage formulations are presented in Table 4.1. Both beverages used the same amount (by weight) of ingredients, except that the control beverage did not contain flaxseed. Therefore, the percentages of ingredients are lower in flaxmilk due to the higher total dry weight of ingredients present, compared to control beverage.
Flaxmilk and control beverages were processed and stored at the same time to reduce confounding factors in time, ingredients and process procedures. Three independent replicates of flaxmilk and control chocolate milk were processed and analyzed for physical, chemical and sensory characteristics.

Table 4.1. Ingredients for chocolate flaxseed-enriched and control dairy-based beverages

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Flaxmilk (% by weight)</th>
<th>Control (% by weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td>87.14</td>
<td>89.34</td>
</tr>
<tr>
<td>Malt milk mix</td>
<td>9.59</td>
<td>9.84</td>
</tr>
<tr>
<td>Flaxseed</td>
<td>2.47</td>
<td>0.00</td>
</tr>
<tr>
<td>Vanilla extract</td>
<td>0.63</td>
<td>0.65</td>
</tr>
<tr>
<td>κ-carageenan</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>guar gum</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>100.00</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

*a Beverage formulations total to 100%. The same weights of ingredients were used in both beverages, except that the control beverage did not contain flaxseed. Percentages are lower in flaxmilk due to higher total dry weight of ingredients present, compared to control beverage.

**Sensory attribute testing**

Flaxmilk and control beverages were processed on day 0 of storage, in three independent replications. Products were stored at 4°C for 14 days and evaluated for sensory characteristics on d 1 and d 7 of storage. Untrained panelists, aged 18 years or older, were recruited from the Virginia Tech community (Blacksburg, VA) and included faculty, staff and students. One sensory panel (3rd replicate, day 1) took place at a health fair (Pulaski, VA), where panelists comprised mostly of senior citizens and Radford University nursing students. The recruited participants (all replicates) were comprised of over 70% females; approximately 50% were aged 18 to 25 years; over 45% have earned a college degree and about half the sample population were students.

Panelists were selected based on willingness to consume the milk-based beverages and no self-reported history of allergic reactions to dairy, flaxseed, cocoa,
chocolate flavoring and/or malted barley. Panelists signed informed consent forms to participate in the sensory panel and completed a demographic survey (Appendix B). A 9-point diagnostic test using a simple intensity scale (Appendix B) was used to characterize product sensory quality for specific attributes. Six attributes (color intensity, thickness, chocolate flavor, malty flavor, sweetness and aftertaste) were evaluated by each panelist. Anchor terms were used to relate the intensity of each product characteristic, where a value of “1” represented “none” and a value of “9” was “very intense” (Meilgaard and others 1999). Fifty panelists completed the analyses per storage day per replicate. Two sensory panels (day 1 and 7 of storage) were held per replication, providing 150 responses total per day of evaluation. Three hundred independent observations were attained per product. There was no tracking of repeat subjects; an estimated 35 individuals participated in more than one panel. The study was approved by the Institutional Review Board for Human Subjects Testing at Virginia Tech.

Flaxmilk and control chocolate milk samples were prepared for consumer evaluation on d 1 after processing. Approximately 22 mL of each beverage were poured into soufflé cups (1 oz), capped, coded with 3-digit numbers and stored at 4°C until served. Sensory evaluation took place in the Sensory Evaluation Laboratory in the Department of Food Science and Technology at Virginia Tech. Panelists were seated in independent sensory booths under white lights. The third replication panel on day 1 of storage (n=50), took place at the Pulaski County Administrative Building in Pulaski, VA. Panelists sampled at a booth set up at the Pulaski Health Fair, but were not isolated from one another.

Each panelist received one sample at a time and the order of samples was presented in a balanced randomized design. A rest period of approximately 1 minute was controlled between evaluations of the two samples. Panelists were given the chance to provide written comments about each sample. Water was provided to cleanse the palate before and after tasting of samples. Panelists were offered candy as compensation for participating in the sensory panels.
Product characterization

Flaxmilk and control chocolate milk products were evaluated on d 1, 7 and 14 of storage to assess product composition and quality. Physical characteristics included color, viscosity and compositional stability. Compositional analysis included total moisture, total ash, total protein, total lipids and fatty acid profile. Microbiological assessment included total aerobic plate count, total coliform count and total yeast and mold counts. These analyses were completed in the Analytical Support Laboratory in the Department of Food Science and Technology at Virginia Tech.

Physical characteristics

Color

L, a and b values for flaxmilk and the control chocolate milk were measured by a tristimulus colorimeter (Model CR-2000 with CR-A70 attachment, Minolta Corporation, Japan). In this color system, L illustrates black to white (0 to 100), a represents green (-) to red (+) and b portrays blue (-) to yellow (+). The colorimeter was calibrated using a white plate with specific values of L = 97.29, a = -0.18, b = +3.75.

Viscosity

Viscosity was measured using a Synchro-lectric viscometer (Model RVT, Brookfield Engineering Laboratories, Inc, Stoughton, MA). Samples (~300mL) of each beverage were poured into 400 mL glass beakers. Spindle number 6 rotating at 50 rpm was used to give readings between 0 and 100 on the scale. Readings of the viscometer were converted to apparent viscosity values (mPa·s) by multiplying with the conversion factor (200), supplied by the manufacturer for spindle number 6, attached to the viscometer.

Compositional stability

The Pennsylvania modified Babcock method (Class B) for chocolate milk was performed to measure total lipid content (expressed as a percentage) in the top and bottom layers of flaxmilk and chocolate control beverages (Wehr and Frank 2004). Each beverage was poured in a 100 mL graduated cylinder, capped and stored until day of analysis. On the day of analysis, the top layer (18 g) was first collected using a 1 mL pipette for each beverage. The bottom layer (18 g) then was collected using a new 1 mL pipette. The total lipid contents of the top and bottom layers were compared to
one another to evaluate compositional stability of each beverage system (Scott and others 2003; Bolling and others 2005).

### Compositional characteristics

**Total moisture**

Total moisture content (expressed as a percentage) was measured using Standard Method 15.114 (moisture/solids, forced draft oven, milk (class A1); Wehr and Frank 2004). After removal of moisture in oven, samples were placed in a desiccator for at least 4 hours until weight was stable, to further remove any moisture from atmospheric humidity.

**Total ash**

Total ash content (expressed as a percentage) was measured using Standard Method 15.040 (Wehr and Frank 2004). After removal of moisture in oven, samples were placed in a muffle furnace at 540°C to incinerate the dehydrated beverage samples to remove their carbon content. The inorganic residual material (i.e. minerals) remained for enumeration.

**Total protein**

Protein concentration (mg/mL) was determined using a dye-binding assay (Bio-Rad RC/DC protein assay, Bio-Rad Laboratories, Hercules, CA). Beverages (1 mL) were diluted 50-fold and prepared according to assay instructions. Samples were centrifuged in a rotor (Model SM-24, Sorvall Centrifuges, DuPont Company, Wilmington, DE) placed in a refrigerated superspeed centrifuge (Model RC-5B, Sorvall Centrifuges, DuPont Company, Wilmington, DE) and centrifuged at 11000 rpm for 5 min.

Protein content (expressed as a percentage) was calculated by

\[
\text{protein conc (mg/mL)} \times \frac{100}{100} = \text{protein conc (mg/100mL)}
\]

\[
\text{protein conc (mg/100mL)} \times \frac{1g}{1000mg} = \text{protein conc (g/100mL)}
\]

\[
\text{protein conc} = \left(\frac{\% \text{weight/volume}}{}\right) = \text{protein} \%.
\]

**Total lipids**

The Pennsylvania modified Babcock method (Class B) for chocolate milk was performed as described for compositional stability (Wehr and Frank 2004). The total lipid content of the top and bottom layers were averaged together to represent the total lipid content (expressed as a percentage) for the beverage for day of storage.
**Lipid extraction**

Lipids were extracted from beverages using chloroform and methanol as described by Bligh and Dyer (1959), modified by Herzallah and colleagues (2005) with additional alterations in the separation and filtration process and time. Approximately 100 mL of beverage were homogenized with 100 mL methanol and 100 mL chloroform in a Waring blender for 2 min (140V, 50% maximum output voltage). An additional 100 mL chloroform were added and the mixture was homogenized again for 2 min. Equal volumes of homogenate were transferred to four 250 mL centrifuge jars (model 422620, Corning, US). Jars were pair-balanced, placed in a rotor (Model SLA-1500, Sorvall Centrifuges, DuPont Company, Wilmington, DE) and centrifuged at 2500 rpm for 20 min using a refrigerated superspeed centrifuge (Model RC-5B, Sorvall Centrifuges, DuPont Company, Wilmington, DE). The upper layer of methanol and water was removed by aspiration. The middle layer (consisting of the stabilizers, protein and flavorings) and the bottom layer (chloroform-lipid extracts) remained. The chloroform-lipid extracts layer was transferred to an Erlenmeyer flask containing anhydrous sodium sulfate and refrigerated overnight. After storage, the chloroform-lipid extract was filtered through new anhydrous sodium sulfate and rinsed with approximately 10 mL chloroform into a round bottom flask. The round bottom flask containing the chloroform-lipid extract was attached to a roto-evaporator (R-3000, Buchi Rotavapor, Switzerland). Chloroform was removed by roto-evaporation, under vacuum at approximately 57°C and 70-95% rpm (increasing speed as lipid became more concentrated). The remaining lipid extracts were stored in 5 mL clear glass vials and nitrogen flushed at 20.5°C. Lipid extracts were immediately capped and stored at -17°C. Extracted lipids then were used for fatty acid profiling.

**Fatty acid profile**

Extracted lipids were transesterified to methyl esters with 0.5 N NaOH in methanol and 14% boron trifluoride in methanol using the *AOCS official method Ce 1b-89* (Park and Goins 1994, Firestone 1998). All samples were analyzed on a Hewlett Packard 5890 gas chromatograph with a split/splitless injector and flame ionization detector (Agilent Technologies, Palo Alto, CA). A 30 m x 0.25 mm (i.d.) column (DB-225, 0.25 μm film thickness, J & W Scientific, Folsom, CA) was used to separate the
fatty acid methyl esters. The temperature program for separation began at 80º C, was maintained for 5 min, then increased to 220º C at 10º C/min and held at 220º C for 4 min. Total analysis time was 23 min. Injector and detector temperatures were 275º C. Ultrapure helium was the carrier gas with a gas velocity of 30 cm/sec. The injection volume was 1.0 μL, and a split ratio of 50:1 was used. Fatty acids were identified by comparison of elution times with standard fatty acids (16A standard, Nu-Check Prep, Elysian, MN) and were normalized as percent area. A linseed oil standard composed of methyl palmitate (16:0), methyl stearate (18:0), methyl oleate (18:1), methyl linoleate (18:2) and methyl linolenate (18:3) was used to identify these fatty acids in the beverages. Data were integrated and quantified using a HP 3393A (Hewlett-Packard, Palo Alto, CA, USA) integrator. Area counts of peaks were evaluated to estimate proportion of saturated, monounsaturated and polyunsaturated fatty acids present in beverages. Saturated fatty acids included 4:0, 6:0, 8:0, 10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 22:0. Unsaturated fatty acids included 14:1, 16:1, 17:1, 18:1, 18:2 and 18:3. All trans fatty acids and long chain polyunsaturated fatty acids (> 20 carbons) were categorized as “unknown”. Averages of fatty acid as percent of total lipid weight of its respective beverage are reported. Lipids of flaxmilk were extracted on d 1, 7 and 14 of storage and from the control on d 1 and 14 of storage from one replicate for analysis.

**Estimation of total carbohydrate and caloric content**

Proximal analysis was performed to estimate total carbohydrate content of flaxmilk, using the following formula:

\[
\text{carbohydrate\%} = 100\% - (\text{fat\%} + \text{protein\%} + \text{moisture\%} + \text{ash\%})
\]

where total fat, protein, moisture and ash contents were evaluated using the respective analytical procedures described above.

Total caloric content (kcal) was calculated by summing up total fat content multiplied by 9 kcal/g fat; total protein content multiplied by 4 kcal/g protein and total carbohydrate content multiplied by 4 kcal/g carbohydrate.

**Microbiological assessment**

All samples were evaluated for total aerobic count, total coliform bacteria count and total yeast and mold count based on standard methods for 3M Petrifilm™ (3M, St
Paul, MN) for aerobic plate count, coliform count, and yeast and mold count (Wehr and Frank 2004). The aerobic count is used to evaluate the level of microorganisms present in the food product. The coliform count measures post-pasteurization contamination by bacteria that attacks protein and lactose; forming acid and gas producing an unclean flavor (Wehr and Frank 2004; Walstra and others 2006). The yeast and mold count is used to enumerate yeast and mold populations present in a food product.

Bacterial counts were performed on d 1, 7, and 14 of storage to determine whether the formulated beverages were of high microbiological quality. These methods are suitable for all dairy products. All test tubes were autoclaved (15 min at 121° C) prior to plating, and sterile disposable pipets were used. Petrifilm plates were stored as instructed by 3M.

Statistical analysis

Two beverages were processed and replicated three times. Demographic information and consumption patterns were expressed in percentages for overall population as well as by gender. Sensory attribute data were analyzed using a 3-way analysis of variance (ANOVA) using the general linear model (GLM) procedure, with main effects of replicate, beverage and day of storage. Means and standard errors are reported. The null hypotheses for the sensory attribute portion of the study state 1) there will be no detectable difference in sensory properties of flaxmilk and the control beverage by untrained panelists on each storage day (day 1 and 7), and 2) there will be no significant changes evaluated by untrained panelists, in sensory properties over a 7-day storage period.

At least two observations per physical or chemical analysis were performed for each replicate with the exception of fatty acid profiling and total aerobic plate count, where only one replicate of each was conducted. Product characteristics of the two beverages were also analyzed using a 3-way ANOVA using the GLM procedure, with the main effects of replicate, beverage and day of storage. Means and standard errors are reported. The null hypotheses for the analytical portion of the study state 1) physical and chemical product characteristics will not differ significantly in the flaxmilk from the control on each storage day (day 1 and 7), and 2) product characteristics will not significantly be altered during the 14-day storage period. All statistical analyses
Results and Discussion

Microbiological assessment

In order to assure that beverages were safe for human consumption, microbiological assessments including total aerobic count, total coliform count and total yeast and mold count, were performed. The bacterial quality of the processed beverages was low initially and remained at the acceptable level throughout the 14-day shelf life. All microbiological counts were well below the required limits for pasteurized fluid chocolate milk beverages (Webster 2007). Although aerobic bacteria, coliform, and yeast and mold are common in chocolate milk powders, the processing of flaxmilk and control beverages reduced these, indicating these beverages were heated sufficiently and recontamination did not occur post-pasteurization.

Overall composition of beverages

The total moisture content of the beverages was significantly higher in the control beverage compared to the flaxmilk over the entire storage period (Table 4.2). The added flaxseed contributed to an increased total solids, therefore decreasing the total moisture content in comparison to the control beverage. Moisture content averaged over the 14 day period of flaxmilk (79%) and control (81%) beverages was influenced by day and replication, but no observable trend was present over the 14-day storage period (4°C). Moisture content was significantly different across replication (p<0.05), indicating some variance in the processing or analysis procedures.

<table>
<thead>
<tr>
<th></th>
<th>Flaxmilk (mean ± SD)</th>
<th>Control (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>78.68 ± 0.76</td>
<td>80.90 ± 0.57</td>
</tr>
<tr>
<td>Day 7</td>
<td>78.61 ± 0.75</td>
<td>80.71 ± 0.76</td>
</tr>
<tr>
<td>Day 14</td>
<td>79.42 ± 0.51</td>
<td>81.44 ± 0.38</td>
</tr>
</tbody>
</table>

Total ash content did not differ between the beverages (p>0.05). Flaxseed is composed of 3% ash (Burrington 2004), which did not play a significant role in the total moisture content.
ash of the flaxmilk. Ash content for flaxmilk averages 0.89% and for control beverage averaged 0.90%. There were no significant differences in the total ash content (p>0.05) between the beverages over the 14-day storage period. Ash content did vary by replication (p<0.05).

Total protein content significantly differed between the flaxmilk and control beverages (p<0.0001). Flaxseed is composed of approximately 20% protein (Burrington 2004), which contributed a significant amount of protein into the beverage in comparison to the control beverage. Protein content for flaxmilk averaged 41 mg/mL compared to 37 mg/mL for chocolate milk. Total protein content also differed significantly for beverages over the 14-day storage period as well as across replication (p<0.05).

Total lipid content of the beverages were significantly different (p<0.05). Flaxmilk had an average 1.11% fat content and the control had an average 0.3% fat content. The fat content of the flaxmilk were significantly greater than the control beverage throughout the storage period (Table 4.3). This demonstrates the higher level of lipids present in flaxmilk due to the addition of the flaxseed, since flaxseed’s nutrient profile is composed of 40% total fat (Burrington 2004). The addition of flaxseed successfully increased the fat content by more than 3-fold in the flaxmilk compared to the control.

Table 4.3. Fat content (%) of chocolate flaxseed-enriched and control dairy-based beverages over a 14-day storage period (4°C)

<table>
<thead>
<tr>
<th></th>
<th>Flaxmilk (mean ± SD)</th>
<th>Control (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1.16 ± 0.11</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Day 7</td>
<td>1.04 ± 0.24</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>Day 14</td>
<td>1.08 ± 0.23</td>
<td>0.28 ± 0.05</td>
</tr>
</tbody>
</table>

Mean values are significantly different (p<0.05) between the two products.

Fatty acid content by percent total weight of each beverage was calculated. The added flaxseed in the flaxmilk altered the composition (total weight of flaxmilk ingredients is 5% greater than the control beverage), negating the direct comparability of the beverages by percent weight. Fatty acid percentages in the chocolate milk control product will be proportionally higher because of this difference.
Low fat (1% milk fat) white milk contains 0.97 g total lipids, broken down to 0.63 g saturated fat, 0.28 g monounsaturated fat and 0.035 g polyunsaturated fatty acids, in a 100 g serving (NDB no. 01082; US Department of Agriculture/Agricultural Research Service 2007). Fatty acids of the beverages in the current study were separated by degree of unsaturation (i.e. saturated, monounsaturated and polyunsaturated fat) (Figure 4.1). The total fat of flaxseed is comprised of 9% saturated fat, 18% monounsaturated fat and 73% polyunsaturated fat (Burrington 2004). With the high amount of polyunsaturated fat present in flaxseed, there was an inverse relationship of saturated fatty acids with polyunsaturated fatty acids in flaxmilk compared to the control chocolate milk. As expected, the mean percent of saturated fatty acids was higher in the control compared to the flaxmilk (54.1% and 20.2%, respectively). On the other hand, the mean percent of total unsaturated fatty acids was lower in the control compared to the flaxmilk (41.6% and 76.4%, respectively). More specifically, the mean monounsaturated fats provided 27.0% of the control beverage’s fatty acid profile, and 23.2% of the flaxmilk’s profile. Polyunsaturated fats provided only 14.6% (mean) of the control beverage’s fatty acid profile, while 53.2% (mean) of the flaxmilk fatty acid profile was polyunsaturated.

![Figure 4.1. Distribution of saturated, monounsaturated and polyunsaturated fatty acids in chocolate flaxseed-enriched and control dairy-based beverages averaged over a 14-day storage period (4°C). Monounsaturated fatty acid content was similar in both beverages. There is an inverse relationship of saturated fatty acids with polyunsaturated fatty acids in flaxmilk compared to chocolate milk.](image)
The composition of flaxseed is estimated to be 40% fat. Of the total fat, 73% is composed of the polyunsaturated variety, more specifically 57% omega-3 (mostly 18:3) and 16% omega-6 fatty acids (mostly 18:2; Burrington 2004). Milk also contains inherent unsaturated fatty acids (14:1, 16:1, 17:1, 18:1, 18:2 and 18:3); however the flaxseed provides most of the unsaturated fatty acids in flaxmilk. Both beverages had similar amounts of saturated and unsaturated fatty acids, but the addition of flaxseed into the flaxmilk dramatically increased the polyunsaturated fatty acid content (Figure 4.1).

Short chain saturated fatty acids (4:0-12:0) are naturally present in milk as well as the malted milk mix. Guar gum, κ-carageenan and vanilla extracts do not contribute fat in this formulation. These lipid sources are present in both flaxmilk and control beverages.

The 18:3 (an omega-3 fatty acid) content in the flaxmilk is significantly greater than in the control beverage, as expected with the addition of flaxseed in the chocolate milk foundation (Figure 4.2). The flaxmilk was composed of an average of 37.0% omega-3 fatty acid content of its total lipid weight; therefore a 100 g sample of flaxmilk is estimated to contain 0.41 g omega-3 fatty acids (Table 4.3). The control beverage was composed of 2.2% omega-3 fatty acid content of its total lipid weight; therefore a 100 g sample of the control beverage is estimated to contain only 0.0065 g omega-3 fatty acids. The 18:2 (an omega-6 fatty acid) content was also significantly greater (p<0.0001) in the flaxmilk compared to the control beverage (Figure 4.2). The flaxmilk was composed of an average of 16.2% omega-6 fatty acid content of its total lipid weight, and the control beverage was 12.5% omega-6 fatty acid of its total lipid weight. The omega-6:omega-3 fatty acid ratio of flaxmilk is calculated to be 1:2.28, while it is 5.74:1 for the control. It is clear that the addition of flaxseed greatly increased the omega-3 fatty acid content in the flaxmilk beverage, compared to the control.

Over the 14-day storage period, significant changes in fatty acid profile of the beverages did not occur. This suggests there were no significant chemical or structural changes of the fatty acids in the beverage’s colloidal system over the study period. This retention of the structural composition of the fatty acids also suggests that limited or
no oxidation occurred, as the percentage of unsaturated fatty acids remained consistent over the 14-day period.

![Graph showing distribution of omega-3 and omega-6 fatty acids in flaxmilk and control beverage over 14 days.]

Figure 4.2. Distribution of omega-3 and omega-6 fatty acids present in chocolate flaxseed-enriched and a control dairy-based beverage averaged over a 14-day storage period (4°C).

An estimated nutrient profile of flaxmilk is reported in Table 4.4 and compared to the nutrient profiles of 1% white and chocolate milks. Flaxmilk had a higher caloric, protein and carbohydrate content than the 1% white and chocolate milks. They each had similar fat content at 1% fat, though fat profile in flaxmilk was mostly comprised of fat inherent in flaxseed rather than milkfat. Omega-3 fatty acid content was greater in the flaxmilk, due to the fatty acids from the flaxseed. Fiber content was not analyzed for flaxmilk, but would be expected to be greater than the fiber content of the 1% white and chocolate milks. Flaxseed is comprised of 28% dietary fiber (Burrington 2004), where milk does not naturally contain any fiber. Therefore the addition of flaxseed into a milk foundation would significantly increase the dietary fiber content.
Table 4.4. Nutrient profiles of 100 g serving of 1% white (reference code #11112210) and chocolate (reference code #11511400) milks (Nutrient profiles compiled from information taken from the USDA Food and Nutrient Database for Dietary Studies) and estimated nutrient profile of flaxmilk

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>1% white milk</th>
<th>1% chocolate milk</th>
<th>flaxmilk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>42.0</td>
<td>63.0</td>
<td>86.54</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>3.37</td>
<td>3.24</td>
<td>4.06</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>4.99</td>
<td>10.44</td>
<td>15.1</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0.97</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Omega-3 fatty acids (g)</td>
<td>0</td>
<td>0.01</td>
<td>0.41</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>0</td>
<td>0.5</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Attempts to modify the fatty acid profile of cow’s milk have previously been carried out. The 1988 Milk Fat Roundtable held by the Wisconsin Milk Board stated that milk fat typically contains 5% polyunsaturated fatty acids, 25% monounsaturated fatty acids and 70% saturated fatty acids (Grummer 1991). An ideal profile for milk fat should be comprised of 10% polyunsaturated fatty acids, 82% monounsaturated fatty acids and 8% saturated fatty acids. Unlike other enriched animal products such as eggs and meat, the lipid profile of milk cannot be enhanced with the modification of cattle feed. When cattle are fed a diet rich in unsaturated lipids, the microorganisms present in the rumen attack the unsaturated fats and convert them to saturated fatty acids, resulting in a high content of saturated fatty acids in milk fat (Mansbridge and Blake 1997). In response to this unsuccessful method of enrichment of the lipid profile, more innovative approaches such as 1) feeding encapsulated unsaturated fatty acids to protect and preserve the lipid within, while present in the rumen (Sabikhi 2004, Carroll and others 2006, Heguy 2006), 2) the infusion of unsaturated fatty acids post-ruminally to decrease short and medium saturated chain fatty acids (Mansbridge and Blake 1997, DePeters and others 2001) and 3) potentially transgenic animals to produce desaturase enzymes to increase unsaturated fatty acid content (Kao and others 2006) have been performed. The unsaturated fat content successfully increased using these mentioned methods to modify the lipid profile of bovine milk. Human consumption of dairy products containing elevated levels of unsaturated fat has been show to successfully reduce

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plasma low-density lipoprotein levels of cholesterol (Ashes and others 1997). Although these approaches to increase the unsaturated fatty acid content of milk were successful, the cost-volume-profit relationship may not be optimal. The fortification of harvested milk may be a more accessible avenue to modify the lipid profile.

**Physical characteristics**

The compositional stability of the beverages was determined by comparison of the fat content of the top and bottom layers of each beverage. There were no significant differences between the top and bottom layers for each beverage throughout the storage period. This indicates there was no significant separation (p=0.09) demonstrating compositional stability in both beverages over the storage period. The type of beverage, day of storage and replicate did not play a factor in the compositional stability of the beverages.

The viscosity of the flaxmilk was significantly greater than the control throughout the 14-day storage period (Figure 4.3). The viscosity measurements of the flaxmilk remained consistent (p=0.36), while the control beverage’s increased over time (p<0.01).

![Bar chart showing viscosity of flaxmilk and control over 14 days](image)

**Figure 4.3. Total viscosity (mPa·s) of chocolate flaxseed-enriched (1% fat) and control dairy-based (0.03% fat) beverages over a 14-day storage period (4°C)**

Instrumental measurements of viscosity agree with the consumer perception of thickness. Panelists participating in the sensory panel rated flaxmilk to be significantly
thicker than control beverage (p<0.0001) as seen in Figure 4.4. The thicker and more viscous rheology is likely an effect of the presence and interaction of the flaxseed (particularly its fiber component), guar gum and κ-carageenan with water present in the flaxmilk matrix. Hydrocolloids were added to help prevent significant dispersion of the beverage emulsions over the storage period. Guar gum was added to serve as an emulsifier and stabilizer. κ-Carageenan was added to keep the cocoa powder dispersed in the emulsion as well as acting as a stabilizer and thickener in the beverages. These hydrocolloids adsorb onto casein present in milk to form a micelle network, which increases viscosity of the entire beverage system (Langendorff and others 2000; Yanes and others 2002). In addition, flaxseed is high in dietary and soluble fiber, which will absorb moisture in solution and swell during processing, likely contributing to the increased viscosity seen in the flaxmilk compared to the control. Overall, the interaction of the added hydrocolloids with the flaxseed appears to produce a thicker and more viscous flaxmilk system compared to the control (Langendorff and others 2000). In addition, the viscosity of milk beverages increases as fat content increases (Phillips and others 1995). Therefore, the added fat from the flaxseed may also have an effect on the viscosity of flaxmilk. Lastly, flaxseed forms suspensions rather than solutions, causing greater viscosity due to the dispersion of the colloidal components in the beverage.

The beverage ingredients were the same, except for the presence of flaxseed in the flaxmilk. This indicates the amount of flaxseed added in the flaxmilk recipe, produced significant changes in the colloidal chemistry and rheological measurements of the beverage, compared to the control beverage as detected by untrained panelists (Figure 4.4).

Color was measured instrumentally using a colorimeter as well as by participants in the sensory panels. L, a and b values derived from the colorimeter were significantly greater in the flaxmilk samples compared to the control throughout the storage period. Both products had a general brown color, however the flaxmilk had more red and yellow hues and was slightly darker (based on the L, a and b values) compared to the control. Some color changes were observed in both beverages. The a and b values of both beverages significantly increased over the 14-day storage period (p<0.0001). The L
values of the beverages decreased significantly over the 14-day storage period (p<0.0001). There was a 3-way interaction between replicate, beverage and day of storage that played an effect for each color value (p<0.05).

Consumer sensory evaluation of color and thickness between the beverages were compared on d 1 and 7 of storage. Although instrumental measurements of color indicated significant differences, participants in the consumer panels were unable to (p = 0.26) as seen in Figure 4.4.

**Flavor evaluation**

Consumer sensory evaluation of the attributes (chocolate flavor, malty flavor, sweetness and aftertaste) between flaxmilk and control beverages was compared on d 1 and 7 of storage. All replicates were combined and analyzed together by storage day. For both storage days, malty flavor and aftertaste were detected as significantly more intense in the flaxmilk compared to the control beverage (Figure 4.4). Chocolate flavor and sweetness were significantly more intense in the control beverage than the flaxmilk (Figure 4.4).

![Figure 4.4. Radar plot of consumers’ perception for the intensity of six sensory attributes for chocolate flaxseed-enriched and control dairy-based beverages on days 1 and 7 of storage (4°C). A value of “1” represented “none” and a value of “9” was “very intense”. * p<0.05](image)

* p<0.05
Perceived differences in the flavor of the beverages occurred, as seen with malty flavor and aftertaste (Figure 4.4). This can be explained by the presence of the flaxseed that contains an inherent malty flavor. The higher intensities of chocolate flavor and sweetness were detected in the control beverage, despite having the same amount in the flaxmilk recipe. This may be due to the presence of the flaxseed that masked the chocolate flavor and sweetness in the flaxmilk recipe, and more cocoa and sugar were added to reach the detectable threshold level in the flaxmilk formulation. However when the flaxseed is not added, the chocolate flavor and sweetness were detected to be more intense (p<0.0001 for both attributes), most likely due to the higher compositional percentage of these ingredients in the control beverage as opposed to the flaxmilk. In addition, the thicker and more viscous texture may have also masked the chocolate and sweetness flavors in flaxmilk, whereas the control beverage did not possess this masking effect with its less viscous texture (Ferry and others 2006).

Consumer panels detected significantly higher intensities of thickness, malty flavor and aftertaste in the flaxmilk compared to the control. Chocolate flavor and sweetness were significantly more intense in the control beverage compared to the flaxmilk. The color of the beverages did not change over the 7-day storage period. This did not support the null hypothesis of no differences in the sensory properties of the beverages on each storage day. However as expected, the consumers' perception of the beverage attributes did not change over the 7-day storage period.

Analysis of physical and compositional characteristics demonstrated no significant differences in total ash content and compositional stability of the beverages; greater moisture content in the control beverage and greater total protein, lipid, viscosity and L, a and b values in flaxmilk. All attributes, with the exception of total ash and compositional stability, did not support the null hypothesis.

Conclusion

Flaxmilk was formulated to serve as a beverage vehicle to increase the intake of omega-3 fatty acids into the diets of consumers by providing 0.41 g of omega-3 fatty acids in 100 g of flaxmilk and an omega-6:omega-3 fatty acid ratio of 1:2.3. Sensory evaluation demonstrated the flaxmilk was detected to be thicker, to possess a maltier flavor and more aftertaste, and less sweet and less chocolate flavor compared to the
control beverage. There were no significant differences detected for color. Instrumental analysis showed significant greater L, a and b values, viscosity and total solids in the flaxmilk compared to the control beverage. The composition of the beverages remained stable throughout the storage period; however flaxmilk had a significantly higher proportion of total fat and unsaturated fats (including omega-3 fatty acids) compared to the control.

This study provides valuable information of characteristics of a novel flaxseed-enriched milk-based beverage in comparison to a control beverage (similar to products currently in the market). Product characterization was limited to the attributes analyzed in the current study, which does not fully describe the physical and chemical components of flaxmilk. Further research may investigate the oxidative stability (both autoxidation and photooxidation) of flaxmilk and determine whether an addition of an antioxidant (i.e. vitamin E) would be necessary to enhance the quality of the beverage. Potential analytical tests would be to examine total solid content of the top and bottom layers of flaxmilk and control beverage and fiber content analysis. This may help indicate whether any separation and/or production of sediment occurs in either beverage. In addition, packaging options to maintain stability of flaxmilk would be an interesting avenue of research.

A prominent limitation in the current study was that for most analytical attributes (L, a and b values, viscosity, total moisture, ash and protein content) tested, replicate had an effect on the recorded observations. This suggests that there was some variability in the processing and analytical procedures that took place. In future research, more standardized methods will need to take place.

In conclusion, flaxmilk did differ from the control beverage for several physical, chemical and sensory characteristics. Many changes in the beverage characteristics also occurred during the 14-day storage period.

Acknowledgment

The authors wish to thank all participants of these sensory panels as well as Walter Hartman, Sean O’Keefe, Kim Waterman and Harriet Williams for assistance in processing and various analytical procedures and Josh Landon for assistance in the statistical analysis.
References


Chapter V
Flax up your health
Introduction

The American population is becoming increasingly concerned about various health conditions including heart disease, overweight and obesity and aging, specifically in the baby boomer population. With this in mind, more individuals are becoming conscious of the quality of their diets. Many individuals evaluate the Nutrition Facts Panel on food packages to compare the nutritional content of products. Eating no longer involves just consumption of foods; eating is also a means to take charge of one's diet, nutrition and general health. In today’s society, there is a growing interest in the use of functional foods and beverages in the daily diet. In 2005, the Institute of Food Technologists defined functional foods as “foods and food components that provide a health benefit beyond basic nutrition for the intended population (Institute of Food Technologists 2005).” Health benefits of functional food products are often supported by structure-function claims and health claims, approved by the Food and Drug Administration (FDA).

Market trends

Retail sales of functional foods and beverages in the United States approached $25 billion in 2006, representing a growth of 9.5% from 2005 (Packaged Facts 2007). As additional functional food and beverage products are formulated by food manufacturers and become available in the marketplace, sales in the United States are predicted to grow to nearly $40 billion by 2011. Research and development of new concepts for functional foods and beverages is on the rising and not expected to slow any time soon.

One of the most currently popular food ingredients being incorporated into food and beverage products are the omega-3 fatty acids. In 2004, the FDA issued a qualified health claim, stating that supportive but not conclusive research suggested that consumption of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may reduce the risk of coronary heart disease. In reaction to this qualified health claim, many food manufacturers have been formulating various enriched foods and beverages with marine-derived fatty acids to increase the omega-3 fatty acids present in food. By early 2006, EPA- and DHA-enriched foods and beverages have entered the mainstream supermarkets in the United States. According to a HealthFocus USA Trend Survey, four
out of 10 adults were actively adding more omega-3 fatty acids into their diets (Horovitz 2007).

**Health benefits**

A primary role of omega-3 fatty acids is serving as the precursor of eicosanoids. Eicosanoids are short-lived signaling molecules that regulate body functions including heart rate, blood pressure, blood clotting, inflammation and the inflammatory response to fight infections in the body.

There are two classes of essential fatty acids: omega-6 and omega-3 fatty acids. Both need to be consumed through the diet, as the human body lacks the enzymes to synthesize these fatty acids from available substrates. The parent compounds of the essential fatty acids are linoleic acid (omega-6) and alpha-linolenic acid (omega-3). Currently, dietary intake of omega-6 fatty acids is approximately ten-fold greater than intake of omega-3 fatty acids, due to the vast consumption of cereal grain-based products, vegetable oils and domesticated animal-derived products. Eicosanoids derived from the omega-6 fatty acids are pro-inflammatory, whereas those from omega-3 fatty acids are anti-inflammatory. A significantly higher intake of omega-6 fatty acids compared to omega-3 fatty acids eventually instigates inflammation in the body, potentially leading to more serious chronic diseases including coronary heart disease, osteoporosis and rheumatoid arthritis. An increased intake of omega-3 fatty acids may help attenuate inflammation, thereby decreasing detrimental effects of a chronic inflammatory process.

**Not all fatty acids are created equal**

Parent compounds in the omega-6 and omega-3 fatty acid classes act as precursors for the elongation process. The parent compounds compete for the delta-6-desaturase enzyme, which initiates metabolic pathways that produce longer-chain fatty acids. Linoleic acid (omega-6 parent compound) undergoes desaturation to form gamma-linolenic acid and arachidonic acid which are precursors to pro-inflammatory eicosanoids. Conversely, alpha-linolenic acid (omega-3 parent compound) desaturates to form EPA and DHA, which produce anti-inflammatory eicosanoids.

Although delta-6-desaturase has a higher affinity for alpha-linolenic acid, the higher intake of omega-6 fatty acids compared to omega-3 fatty acids in the American
diet increases the availability of linoleic acid in the body to undergo desaturation. This results in the favored production of pro-inflammatory eicosanoids, initiation inflammation. The delta-6-desaturase enzyme is a slow and rate-limiting step. If delta-6-desaturation is impaired for any reason, production of further metabolites will decline. Therefore, the increased intake of alpha-linolenic acid can react with the delta-6 desaturase and shift the metabolic pathway toward elongation of the omega-3 fatty acids and away from the production of pro-inflammatory omega-6 fatty acids.

Studies have show that the optimal dietary approach to attaining heart healthy benefits is to consume fish and fish products to increase EPA and DHA intake. Alpha-linolenic can metabolize to produce these longer-chain fatty acids; however, the conversion rates in humans are modest at best. Although it is the longer-chain omega-3 fatty acids that hold the functional anti-inflammatory role, their precursor, alpha-linolenic acid is still important. Most research has focused on EPA and DHA. There is a lack of research supporting the potentially positive benefits of alpha-linolenic acid intake and attenuation of inflammation in the body.

**Currently available products**

The American Heart Association recommends that individuals consume fish and fish products to provide omega-3 fatty acids (specifically EPA and DHA) in the diet. However, most modern Western societies seldom consume large amounts of fish and/or fish products due to the high cost, inconvenience or aversion to fishy flavors. This is where functional foods and beverages play a role. The incorporation of omega-3 fatty acids into food and beverage products is not a new concept. Various products, including table spreads, baked goods, pasta, ice cream and shelf-stable milks, have been formulated, but not all are commercially available.

The use of milk as the base for a functional beverage may aid in increasing milk consumption in the United States, which has declined over the past decades, particularly due to competition with juices and sodas. Milk serves as an efficient vehicle for fat absorption due to the presence of very small micelles which increase the surface area for absorption of fats and fat-soluble compounds. The addition of omega-3 fatty acids into a milk-base beverage is feasible.
Fish-oil enriched milk-based beverages have been previously formulated for use in human clinical trials. Fatty acids in milk-based beverages were absorbed and reached the plasma as demonstrated by significant increases in plasma concentrations of EPA and DHA after daily consumption of omega-3 enriched milk-based beverages for at least several weeks.

There is a consensus that the longer-chain fatty acids are more effective than alpha-linolenic acid in the attenuation of inflammation. However, the use of marine-derived oils is limited by their oxidative susceptibility leading to pungent fishy and metallic off-flavors. EPA and DHA are more susceptible to lipid oxidation than alpha-linolenic acid. In addition, groups who do not consume seafood avoid these fish-oil enriched beverages. An alternative is to use plant-derived omega-3 fatty acid sources in the formulation of foods and beverages as delivery vehicles to increase the omega-3 fatty acid intake in the diet.

**Novel product development**

Flaxseed (*Linum usitatissimum*) is a blue flowered crop, where the seeds can be used for food and animal feed. The seed itself is dark brown in color, flat, oval with a pointed tip and slightly larger than a sesame seed. It has a nutty and earthy flavor. Flaxseed is the richest source of alpha-linolenic acid composed of 37% total fat, where 57% of the total fat is alpha-linolenic acid. It is also a dietary source for small amounts of some fat-soluble vitamins, including vitamin E compounds (i.e. tocopherols) which provide antioxidant properties and may help promote oxidative stability.

Flaxseed is generally used as an ingredient in baked goods, where the baking heat does not cause any significant detrimental effects on the nutrient content of the flaxseeds. Researchers at Virginia Tech have successfully formulated a flaxseed-enriched milk-based beverage (“flaxmilk”). Flaxseed was incorporated into a chocolate milk base with other flavorings to produce a palatable and acceptable beverage. The main advantage of flaxmilk is the combination of the health benefits of nutrients contained in flaxseed (omega-3 fatty acids, vitamin E, lignans, fiber) and milk (calcium, protein, vitamins A, B₁₂, D, riboflavin, niacin, potassium, phosphorus). This novel concept of combining milk and flaxseed may produce a beverage system that provides health benefits of both food, but in one beverage. There is also concern, however, for
potential disadvantages from the combination of these ingredients. For example, additional fiber inherent in flaxseed may hinder the absorption of calcium naturally contained in the milk.

**Consumer acceptance**

Different formulations were developed in a test kitchen at Virginia Tech to produce a palatable, compositionally stable and desired nutrient profile beverage. Consumer sensory panels using a 9-point hedonic scale were conducted to evaluate the acceptability of flaxmilk beverages. A numeric score of “6” (translated to “like slightly”) was targeted and achieved for the final recipe of flaxmilk. Demographics characteristics of the sensory panelists were also collected.

Acceptance of this novel flaxmilk beverage was likely related to these consumers having a high level of education (at least a high school degree) which has been associated with less neophobia when faced with new foods or beverages. In addition, knowledge of positive health benefits in the beverage significantly increased consumer willingness to consume flaxmilk on a daily basis. Trust and familiarity in health-related information plays an important role in choosing functional foods and/or beverages. Positive health benefits of functional foods are the most critical factor that affects acceptance of products by consumers.

The acceptance of flaxmilk in the study demonstrated that flaxseed can be incorporated into a milk base to produce a beverage that provides both nutrients inherent in milk as well as in flaxseed. Willingness of panelists to consume flaxmilk daily may suggest that this novel beverage may be a potential source of omega-3 fatty acids.

**Analysis of flaxmilk**

Product characterization and sensory attribute testing were conducted on flaxmilk and compared to a chocolate milk beverage control (same recipe without flaxseed). Three replicates were performed to ensure reliability of beverage formulation, processing and laboratory procedures. Six attributes (color, intensity, thickness, chocolate flavor, malty flavor, sweetness and aftertaste) were evaluated by a panel of consumers using a 9-point intensity scale. Beverages were evaluated on day 1 and 7 of the storage period. Sensory evaluation demonstrated that flaxmilk was detected to be thicker, to possess a maltier flavor and more aftertaste, and less sweet and less
chocolate flavor compared to the control beverage. There were no significant differences detected for color.

Physical characteristics included color, viscosity, compositional stability, total moisture and total ash. Chemical characteristics included total protein content and fatty acid profiling. Microbiological assessment, including total aerobic, total coliform and total yeast and mold counts, were also performed on the beverages. Instrumental analysis showed significant differences in color, viscosity and total solids in the flaxmilk compared to the control beverage. The composition of the beverages remained stable throughout the 14-day storage period; however, flaxmilk had a significantly higher proportion of total fat and unsaturated fats (including omega-3 fatty acids) than the control. Although there were significant changes in the beverages during the storage period, the nutritive properties of flaxmilk did not decline, indicating a stable beverage to sufficiently deliver omega-3 fatty acids into the diet.

**Future research**

This cutting-edge research performed at Virginia Tech demonstrates that the incorporation of flaxseed into a milk base to formulate flaxmilk is a feasible method to provide a beverage that delivers omega-3 fatty acids into the diet. Further chemical analysis is necessary to estimate a more complete nutrient profile of flaxmilk, as well as to gain a further insight about the colloidal chemistry and oxidative stability of the beverage.

There is a gap in the knowledge of the effects of increased dietary flaxseed consumption on inflammation. It would be valuable to conduct a human feeding trial to determine whether (1) flaxmilk is palatable for daily consumption over a period of time; (2) regular and sustained consumption of flaxmilk is effective in increasing plasma omega-3 fatty acid content; and (3) biomarkers of inflammation change in response to flaxmilk consumption. These results will provide a clearer understanding of the health benefits of regular consumption of flaxmilk.
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CHAPTER VI
Functional Food Ingredients with Health Benefits on Bone in Postmenopausal Women*

* Sections of this chapter have been submitted to Nutrition Research Reviews.
Abstract

Osteoporosis affects an estimated 55% of individuals 50 years of age and older in the United States. Due to adverse side effects associated with hormone replacement therapy, many postmenopausal women seek more natural alternative therapies to attenuate bone loss associated with menopause. The specific aim of this review is to evaluate the effectiveness of the functional food ingredients – specifically essential fatty acids, non-digestible oligosaccharides, and sugar alcohols – on bone health in postmenopausal women. This review critically assesses available information on effects of these food substances, places them in context of human physiology (specifically in postmenopausal women) and presents specific recommendations to clinicians, food manufacturers, educators and policy makers. Omega-3 fatty acids appear to reduce production of pro-inflammatory cytokines in postmenopausal women, thereby decreasing bone loss. In older postmenopausal women, omega-3 fatty acids increase the concentration of osteocalcin, a biomarker of bone formation, as well as increase bone density. Non-digestible oligosaccharides increase calcium absorption in women at least six years beyond menopause. Xylitol increases calcium absorption in animal models of postmenopause. More research is necessary to better understand how these functional food ingredients are beneficial to bone health. With this knowledge, clinicians, nutrition educators and policy makers may communicate with the community to improve the status of bone health in postmenopausal women. In the future, interest by food manufacturers to formulate and modify functional food products for nutritional and health purposes will continue to be stimulated, and new health claims may be issued.

Keywords: Functional foods, non-digestible oligosaccharides, omega-3 fatty acids, osteoporosis, sugar alcohol
Introduction

Osteoporosis is a major health threat for an estimated 10 million people in the United States (U.S.) (United States Department of Health and Human Services 2004). This skeletal disorder can lead to an increased risk of bone fracture, more commonly seen among older adults, and costs over $15 billion to treat each year. Osteoporosis is defined by the standard deviation in bone mineral density (BMD) relative to the young healthy female reference. A BMD ≥ 2.5 standard deviations below the reference population is categorized as osteoporosis (Bates and others 2002; Gass and Dawson-Hughes 2006) and characterized by low bone mass and microarchitectural deterioration of bone tissue in which calcium (Ca) and other minerals are depleted. Osteoporosis is three times more common in women than in men, largely due to the hormonal changes of menopause.

Each year, 3.5 million U.S. women experience menopause (Warren and Halpert 2004) and the associated changes in bone turnover, resulting in an increased risk of bone loss. Bone turnover or remodeling is a coupled process of bone formation and resorption. During periods of stable bone mass, bone formation equals resorption. The loss of estrogen in postmenopause, leads to an “uncoupling” of bone turnover, where osteoblast (bone formation cells) activity is less than osteoclast (bone resorption cells) activity (Troen 2003). Biomarkers of bone turnover activity demonstrate that while bone formation accelerates in postmenopause, bone resorption surpasses osteoblastic activity (Rogers and others 2000). Consequently, the microarchitectural deterioration of bone tissue produces porous and brittle bones susceptible to fracture.

Hormone replacement therapy (HRT) has been found to be effective in the attenuation of bone loss by reversing the effects of menopause on the skeleton. Due to the adverse health consequences associated with HRT use, such as breast and uterine cancer and uterine bleeding (Warren and Halpert 2004), many postmenopausal women seek out alternate therapies for the preservation of bone.

Interest in strategies to reduce risks of osteoporosis and related health care costs is developing. As life expectancy of the population increases, maintenance of a high quality of life becomes a greater concern. The increasing costs of prescription drugs and health care are accompanied by a growing trend towards self-medication with
natural products, such as food-based ingredients (Brouns and Vermeer 2000). The market for functional foods is growing, and the food industry is continually and intensely searching for natural food ingredients that may have positive effects on health, disease prevention or management (Brouns and Vermeer 2000). Numerous studies have been conducted on the effect of essential nutrients of bone (Ca and vitamin D), and supplements of these nutrients have been shown to be somewhat effective (Dawson-Hughes and others 1990, 1997; Jackson and others 2006). However, it is becoming more desirable to consume whole foods containing certain food substances, as opposed to dietary supplements. New avenues of research are focusing on the benefits of food ingredients that can be incorporated into functional foods for the enhancement of bone health and prevention of osteoporosis.

The term *functional food* has many definitions ranging from simple to elaborate, without an established consensus in the U.S. The Institute of Food Technologists’ Expert Panel defines functional foods as “foods and food components that provide a health benefit beyond basic nutrition (for the intended population)” (Institute of Food Technologists 2005).

During the late 20th century, functional foods and beverages with suggested health benefits, based on scientific evidence, emerged into the market. The most popular foods were sport drinks, probiotic dairy products, heart-healthy spreads and ready-to-eat cereals (Weststrate and others 2002). The functionality claims of these products include improved gut and heart health, better immunity and higher energy levels. Interest in developing functional foods targeted at chronic diseases, such as osteoporosis, is currently growing. The consumer demand for buying healthful foods and beverages coupled with scientific evidence has further stimulated the functional foods and beverage market (Milner 2002). In 2000, the total market for functional ingredients, foods, beverages and dietary supplements was estimated at $50 billion. This was a 7% increase over the previous year. An overall growth rate of 10% per year, potentially outperforming the standard foods and beverage market growth rate of 2% per year was estimated in 2002 (Weststrate and others 2002).

Functional foods designed to prevent osteoporosis may work by providing key nutrients for bone development or maintenance, enhancing Ca absorption and/or
retention, or by building peak bone mass or suppressing bone loss (Weaver and Liebman 2002). There is already a developed market for health-driven foods in the form of vitamin and mineral fortification of popular foods for the general public (Weststrate and others 2002). It is predicted that advanced functional foods targeting chronic diseases will soon emerge. Weststrate and others (2002) state that the area of functional foods is beginning to come of age and its development will be driven by exciting nutritional scientific discoveries which are currently taking place.

The specific aim of this review is to critically evaluate the effectiveness of the following functional food ingredients – essential fatty acids (FA), non-digestible oligosaccharides, and sugar alcohols – on bone health in postmenopausal women. Cellular and animal studies are presented when relevant human studies are not available. These components move beyond the traditional vitamins and minerals known to impact bone and were selected based on their novelty and potential emergence as added food ingredients that may attenuate post-menopausal bone loss. This review also presents specific recommendations to clinicians, food manufacturers, educators and policy makers.

**Essential fatty acids**

There are two classes of essential FA – omega-3 (n-3) and omega-6 (n-6). Biosynthesis enzymes in humans can only insert a double bond at the n-9 or higher position (Albertazzi and Coupland 2002). Therefore, the essential FA (n-6 and n-3) are not synthesized in the body and must be consumed through the diet. Omega-6 FA can be found in most plant oils, meat and poultry products. Omega-3 FA can be found in higher concentrations in fatty coldwater fish, walnuts, and flaxseed oil. The parent compound in the n-6 FA family is linoleic acid (LA, 18: 2n-6), while the parent compound in the n-3 FA family is alpha-linolenic acid (ALA, 18: 3n-3). Both LA and ALA precursors compete for delta-6-desaturase, which is the enzyme needed to form elongated FA metabolites with more specific roles in the body. Aging is one of the known factors that inhibit FA desaturation (Kruger and others 1998).

The introduction of cereal grains into the food supply, the sophistication of the modern oil industry, and food processing advances have been cited as contributors to the increased intake of corn, sunflower and sesame oils, and cereal grains, all rich
sources of \textit{n-6} FA. In addition, the intake of \textit{n-3} FA has declined, primarily due to the significant decrease in regular consumption of fish and fish products (Simopoulos 1999; 2002; James and others 2000; Saldeen and Saldeen 2004). Observational studies have reported the average \textit{n-6}:\textit{n-3} FA ratio to be between 9.8:1 to 16.7:1 (Kris-Etherton and others 2000; Wahrburg 2004; Simopoulos 2006). The high intake of \textit{n-6} FA raises concern as it interferes with the conversion of ALA to eicosapentaenoic acid (EPA) and docosahexanenoic acid (DHA), both of which have beneficial health effects in prevention of cancer, cardiovascular diseases and inflammatory diseases (Horrocks and Yeo 1999; Ruxton and others 2004; Siddiqui and others 2004). High \textit{n-6} FA intake favors arachidonic acid production, a precursor to prostaglandin \textit{E$_2$} (PGE$_2$). PGE$_2$ is the predominant bone-cell derived prostaglandin which mediates the action of pro-inflammatory cytokines on bone cells and assists in regulation of bone remodeling (Miller and Marks 1994; Darlington and Stone 2001). PGE$_2$ activity on bone formation may be biphasic and dose-dependent – stimulatory at low concentrations and inhibitory at high concentrations. At lower concentrations, PGE$_2$ stimulates DNA and type 1 collagen synthesis (Chyun and Raisz 1984), while at higher concentrations; PGE$_2$ hampers bone collagen synthesis (Raisz and Koolemans-Beynen 1974). High intake of \textit{n-3} FA has been associated with an increased rate of bone formation, while high \textit{n-6} FA intake has demonstrated the opposite effect. Because \textit{n-3} and \textit{n-6} FA both serve as substrates for delta-6-desaturase, PGE$_2$ production can be reduced and bone formation enhanced, by lowering the \textit{n-6}:\textit{n-3} FA ratio (Watkins and others 2001).

A lower \textit{n-6}:\textit{n-3} FA ratio may also lead to a decreased production of pro-inflammatory cytokines, producing beneficial effects on bone metabolism. A reduction in pro-inflammatory cytokine synthesis is correlated with increased Ca absorption (Kettler 2001) and Ca deposition in bones of rats (Sakaguchi and others 1994). The increased \textit{n-3} FA intake, therefore, is deemed to enhance Ca absorption, reduce calcium excretion, and increase bone Ca (Weiss and others 2005). These speculations are based on previous animal studies, but have not been validated in clinical trials involving postmenopausal women.

An epidemiologic study with older Rancho Bernardo residents (mean age = ~72.7 y) was conducted to examine relationships between the dietary \textit{n-6}:\textit{n-3} FA ratio
and BMD at the hip and lumbar spine (Table 6.1) (Weiss and others 2005). Subjects completed a food frequency questionnaire, and BMD was measured using dual-energy X-ray absorptiometry. The average $n$-$6:n$-$3$ FA ratio was 7.9:1. A significant inverse association between the $n$-$6:n$-$3$ FA ratio and BMD at the hip was reported, independent of age, body mass index (BMI), and lifestyle factors (Weiss and others 2005).

Very few clinical studies have been conducted on the effect of $n$-$3$ FA supplementation on bone, specifically in postmenopausal women. A small study compared 6 premenopausal women to 6 postmenopausal women (Meydani and others 1991). All subjects consumed 1.68 g EPA and 0.72 g DHA daily for 12 weeks. The additional intake of $n$-$3$ FA suppressed production of pro-inflammatory cytokines, where a more drastic reduction of cytokine production was seen in the postmenopausal women compared to the premenopausal women.

Van Papendorp and others (1995) conducted a pilot study with osteoporotic women residing in an extended care facility. Subjects were divided into four treatment groups and fed 4 g of: (1) evening primrose oil ($n$-$6$ FA); (2) fish oil ($n$-$3$ FA); (3) mixture of evening primrose and fish oils; or (4) olive oil, for 16 weeks. A modest increase in osteocalcin, a bone formation biomarker, was found in both fish oil groups as opposed to the evening primrose oil only and olive oil groups. Fish oil intake significantly decreased the $n$-$6:n$-$3$ FA ratio which paralleled the increase in osteocalcin. Evening primrose oil alone did not have a significant effect on the bone cell response but may have had a synergistic effect with fish oil on bone. In a follow-up study, Kruger and others (1998) supplemented postmenopausal women with low BMD with 6 g of a mixture of evening primrose and fish oil or 6 g of coconut oil (control). All women also received 600 mg Ca carbonate daily for 18 months to keep Ca intake constant between groups. Lumbar spine and femoral neck BMD decreased in the coconut oil group, while lumbar spine BMD remained the same, and interestingly, femoral neck BMD increased in the oil mixture group (Kruger and others 1998). These clinical studies in older adults suggest that an increase in $n$-$3$ FA to lower the current $n$-$6:n$-$3$ FA ratio may improve bone health.
Table 6.1. Effects of the omega-6:omega-3 fatty acid ratio and non-digestible oligosaccharides on indicators of bone in human studies

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Study Design</th>
<th>Level of Ingredient</th>
<th>Subjects</th>
<th>Methods</th>
<th>Statistical Adjustments</th>
<th>Statistically Significant Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omega-6:omega-3 fatty acid ratio (n-6:n-3 FAR)</td>
<td>Cross-sectional cohort</td>
<td>n-6:n-3 FAR of • ~8.4:1 in men • ~7.9:1 in women not using hormone replacement therapy (HRT) • ~7.8:1 in women using HRT</td>
<td>N = 1532 • Men and women • Aged 45 to 90 years • Mean age ≈ 72.7 years • No known osteoporosis</td>
<td>• n-6:n-3 FAR estimated by food frequency questionnaire • Bone mineral density (BMD; g/cm²) by dual-energy X-ray absorptiometry (DXA)</td>
<td>• Age (years) • Body mass index (kg/m²) • Calcium (Ca) intake (mg/day) • Alcohol intake (g/day) • Exercise (≥ 3 times/week or &lt; 3 times/week) • Smoking history (never, past, or current) • Thiazide use (yes or no) • Thyroid hormone use (yes or no)</td>
<td>Men • Inverse association between n-6:n-3 FAR and total hip BMD Women • Not using HRT, inverse association between n-6:n-3 FAR and total hip BMD and lumbar spine (LS) BMD • Using HRT, inverse association between n-6:n-3 FAR and total hip BMD</td>
<td>Weiss, Barrett-Conner &amp; von Muhlen (2005)</td>
</tr>
</tbody>
</table>

120
<table>
<thead>
<tr>
<th>n-6:n-3 FAR</th>
<th>Duration</th>
<th>Description</th>
<th>Intervention Details</th>
<th>Outcome Measures</th>
<th>Outcome Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-week randomized controlled trial (RCT)</td>
<td>Four oil-based supplement treatments with n-6:n-3 FAR of • ~7.3:1 (Group 1) • ~4.0:1 (Group 2) • ~4.3:1 (Group 3) • ~10.0:1 (Group 4, Control)</td>
<td>N = 40 • Women • Mean age = 80.0 years • Osteoporotic</td>
<td>Oil-based supplement intervention for 16 weeks • Serum osteocalcin (ng/mL) measured at baseline and end of treatment</td>
<td>None</td>
<td>Serum osteocalcin increased in Groups 2 and 3 compared to Group 1</td>
</tr>
<tr>
<td>18-month RCT</td>
<td>Two oil-based supplement treatments with n-6:n-3 FAR of • ~4.0:1 (Group 1) • ~10.0:1 (Group 2, Control)</td>
<td>N = 60 • Women • Mean age = 79.5 years • Osteopenic or osteoporotic</td>
<td>Oil-based supplement intervention for 18 months • Dietary Ca intake controlled between treatment groups • Serum osteocalcin, bone alkaline phosphatase (BALP; μg/L), and urinary deoxypyridinoline (DPD; nmol/mmol creatinine) measured at baseline and end of treatment • BMD by DXA at baseline and end of treatment</td>
<td>None</td>
<td>Serum osteocalcin and urinary DPD decreased and serum BALP increased in Groups 1 and 2 • LS BMD did not change in Group 1, while LS BMD decreased by 3.2% in Group 2 • Femoral neck (FN) BMD increased by 1.3% in Group 1, while FN BMD decreased by 2.1% in Group 2</td>
</tr>
</tbody>
</table>

Van Papendorp, Coetzer & Kruger (1995)
| Fructo-oligosaccharides (FOS) | 5-week RCT, crossover, double-blind | 10 g of FOS | N = 12 | • Women  
• Mean age = 59.8 years  
• No known osteoporosis  
• n = 6 women of menopause duration of 2 to 6 years  
• n = 6 women of menopause duration of > 6 years | • 5-week FOS supplement intervention, 3-week washout, and 5-week placebo intervention (random ordered)  
• Ca intake controlled  
• $^{44}$Ca recovery for Ca absorption assessment  
• Serum osteocalcin and urinary DPD | None | • Ca absorption not different between FOS and placebo groups  
• Ca absorption with FOS treatment higher compared to placebo only in 6 women of menopause duration of > 6 years  
• No change in serum osteocalcin or urinary DPD with either FOS or placebo | Tahiri, Tressol, Arnaud, et al. (2003) |
|---|---|---|---|---|---|---|---|
| FOS-enriched inulin | 6-week RCT, crossover, double-blind | 12 g of FOS-inulin | N = 15 | • Women  
• Menopause duration of > 10 years  
• No known osteoporosis | • 6-week FOS-inulin supplement intervention, 6-week washout, 6-week placebo intervention (random ordered)  
• Ca intake controlled  
• $^{44}$Ca/$^{42}$Ca recovery for Ca absorption assessment  
• Serum osteocalcin and urinary DPD | None | • Ca absorption increased with FOS-inulin treatment compared to placebo  
• Increased osteocalcin and DPD after FOS-inulin treatment but not greater than placebo effect | Holloway (unpublished results, 2004) |
The major source of EPA and DHA in the American diet is fatty fish, such as salmon, halibut, mackerel and herring, which can be taken in the form of fish oils. However, the oils need to be consumed in large doses to be effective, which can be both expensive and unpalatable. The current daily intake is estimated to be 0.1 to 0.2 g of a combination of EPA and DHA (California Olive Industry); however, based on previous clinical research, a minimal intake of 0.5 g/d of EPA and DHA is recommended (Gebauer and others 2006). The 2000 American Heart Association Dietary Guidelines recommend at least two servings of fatty fish per week for healthy adults. Specifically for ALA, a daily intake of 1.5 to 3 g seems beneficial, although definitive data from prospective, randomized clinical trials are still necessary to confirm this (Kris-Etherton and others 2003). If over consumed, \( n \)-3 FA may suppress immunity and inflammation responses in the body; however, an overdose is difficult to achieve. From the latter two studies, a small amount of evening primrose oil may also be beneficial in bone metabolism. Moderate clinical promise of an attenuation of inflammation has been shown with supplementation of \( n \)-3 FA containing oils and evening primrose oil in the elderly. Additional clinical trials to assess the effects of these oils on bone related parameters are essential.

**Non-digestible oligosaccharides**

*Inulin and oligofructose*

Fermentable substances such as prebiotics can alter Ca absorption and modify its retention in the body. A prebiotic substance is defined as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity” of potentially health-promoting indigenous microflora in the colon (Cashman 2003). The existing Ca absorption enhancing prebiotics are the non-digestible oligosaccharides – inulin and oligofructose.

Inulin and oligofructose occur naturally in common foods including vegetables (such as Jerusalem artichokes, onions and chicory), fruits, and to a lesser extent, cereals (Coxam 2005). These carbohydrates can also be industrially produced in larger quantities. The processing procedure involves hot water extraction of naturally occurring inulin from chicory roots followed by technological refinement similar to the production of sucrose from sugar beets (Franck 2002; Coxam 2005). Inulin is then
exposed to evaporation and spray drying (Franck 2002). Chicory inulin is primarily composed of a mixture of oligomers and polymers of fructose (Roberfroid 2002). Inulin can be enzymatically hydrolyzed by endo-inulase, followed by spray drying to produce oligofructose (Franck 2002). Oligofructose can also be synthesized from sucrose using fructosyl-transferase (Bornet 1994). Inulin has an average chain length of 10 units, while oligofructose is more soluble with shorter chain lengths (~ 5 units).

Inulin and oligofructose are linear \( \beta \) 2-1 fructans, where the anomeric \( C_2 \) in their fructose monomer form \( \beta \) 2-1 glycosidic linkages. These \( \beta \) linkages are resistant to hydrolysis by digestive enzymes in the human small intestine, which are specific for \( \alpha \) glycosidic linkages. Therefore, these carbohydrates are non-digestible and classified as dietary fiber.

These oligosaccharides are speculated to enhance mineral absorption, due to their prebiotic nature. As previously mentioned the oligosaccharides are resistant to hydrolysis in the small intestine and are selectively fermented by the microflora in the colon. The colonic fermentation produces the short-chain fatty acids (SCFA) – acetate, propionate and butyrate. It is generally accepted that these SCFA then lower the luminal pH in the large intestine, thereby enhancing mineral solubility and leading to greater passive Ca absorption (Cashman 2003; Coxam 2005; Weaver 2005). In addition, the acidic environment stimulates the intestinal epithelium, which increases the absorptive capacity, also aiding in the enhancement of Ca absorption.

Across growth stages of the lifespan, and particularly during puberty when estrogen significantly influences bone regulation in girls, bone formation exceeds resorption. Hormonal control over skeletal development is high during this life stage, and any influence of added functional food ingredients, such as non-digestible oligosaccharides, may not be beneficial despite enhancement of Ca absorption (Saggese and others 2002). During postmenopause when bone resorption outpaces bone formation due to a decrease in estrogen, functional food ingredients may have very beneficial effects. Several studies have shown that inulin and oligofructose administration effectively stimulates Ca absorption in the rat, particularly in aged animals and in ovariectomized (OVX) models (Morohashi and others 1998; Scholz-Ahrens and others 2002; Zafar and others 2004; Coudray and others 2005; Raschka
and Daniel 2005). If inulin and oligofructose increase Ca absorption in postmenopausal women, the adverse effects of low estrogen on bone may be attenuated.

Only two human studies have been conducted on the use of inulin and oligofructose as dietary approaches to reducing bone loss in postmenopausal women (Table 1). In a randomized, double-blind crossover study, 12 healthy postmenopausal women (not receiving HRT and postmenopause for > 2 y) were fed either 10 g/d of short chain fructooligosaccharides or sucrose (placebo) for 5 weeks, with a washout period of ≥ 3 weeks (Tahiri and others 2003). Subjects averaged approximately 900 mg Ca daily. Subjects consumed an oral $^{44}$Ca isotope solution and radiopaque pellets (a feces marker) to measure $^{44}$Ca plasma enrichment, urinary enrichment and fecal enrichment as well as fractional Ca absorption of $^{44}$Ca. Mean Ca absorption between treatments was not significantly different. Bone biomarkers (osteocalcin and deoxypyridinoline) did not significantly differ between treatments. The subjects’ menopause duration ranged widely (2 to 22 y). Thus, subjects were divided into two subgroups according to menopause, including duration 2 to 6 y (n=6) or > 6 y (n=6). Enhanced Ca absorption due to the short-chain fructooligosaccharides in women who were at least 6 years postmenopause showed an interesting positive trend. This suggested that the drastic change in estrogen concentration that occurred in early postmenopause masked any beneficial effect of diet. Thus, the use of this prebiotic would be more effective after hormones have stabilized as in late postmenopause, similar to findings with Ca supplementation (Weaver 2005).

In a more recent randomized, placebo-controlled, double-blind crossover study, 15 postmenopausal women (not receiving HRT within the last year and > 10 y postmenopause, mean age = 72.2 years) were treated daily with 12 g oligofructose-enriched inulin (Synergy1) or maltodextrin (placebo) for 6 weeks, with a washout period of 6 weeks. Subjects consumed between 800 to 1200 mg Ca daily. True fractional Ca absorption measured by dual isotopes before and after treatment, as well as absolute change in Ca absorption, showed a significant increase in the Synergy1 treatment compared to the placebo (Holloway and others 2007). Serum osteocalcin and urinary deoxypyridinoline crosslinks concentrations were significantly greater after the Synergy1 treatment compared to baseline, but were not significantly greater than the placebo
group after 6 weeks. This suggests a potential impact on bone turnover with oligofructose-enriched inulin by improvement of mineral absorption in postmenopausal women. A synergistic effect may be present and, therefore, opens a further line of investigation.

The consumption of inulin has been predicted to have more of an effect on the enhancement of Ca absorption, because the longer chains are fermented at a slower rate than oligofructose. Thus, these longer chains reach more distal regions of the colon, where selective metabolic activity can occur (Tuohy and others 2001; Harmsen and others 2002). It has been shown that 10 g/d of inulin is fermented in its entirety in the intestinal tract of human subjects, with none recovered in the feces (Van Loo 2004). The current average intake is 1 to 4 g/d in the U.S. (Kaur and Gupta 2002). Too high of an intake may lead to undesirable side effects such as flatulence, abdominal bloating, cramping, and pain, and diarrhea, due to microfloral stimulation (Coussement 1999). There is no uniform upper tolerance of inulin or oligofructose, because intestinal acceptability is a subjective measure (Coussement 1999). However, Briet and others (1995) reported that an intake of 30 g/d of oligofructose led to excess flatus, with higher doses resulting in abdominal bloating and cramping and diarrhea. It is not clear whether a recommended intake of 10 to 12 g/d should be established to enhance Ca absorption for maintaining bone density. Presently, there is insufficient evidence in postmenopausal women to suggest that an addition of dietary inulin and oligofructose in the diet enhances Ca absorption and whether this extra Ca would be deposited in bones. Long term studies are needed to confirm such a hypothesis.

Sugar alcohol

Xylitol

Xylitol is a 5-carbon sugar alcohol that is found widely distributed in nature, specifically in fruits. Xylitol is a noncariogenic sugar substitute often used in candies and chewing gums. It is also an intermediate in the metabolism of carbohydrates, with 5 to 15 g/d of xylitol formed in humans (Mattila and others 2005).

Orally administered xylitol has shown some interesting properties in rats that may be useful for the prevention of osteoporosis in humans. However, clinical research is still being performed on rodents and has not yet advanced into human subjects.
Previous studies performed on rats have shown that dietary xylitol was effective in protecting against changes in bone structure and bone biomechanical properties in OVX rats. Supplementation of 5 to 20% dietary xylitol by weight of diet has been shown to increase intestinal Ca absorption and bone recalcification after a 3-week Ca-deficient period (Hamalainen and others 1985; Hamalainen and Makinen 1989). Dietary xylitol supplementation has significantly diminished ovariectomy-induced bone resorption in rats (Svanberg and others 1997; Mattila and others 1998), likely due to an increase in Ca absorption.

An enhancement of intestinal absorption of Ca in rats with the consumption of simple sugars and sugar alcohols was first observed in the mid 1950s (Fournier and others 1955). Subsequent research suggested that increased Ca absorption occurs when slowly absorbing carbohydrates reach the lower gut, keeping Ca soluble for a longer period (Wasserman and Comar 1959; Vaughan and Filer 1960). A xylitol:Ca molar ratio of 1.5:1 supplement was found to have the greatest Ca retention in 11-week-old rats (Hamalainen 1994). It is not clear whether this ratio would be effective in adult humans. Xylitol may have an interactive effect with the Ca mineral, and the two could be consumed together for optimal absorption. Yet, even if xylitol enhances Ca absorption, this Ca may or may not be deposited in bone, although this potential exists (Svanberg and others 1997; Mattila and others 1998).

This process is independent of vitamin D action, indicating that the paracellular Ca absorption route is involved (Hamalainen 1994; Mattila 1999; Mattila and others 2005). The vitamin D dependent Ca absorption rate is lower in the elderly, so a functional food that increases passive Ca absorption would be advantageous. However, in spite of promising results in animal studies, there is currently no compelling human evidence (Forster and others 1981; Makinen and others 1982; Bar 1985). This lack of evidence may result from the low level of dietary xylitol in humans, compared to experimental rats. It has been assumed that a reasonable amount of xylitol (< 100 g/d) will cause no harm in Ca metabolism in adult humans (Culbert and others 1986). Although dietary xylitol was effective in increasing bone mass of healthy rats and preventing bone loss in OVX rats, more evidence is necessary to confirm whether similar results will be seen in humans.
Discussion

Possibilities exist for a role of n-3 FA, inulin, oligosaccharides, and xylitol in attenuating bone loss in postmenopausal women. Current studies suggest that n-3 FA intake may be effective in the early years after menopause when inflammation is greatest due to the rapidly decreasing estrogen concentration (Meydani and others 1991). n-3 FA may also be effective in later postmenopause by impacting bone cell regulation and bone density (Van Papendorp and others 1995; Kruger and others 1998). Non-digestible oligosaccharides may be most beneficial for older postmenopausal women, after hormone concentrations have stabilized (Tahiri and others 2003; Holloway and others 2007).

Mechanisms of action should be considered when evaluating these functional food components. n-3 FA may lower the n-6:n-3 FA ratio and prevent the production of pro-inflammatory cytokines, producing a secondary effect of attenuating bone density loss. In contrast, non-digestible oligosaccharides, and possibly sugar alcohols, may enhance Ca absorption in the colon. Clearly, extensive research is necessary in this targeted group of women to provide a compelling body of evidence that would support any health claims between these functional food ingredients and preservation of the skeleton.

Applications

As more research is conducted on these three functional food ingredients for effects on bone health, it is important to address how to utilize this information by clinicians, food manufacturers, educators and policy makers to communicate this to the targeted population.

Clinicians

It is imperative for clinicians to educate themselves about bone health and pass this knowledge to their patients. As functional foods with claimed bone health benefits emerge in the market, clinicians are responsible to understand the nutritional foundation of products and feel confident in advising elderly patients regarding incorporation of these products into a healthy diet. Clinicians need to have a strong understanding of the nutritional science of the food ingredients to be able to distinguish valuable functional food products from those that are ineffective or harmful. With the information
presented in this review, along with other information available on potentially beneficial functional food ingredients, clinicians should feel comfortable relaying this information to their patients. The clinician should be able to determine which food ingredient (if any) would maximize the prevention of bone loss, based on the health condition of the patient. It is crucial for the clinician to understand that these functional food ingredients are not universally beneficial in all patients due to differences in their genetic makeup. It is also important for clinicians to effectively communicate this information about these functional food ingredients to patients without encouraging unrealistic belief that these functional food ingredients are magic bullets to cure osteoporosis. Clinicians are the first contact for most postmenopausal patients about bone health and nutrition; therefore, it is important to provide accurate current information and to recommend the best treatments for prevention or attenuation of osteoporosis.

**Food Manufacturers**

Many adults in the U.S. select foods for health purposes, regardless of age or gender. Older individuals tend to select foods based on potential merits for reducing disease risk or improving quality of life (Milner 2002). The desire to stay healthy and look good in a fast-paced environment is becoming more difficult to fulfill. Consumers are attracted to quick fixes and shortcuts, in reference to food preparation, weight loss, or disease prevention (Niness 1999). The key to success for food manufacturers is to develop products that are acceptable by consumer standards and are consistent with their familiarity of the health benefits of the novel product (Weststrate and others 2002). An approach to integrate the needs and demands of the consumer with the scientific research will lead to innovative functional food products. A developing market of health-based foods will flourish. This is already occurring in the vitamin and mineral fortification of popular foods, such as cereals. It is expected that an emergence of more advanced functional foods will occur, possibly containing some combination of the previously reviewed ingredients that target chronic diseases such as osteoporosis. In the meantime, food manufacturers will apply new scientific findings to develop novel food products that will have specific health benefits.

The food industry can use present knowledge to formulate novel functional food products that incorporate one or all of these specific ingredients. The best candidates
for the addition of \( n \)-3 FA are foods that already contain fat and oil, such as spreads or baked goods. This will lessen drastic alterations in fat composition of the product while maintaining palatability and texture. Because the current intake of EPA and DHA is quite low, relative to recommended intakes, it is practical to develop novel products to increase intakes of essential FA.

Non-digestible oligosaccharides and xylitol may be incorporated into baked goods as well as other food products that can be sweetened with these carbohydrates. For example, xylitol is often used in confectionary products as a sucrose replacement, while inulin is used as a source of dietary fiber in baked goods. Although the enhancement of Ca absorption by these products appears promising, it is important to incorporate only a moderate amount (\( \leq 10 \) g) of these carbohydrates into food products to allow for differences in intestinal tolerance to these ingredients by consumers. Adding these carbohydrates into food systems that are naturally rich in Ca in order to facilitate maximal Ca absorption may be most beneficial. Although there is no guaranteed formula for a successful functional food product, these suggested uses of \( n \)-3 FA, non-digestible oligosaccharides, and sugar alcohols may be helpful in the development of novel products aimed at prevention or attenuation of bone loss in postmenopausal women.

**Educators**

Nutrition educators have the opportunity to work in the community and provide personal contact with patients who are at high risk or already have been diagnosed with osteoporosis. The International Food Information Council (International Food Information Council 2005) reported that 90% of adults in the U.S. were able to name a specific food and its associated health benefit. The most common foods mentioned were Ca rich dairy foods and bone health. This indicates that osteoporosis is a well known health disorder and that there is concern among the general public. Therefore, it is important for nutrition educators to communicate with the population to minimize any gaps of information present between scientific knowledge and the public awareness of bone health and food products. As more research is performed on the specific functional food ingredients reviewed here, nutrition educators including public interest...
groups will spread messages about whether these ingredients are beneficial in influencing bone health.

Previous education programs have been developed and presented to populations in the U.S. on functional food intake as well as specific ingredients such as n-3 FA. The Functional Foods for Health Program developed at the University of Illinois recruited registered dietitians to communicate information about functional foods (via an educational kit) to consumers (Pelletier and others 2002). Surveys were distributed before and after the information session, where 530 consumers provided responses for analysis. Most participants indicated interest in increasing their functional food intake of various foods including tomatoes, purple grapes, oats, and soy. The older participants (> 65 years old), especially women, were more likely to already be consuming these common functional foods but were also more interested in consuming additional functional food products. This is encouraging, because a viable target population for new functional food products with possible benefits for bone health is postmenopausal women. The Diet and Omega-3 Intervention Trial was conducted in Denmark in 2005 with 563 elderly men (Hjerkinn and others 2005). Subjects were divided in four treatment groups: (1) dietary counseling to promote n-3 FA consumption (alone); (2) dietary counseling with n-3 FA supplements; (3) dietary counseling with placebo supplements; and (4) n-3 FA supplements without dietary counseling. The researchers concluded that participants in the dietary counseling alone and n-3 FA supplements alone groups had significantly reduced serum biomarkers of atherosclerosis. This indicates that dietary counseling encouraging greater intake of n-3 FA was as effective as providing n-3 FA supplementation to these subjects.

Programs such as the two mentioned above, seem to have positive impacts. Programs that focus on the consumption of functional foods should also be emphasized to increase the overall intake of healthier foods. Programs targeted at an elderly population seemed to be most effective, as these participants are most concerned about their diets and overall health. When sufficient data on the bone health-enhancing properties of the reviewed functional food ingredients are available, new programs may be developed and introduced to the community by nutrition educators.
Policy makers

Current legislative priorities in bone health are to prevent osteoporosis, to promote lifelong bone health, to improve the lives of those affected by osteoporosis and related fractures and to find a cure (National Osteoporosis Foundation 2006). Although these goals are not anticipated to be fully achieved in the near future, the National Osteoporosis Foundation diligently develops programs of awareness, public and health professional education, advocacy and research to make advances toward their goals. The Surgeon General’s report on bone health and osteoporosis also calls for federal, state and local governments to collaborate with the private sector and community to promote bone health. An increase in awareness is necessary among consumers and clinicians to advocate for the best methods of prevention, assessment and treatment of bone disease. A better informed and more concerned public will adhere to recommendations to attain healthy bones, which currently consist of Ca and vitamin D intake combined with physical activity. As more clinical research is being conducted on the reviewed functional food ingredients, other food substances may eventually be added to the current recommendations.

The Dietary Guidelines for Americans emphasizes the importance of foods to provide essential nutrients to prevent deficiency diseases as well as the other components vital for health (Schneeman 2000). It would be expected that with more sound scientific evidence of these functional food ingredients having a positive effect on bone health, new health claims may be approved by the Food and Drug Administration (FDA). The approved health claim between Ca intake and osteoporosis has been established. Health claims have been approved by the FDA on non-cariogenic carbohydrate sweeteners (such as xylitol) and dental caries and n-3 FA and coronary heart disease. However, claims linking these food components to bone health or osteoporosis are not currently approved by the FDA due to lack of scientific evidence. The developments of new health claims and authoritative statements as substantiation for health claims on food products may soon occur, when sufficient evidence is available for the reviewed functional food ingredients on bone health. This will stimulate interest in the formulation and modification of functional food products for nutritional and health purposes (Schneeman 2000). These novel products will require consumer
acceptability testing to ensure an opportunity to survive in the market. Before these novel products are commercially available, clinical trials are necessary to assure safety in consumption of product as well as efficacy in producing a positive effect on bone health in the consumers.

**Conclusion**

Many postmenopausal women in the U.S. seek alternative therapies to HRT for the prevention or attenuation of bone loss associated with osteoporosis. Alternative therapies may include increasing the consumption of functional foods containing bioactive ingredients that affect calcium absorption in addition to the direct consumption of calcium-rich food sources. Initial studies of n-3 FA, inulin and oligosaccharides, and xylitol on bone health suggest that these compounds may have different mechanisms for affecting calcium absorption and bone responses. More research will need to be performed with these functional food ingredients to gain a better understanding of what ingredients are beneficial to bone health and which ones are ineffective.

With present knowledge and future findings, clinicians, nutrition educators, and policy makers must communicate with the public to improve treatment of bone health in postmenopausal women through the consumption of food products containing these functional food ingredients. It is a reasonable goal to improve the bone health of postmenopausal women through the consumption of food products containing these functional food ingredients.
References


Chapter VII
Association Between Estimated Dietary Omega-6:Omega-3 Fatty Acid Ratio and Bone Mineral Measurements in Young-Adult and Older Women

To be submitted to Osteoporosis International
Abstract

There is growing evidence that a lower dietary omega-6 (n-6) to omega-3 (n-3) fatty acid (FA) ratio may benefit bone health. Research has been conducted primarily in animal models and older postmenopausal women with promising results. It has been proposed that a diet consisting of a low n-6:n-3 FA ratio may be associated with a higher bone mass in elderly individuals, but this relationship has not been evaluated in a younger age group. It was hypothesized that an inverse association between the dietary n-6:n-3 FA ratio and total body and site-specific bone mineral measurements would be found in young-adult and postmenopausal women. Secondary analyses from dietary intake and bone mineral density data were conducted. The n-6:n-3 FA ratio had no appreciable association with total body or site-specific bone mineral measurements in either young-adult or postmenopausal women.

Keywords: essential fatty acid ratio, bone mineral density, bone mineral content, women
Introduction

Osteoporosis is a problematic skeletal condition, more commonly seen among older adults, characterized by low bone mass and microarchitectural deterioration of bone tissue in which calcium (Ca) and other minerals are depleted. In healthy bone, osteoclasts continuously remove, while osteoblasts build matrix. When this coupled process becomes unsynchronized, the bone matrix weakens, eventually leading to low bone mass.

Osteoporosis may occur simultaneously with inflammation as observed in aging and menopause (Arron and Choi 2000; Yun and Lee 2004; Ginaldi and others 2005). Omega-3 (n-3) fatty acids (FA) attenuate inflammation (Caughey and others 1996; Calder 2006; Nannicini and others 2006; Smith and others 2006; von Schacky and Harris 2007) and may serve to promote bone health. For example, bone growth may be optimized by alteration of the dietary omega-6 (n-6):n-3 FA ratio (Watkins and others 2000).

High n-6 FA intake favors arachidonic acid production, a precursor to prostaglandin E2 (PGE2) which, in turn, mediates the action of pro-inflammatory cytokines on bone cell and regulation (Miller and Marks 1994; Darlington and Stone 2001). High intake of n-3 FA has been associated with an increased rate of bone formation, while high n-6 FA intake has demonstrated the opposite effect. Because n-3 and n-6 FA both serve as substrates for delta-6-desaturase, PGE2 production is reduced and bone formation may be enhanced by lowering the n-6:n-3 FA ratio (Watkins and others 2001). Rodent studies demonstrated that diets with a high n-6:n-3 FA ratio, were correlated with low bone formation and high bone resorption biomarker concentrations (Watkins and others 2000, 2006). A low dietary n-6:n-3 FA ratio may also produce beneficial effects on bone metabolism by increasing intestinal Ca absorption (Kettler 2001), reducing renal Ca excretion (Claassen and others 1995) and skeletal Ca deposition (Sakaguchi and others 1994). These speculations are based on animal studies.

Few human studies have explored the relationship between the dietary n-6:n-3 FA ratio and bone health. A significant inverse association between the n-6:n-3 FA ratio and bone mineral density (BMD) at the hip was reported in elderly male and female
Rancho Bernardo residents (Weiss and others 2005). In a pilot study with elderly osteoporotic women, nutritional supplement treatments containing fish oil increased bone formation biomarkers (van Papendorp and others 1995). In a follow-up study, elderly postmenopausal women who consumed an evening primrose and fish oil mixture had attenuation of BMD loss at the lumbar spine compared to same-aged women who received a coconut oil control (Kruger and others 1998). In a randomized, double-blind, balanced order, three-period crossover study, moderately hypercholesterolemic men and women in mid-life consumed three diets with varying \( n-6:n-3 \) FA ratios (Griel and others 2007). Bone resorption biomarkers were significantly lower after consumption of the diet with the lowest \( n-6:n-3 \) FA ratio compared to the moderate and highest \( n-6:n-3 \) FA ratios.

A diet consisting of a low \( n-6:n-3 \) FA ratio may be associated with high bone mass in older individuals (Weiss and others 2005). Rodent studies also suggest that a low dietary \( n-6:n-3 \) FA ratio promotes high BMD at the time of bone mass consolidation (Watkins and others 2000, 2006). The direction of this relationship is unclear, however, in young adults. The purpose of this study was, therefore, to examine the relationship between the dietary \( n-6:n-3 \) FA ratio and total body (TB) and site-specific bone mineral content (BMC) and BMD in women. It was hypothesized that an inverse association between the dietary \( n-6:n-3 \) FA ratio and bone mineral measurements would be found in all women. Furthermore, this negative association would continue to exist when women were categorized by age group (i.e., young-adult and postmenopausal).

**Materials and Methods**

*Study population*

This investigation was a secondary analysis of data previously collected from studies related to bone mineral status in young-adult (aged 18 to 25 years), mid-life (aged 27 to 50 years) and postmenopausal (aged 50+ years) women. Subjects were recruited from the Virginia Tech community and surrounding locations by use of paper flyers, electronic mail notices, and personal contacts. All original studies were approved by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech (Blacksburg, VA). Each woman, before participating in any procedure, provided written informed consent.
Exclusion criteria for two of the original studies were: aged < 18 and > 25 years, weekly participation in > 5 h of hard and very hard physical activity; amenorrhea, oligomenorrhea, or any disruption of menstrual cycles within the previous years; use of oral contraceptives for < 18 mo (if used); use of medications to correct metabolic disorders; history of eating disorders, metabolic disorders, or chronic diseases; parity ≥1; cigarette smoking or previous/current bone fracture. Individuals with a body mass index (BMI) of < 18 or > 25 kg/m^2 were excluded as well as women with body weight fluctuations of > 2.27 kg, ≥ 3 times in the past 2 years from time of examination. There were no exclusion criteria for women (aged 26+ years) in the other studies.

For the current examination, exclusion criteria included missing anthropometric measurements, body composition measurements or dietary records. Thus, data from 202 of the original 209 subjects were included in this current study. Women were aged 18 to 79 years.

All subjects were analyzed as one sample (n=202) and then separated into two subsamples based on age. The young-adult subsample included all subjects aged 18 to 26 years (n=136) and the postmenopausal subsample included women aged ≥ 50 years (n=46). Women between 27 to 49 years of age (n=20) were not included in subsample analyses. The young-adult subsample represented a group of women approaching peak bone mass, while the postmenopausal subsample represented women experiencing estrogen-related loss of bone mass.

**Assessment of dietary intake**

Each subject completed a 4-day (3 weekdays, 1 weekend day) dietary intake record. Subjects recorded all foods and beverage intake during the 4-day period. Written instructions and handouts with portion size information and pictures were provided to subjects to maximize accuracy of records. Subjects returned their dietary intake records by postal mail. An investigator contacted a subject, if needed, to clarify intake and/or portion sizes. Food records were analyzed to estimate total energy, n-6 FA and n-3 FA intakes, using the Food Processor® dietary analysis software (ESHA Research, Version 8.1; 2003, Salem, OR, USA). Any unlisted foods and beverages were added to the database by the investigator to ensure accurate results. Dietary
supplements were not included in the dietary intake assessment. The \( n-6:n-3 \) FA ratio was computed from analysis outputs.

**Anthropometric and bone mineral content and density measurements**

Body height was measured to the nearest 0.1 cm with stadiometer (Detecto, Webb City, MO, USA), body weight was measured to the nearest 0.1 kg on an electronic scale (Scaletronix, Wheaton, IL, USA). Each subject’s BMI was calculated from these height and weight measurements by an investigator.

Each subject underwent dual-energy x-ray absorptiometry (DXA) scans (QDR4500A, Hologic, Inc., Bedford, MA, USA) of the TB, lumbar spine (LS; \( L_1-L_4 \)), non-dominant total proximal femur (TPF), including the femoral neck (FN), and non-dominant total forearm (TF) for measurement of BMC (g) and BMD (g/cm\(^2\)). Total body fat mass (FM; kg), fat-free soft tissue mass (FFSTM; kg), and body fat percentage (BF\%\) measurements were provided by TB DXA scans. Standard measurement and analysis protocols were used with all DXA scans. Quality control procedures were conducted prior to any scan by use of an anthropomorphic phantom LS. The coefficient of variation (CV) for quality control monitoring with this DXA was 0.36\%. Quality control for soft tissue mass was ensured by scans of an external soft tissue bar (Hologic, Bedford, MA, USA). Test-retest reliability CVs for this DXA are presented elsewhere (Miller and others 2004; Nickols-Richardson and others 2005).

**Statistical Analyses**

Descriptive statistics were calculated for sample characteristics and reported as means ± standard deviations (SD). The means of the subsamples were compared to each other using the Welch approximate \( t \)-test, due to unequal sample sizes and unequal variances. Bivariate Pearson correlation coefficients were calculated to examine simple relationships between TB and site-specific bone measurements (BMC and BMD) with variables of interest, specifically \( n-3 \) FA and \( n-6 \) FA intake and the \( n-6:n-3 \) FA ratio. Correlations were determined for the entire sample as well as the two subsamples. Statistical significance was set at the \( p < 0.05 \) level. All statistical procedures were completed using Statistical Analysis Software (version 9.1, SAS Institute Inc, Cary, NC, USA).
Results

The subjects’ characteristics for all 202 women are presented in Table 7.1. Characteristics for women by age group are also provided in Table 7.1.

Postmenopausal women were heavier, of greater BMI, and possessed more FM, FFSTM and BF% compared to young-adult women (Table 7.1). Although postmenopausal women had significantly lower TPF BMC and BMD and FN BMC and BMD compared to young-adult women, they were not significantly different in TB, LS or TF BMC and BMD measurements (Table 7.1).

Estimated total energy intake was significantly greater in the young-adult women compared to the postmenopausal women. Absolute intakes of estimated dietary \( n\)-6 FA and \( n\)-3 FA did not differ between age groups; however, postmenopausal women had a significantly lower dietary \( n\)-6:\( n\)-3 FA ratio compared to young-adult women.

Tables 2, 3 and 4 display Pearson correlation coefficients for comparisons between bone mineral content and bone mineral density with anthropometric and soft tissue mass measures for the entire sample, and the young-adult and postmenopausal subsamples, respectively. Pearson correlation coefficients for comparisons between bone mineral measurements and dietary intake estimates of total energy, \( n\)-6 FA and \( n\)-3 FA are also presented in Tables 7.2, 7.3 and 7.4. In all women (Table 7.2), body height, body weight and fat-free soft tissue were significant and positively associated with all BMC and BMD measurements \((p \leq 0.05 – p \leq 0.001)\) with the exception of total forearm BMD, where only weight was related. Similar associations are seen within the young-adult women (Table 7.3), with the exception of TPF BMD, FN BMD and body height. Fewer significant associations are seen within the postmenopausal women (Table 7.4).
Table 7.1. Subject characteristics for all women (n=202) and by age group of women

<table>
<thead>
<tr>
<th></th>
<th>All Women (n=202)</th>
<th>Young-adult women (n=136)</th>
<th>Postmenopausal women (n=46)</th>
<th>p value between age groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>31.7 ± 16.8</td>
<td>20.7 ± 2.2</td>
<td>59.0 ± 6.7</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>164.8 ± 6.5</td>
<td>165.4 ± 6.2</td>
<td>163.5 ± 6.7</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>62.4 ± 11.2</td>
<td>59.7 ± 7.5</td>
<td>69.2 ± 15.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>23.0 ± 4.2</td>
<td>21.9 ± 2.6</td>
<td>26.0 ± 6.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Fat mass (kg)</strong></td>
<td>19.2 ± 8.0</td>
<td>17.0 ± 6.0</td>
<td>24.4 ± 10.4</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Fat-free soft tissue (kg)</strong></td>
<td>42.2 ± 5.3</td>
<td>41.6 ± 4.6</td>
<td>43.7 ± 6.7</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Body fat %</strong></td>
<td>29.26 ± 6.52</td>
<td>27.46 ± 5.20</td>
<td>33.66 ± 7.66</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>TB BMC (g)</strong></td>
<td>2148.73 ± 278.59</td>
<td>2158.06 ± 234.17</td>
<td>2105.10 ± 306.33</td>
<td>NS</td>
</tr>
<tr>
<td><strong>TB BMD (g/cm²)</strong></td>
<td>1.11 ± 0.08</td>
<td>1.11 ± 0.07</td>
<td>1.10 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td><strong>LS BMC (g)</strong></td>
<td>57.59 ± 10.26</td>
<td>57.53 ± 8.35</td>
<td>56.18 ± 12.73</td>
<td>NS</td>
</tr>
<tr>
<td><strong>LS BMD (g/cm²)</strong></td>
<td>1.00 ± 0.12</td>
<td>1.01 ± 0.09</td>
<td>0.97 ± 0.16</td>
<td>NS</td>
</tr>
<tr>
<td><strong>TPF BMC (g)</strong></td>
<td>4.14 ± 0.67</td>
<td>4.32 ± 0.55</td>
<td>3.74 ± 0.65</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><strong>TPF BMD (g/cm²)</strong></td>
<td>0.83 ± 0.12</td>
<td>0.87 ± 0.09</td>
<td>0.74 ± 0.16</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>FN BMC (g)</strong></td>
<td>31.07 ± 5.70</td>
<td>32.25 ± 4.70</td>
<td>29.27 ± 5.54</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>FN BMD (g/cm²)</strong></td>
<td>0.93 ± 0.12</td>
<td>0.97 ± 0.10</td>
<td>0.85 ± 0.12</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>TF BMC (g)</strong></td>
<td>11.82 ± 1.70</td>
<td>11.89 ± 1.32</td>
<td>11.40 ± 2.34</td>
<td>NS</td>
</tr>
<tr>
<td><strong>TF BMD (g/cm²)</strong></td>
<td>0.56 ± 0.05</td>
<td>0.56 ± 0.04</td>
<td>0.55 ± 0.07</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Total energy intake (kcal/day)</strong></td>
<td>1845.47 ± 510.24</td>
<td>1919.59 ± 518.63</td>
<td>1631.14 ± 463.09</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>n-6 FA intake (g/day)</strong></td>
<td>9.26 ± 4.85</td>
<td>9.22 ± 4.97</td>
<td>8.53 ± 3.75</td>
<td>NS</td>
</tr>
<tr>
<td><strong>n-3 FA intake (g/day)</strong></td>
<td>1.04 ± 0.51</td>
<td>1.01 ± 0.48</td>
<td>1.06 ± 0.49</td>
<td>NS</td>
</tr>
<tr>
<td><strong>n-6:n-3 FA ratio</strong></td>
<td>9.66 ± 3.85</td>
<td>9.85 ± 3.89</td>
<td>8.85 ± 3.72</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

*a Means ± standard deviations; TB = total body; BMC = bone mineral content; BMD = bone mineral density; LS = lumbar spine (L1-L4); TPF = total proximal femur; FN = femoral neck; TF = total forearm; BMI = body mass index; n-3 = omega-3; n-6 = omega-6; NS = Not significant
Table 7.2. Pearson's correlation coefficients for total body and site-specific bone mineral measurements with anthropometric and soft tissue mass measurements and dietary intake estimates in all women

<table>
<thead>
<tr>
<th></th>
<th>TB BMC (g)</th>
<th>TB BMD (g/cm²)</th>
<th>LS BMC (g)</th>
<th>LS BMD (g/cm²)</th>
<th>TPF BMC (g)</th>
<th>TPF BMD (g/cm²)</th>
<th>FN BMC (g)</th>
<th>FN BMD (g/cm²)</th>
<th>TF BMC (g)</th>
<th>TF BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Height (cm)</strong></td>
<td>0.65***</td>
<td>0.31***</td>
<td>0.46***</td>
<td>0.29***</td>
<td>0.46***</td>
<td>0.24**</td>
<td>0.45***</td>
<td>0.25**</td>
<td>0.59***</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>0.36***</td>
<td>0.14*</td>
<td>0.24**</td>
<td>0.27***</td>
<td>0.27***</td>
<td>0.19**</td>
<td>0.24**</td>
<td>0.18*</td>
<td>0.33***</td>
<td>0.17*</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>0.06</td>
<td>-0.01</td>
<td>0.02</td>
<td>0.13</td>
<td>0.06</td>
<td>0.08</td>
<td>0.04</td>
<td>0.07</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>FM (kg)</strong></td>
<td>0.16*</td>
<td>-0.01</td>
<td>0.12</td>
<td><strong>0.19</strong> **</td>
<td>0.12</td>
<td>0.11</td>
<td>0.13</td>
<td>0.07</td>
<td><strong>0.14</strong></td>
<td>0.08</td>
</tr>
<tr>
<td><strong>FFST (kg)</strong></td>
<td>0.58***</td>
<td><strong>0.33</strong>*</td>
<td>0.36***</td>
<td>0.32***</td>
<td><strong>0.44</strong>*</td>
<td><strong>0.33</strong>*</td>
<td>0.43***</td>
<td>0.31***</td>
<td><strong>0.52</strong>*</td>
<td><strong>0.24</strong></td>
</tr>
<tr>
<td><strong>BF%</strong></td>
<td>-0.05</td>
<td><strong>-0.16</strong></td>
<td>-0.01</td>
<td>0.09</td>
<td>-0.06</td>
<td>-0.44</td>
<td>-0.05</td>
<td>-0.06</td>
<td>-0.04</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Total energy (kcal)</strong></td>
<td><strong>0.18</strong></td>
<td><strong>0.15</strong></td>
<td>0.07</td>
<td>0.03</td>
<td><strong>0.15</strong></td>
<td>0.13</td>
<td><strong>0.18</strong></td>
<td>0.11</td>
<td><strong>0.15</strong></td>
<td>0.09</td>
</tr>
<tr>
<td><strong>n-3 fatty acid (g)</strong></td>
<td>0.13</td>
<td>0.08</td>
<td>0.16*</td>
<td>0.05</td>
<td>0.04</td>
<td>-0.02</td>
<td>0.03</td>
<td>-0.06</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>n-6 fatty acid (g)</strong></td>
<td>0.13</td>
<td>0.12</td>
<td><strong>0.08</strong></td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.13</td>
<td>0.02</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>n-6:n-3 fatty acid ratio</strong></td>
<td>0.01</td>
<td>0.08</td>
<td>-0.11</td>
<td>-0.03</td>
<td>0.003</td>
<td>0.07</td>
<td>0.08</td>
<td>0.11</td>
<td>0.05</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*a n=202. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.0001; TB = total body; BMC = bone mineral content; BMD = bone mineral density; LS = lumbar spine (L₁-L₄); TPF = total proximal femur; FN = femoral neck; TF = total forearm; BMI = body mass index; FM = fat mass; FFSTM = fat-free soft tissue mass; BF% = body fat percentage; n-3 = omega-3; n-6 = omega-6.
Table 7.3. Pearson's correlation coefficients for total body and site-specific bone mineral measurements with anthropometric and soft tissue mass measurements and dietary intake estimates in young-adult women

<table>
<thead>
<tr>
<th></th>
<th>TB BMC (g)</th>
<th>TB BMD (g/cm²)</th>
<th>LS BMC (g)</th>
<th>LS BMD (g/cm²)</th>
<th>TPF BMC (g)</th>
<th>TPF BMD (g/cm²)</th>
<th>FN BMC (g)</th>
<th>FN BMD (g/cm²)</th>
<th>TF BMC (g)</th>
<th>TF BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>0.63***</td>
<td>0.22**</td>
<td>0.47***</td>
<td>0.28**</td>
<td>0.52***</td>
<td>0.15</td>
<td>0.39***</td>
<td>0.12</td>
<td>0.60***</td>
<td>0.01</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.46***</td>
<td>0.17*</td>
<td>0.30**</td>
<td>0.37***</td>
<td>0.39***</td>
<td>0.24**</td>
<td>0.33***</td>
<td>0.22**</td>
<td>0.44***</td>
<td>0.21*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.08</td>
<td>0.04</td>
<td>0.01</td>
<td>0.21*</td>
<td>0.08</td>
<td>0.16</td>
<td>0.10</td>
<td>0.16</td>
<td>0.08</td>
<td>0.22*</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>0.25*</td>
<td>0.02</td>
<td>0.16</td>
<td>0.27**</td>
<td>0.19</td>
<td>0.21*</td>
<td>0.24**</td>
<td>0.14</td>
<td>0.21*</td>
<td>0.12</td>
</tr>
<tr>
<td>FFST (kg)</td>
<td>0.62***</td>
<td>0.31**</td>
<td>0.35***</td>
<td>0.37***</td>
<td>0.55***</td>
<td>0.39***</td>
<td>0.50***</td>
<td>0.30**</td>
<td>0.58***</td>
<td>0.22**</td>
</tr>
<tr>
<td>BF%</td>
<td>0.02</td>
<td>-0.13</td>
<td>0.04</td>
<td>0.15</td>
<td>-0.01</td>
<td>0.05</td>
<td>0.06</td>
<td>0.03</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Total energy (kcal)</td>
<td>0.19*</td>
<td>0.11</td>
<td>0.17</td>
<td>0.14</td>
<td>0.15</td>
<td>0.05</td>
<td>0.11</td>
<td>-0.05</td>
<td>0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>n-3 fatty acid (g)</td>
<td>0.12</td>
<td>0.08</td>
<td>0.12</td>
<td>0.06</td>
<td>0.15</td>
<td>0.06</td>
<td>0.16</td>
<td>-0.02</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>n-6 fatty acid (g)</td>
<td>0.13</td>
<td>0.02</td>
<td>0.25**</td>
<td>0.11</td>
<td>0.06</td>
<td>-0.01</td>
<td>0.09</td>
<td>-0.08</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>n-6:n-3 fatty acid ratio</td>
<td>-0.1</td>
<td>0.10</td>
<td>-0.18*</td>
<td>-0.07</td>
<td>0.07</td>
<td>0.09</td>
<td>0.04</td>
<td>0.06</td>
<td>0.02</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*a n=136. *p < 0.05; **p < 0.01; ***p < 0.0001; TB = total body; BMC = bone mineral content; BMD = bone mineral density; LS = lumbar spine (L₁-L₄); TPF = total proximal femur; FN = femoral neck; TF = total forearm; BMI = body mass index; FM = fat mass; FFSTM = fat-free soft tissue mass; BF% = body fat percentage; n-3 = omega-3; n-6 = omega-6.
Table 7.4. Pearson's correlation coefficients for total body and site-specific bone mineral measurements with anthropometric and soft tissue mass measurements and dietary intake estimates in postmenopausal women

<table>
<thead>
<tr>
<th></th>
<th>TB BMC (g)</th>
<th>TB BMD (g/cm²)</th>
<th>LS BMC (g)</th>
<th>LS BMD (g/cm²)</th>
<th>TPF BMC (g)</th>
<th>TPF BMD (g/cm²)</th>
<th>FN BMC (g)</th>
<th>FN BMD (g/cm²)</th>
<th>TF BMC (g)</th>
<th>TF BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>0.61***</td>
<td>0.30*</td>
<td>0.35*</td>
<td>0.14</td>
<td>0.46**</td>
<td>0.14</td>
<td>0.32*</td>
<td>0.14</td>
<td>0.52**</td>
<td>0.20</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.21</td>
<td>0.01</td>
<td>0.07</td>
<td>0.19</td>
<td>0.50**</td>
<td>0.57***</td>
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<td>0.55***</td>
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<td>BMI (kg/m²)</td>
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<td>-0.10</td>
<td>-0.07</td>
<td>0.11</td>
<td>0.32*</td>
<td>0.50**</td>
<td>0.48**</td>
<td>0.48**</td>
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<td>0.17</td>
<td>0.63***</td>
<td>0.54***</td>
<td>0.47**</td>
<td>0.50**</td>
<td>0.41**</td>
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<td>BF%</td>
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<td>Total energy (kcal)</td>
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<td>n-3 fatty acid (g)</td>
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<td>n-6 fatty acid (g)</td>
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<td>n-6:n-3 fatty acid ratio</td>
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<td>-0.15</td>
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<td>-0.04</td>
<td>-0.05</td>
<td>-0.003</td>
<td>0.02</td>
<td>0.01</td>
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\(^{a}\) n=46. *p \leq 0.05; **p \leq 0.01; ***p \leq 0.0001; TB = total body; BMC = bone mineral content; BMD = bone mineral density; LS = lumbar spine (L₁-L₄); TPF = total proximal femur; FN = femoral neck; TF = total forearm; BMI = body mass index; FM = fat mass; FFSTM = fat-free soft tissue mass; BF% = body fat percentage; n-3 = omega-3; n-6 = omega-6.
Estimated total energy intake of the entire sample showed significant, albeit weak positive associations with BMC of TB, TPF, FN and TF as well as TB BMD (Table 7.2). These associations were not as prominent in the young-adult (Table 7.3) or postmenopausal women (Table 7.4). Weak associations (Pearson correlation coefficients < 0.25; \( p \leq 0.05 \)) were seen within the entire sample as well as subsamples for any bone mineral measurement and estimated dietary intake of \( n-3 \) fatty acid, \( n-6 \) fatty acid and \( n-6:n-3 \) fatty acid ratio.

**Discussion**

The estimated average total energy intake was 1845 kcal/day for all women and slightly higher in the young-adult women (1920 kcal/day) and slightly lower in the postmenopausal women (1631 kcal/day). These estimated intakes all fall within the referenced 2000 kcal diet recommended for an average American.

The estimated average intake of \( n-3 \) FA was 1.04 g/day in the entire sample, 1.01 g/day in the young-adult women and 1.06 g/day in the postmenopausal women. This was slightly lower than the recommended adequate intake of 1.1 g/day for women of these ages (National Research Council 2005). The estimated average intake of \( n-6 \) FA was 9.26 g/day in the entire population, 9.22 g/day in young-adult women and 8.53 g/day in postmenopausal women. This was lower than the recommended adequate intake of 11 g/day for women aged 18 to 50 years and 12 g/day for women aged >50 years (National Research Council 2005).

The mean estimated dietary \( n-6:n-3 \) FA ratio was 9.66:1 for the full sample, 9.85:1 in the young-adult women and 8.85:1 in the postmenopausal women. The average \( n-6:n-3 \) FA ratio in the United States has been estimated at 9.8:1 (Kris-Etherton and others 2000). Postmenopausal women had a significantly lower \( n-6:n-3 \) FA ratio, due to a lower (but not significantly lower) intake of \( n-6 \) FA compared to the young-adult women.

Contrary to expected, there was virtually no association between the estimated dietary \( n-6:n-3 \) FA ratio and measures of bone mineral in women included in this study. Although the LS BMC was weakly associated with the estimated \( n-6 \) FA intake (Pearson correlation coefficients = 0.25; \( p \leq 0.01 \)) and the \( n-6:n-3 \) FA ratio (Pearson correlation...
coefficient = -0.18 ; p ≤ 0.05) of the young-adult women; the relationships were not appreciable.

Older postmenopausal women have been the main focus for studies examining any relationship between the dietary n-6:n-3 FA ratio and bone mineral measurements. This leaves the unexplored potential of a similar association in younger women.

Postmenopausal women are post peak bone mass where bone growth no longer occurs. In many older women, bone loss is taking place. Studies using animal models have shown beneficial effects of diets high in n-3 FA compared to diets high in n-6 FA on bone. Ovariectomized rats fed a diet with 10:1 n-6:n-3 FA ratio had significantly greater pyridinoline and deoxypyridinoline concentrations and lower osteocalcin concentration compared to ovariectomized rats fed a diet with a 5:1 n-6:n-3 FA ratio (Watkins and others 2005). This suggests that a high n-6:n-3 FA ratio reduces bone formation and enhances bone resorption activity in a simulated model of postmenopause.

A few human studies support results of animal work. In an epidemiologic study, older Rancho Bernardo residents (mean ages: male = 72.9 years and female = 74.0 years) demonstrated a significant inverse association between dietary n-6:n-3 FA ratio and TPF BMD (Weiss and others 2005). These results were independent of age, BMI and lifestyle factors. Previously, two clinical trials were conducted with older postmenopausal women residing in a South African extended care facility. In the pilot study (van Papendorp and others 1995), 40 osteoporotic women (mean age = 80 y) who consumed four grams of fish oil (n-3 FA) or a mixture of evening primrose oil (n-6 FA) and fish oil daily for 16 weeks had a significant increase in osteocalcin compared to women who consumed equivalent amounts of only evening primrose oil or olive oils. In a follow-up study (Kruger and others 1998), 65 osteoporotic women consumed either six grams of a fish oil and evening primrose oil mixture or six grams of coconut oil, daily for 18 months. All women were supplemented with 600 mg calcium to maintain consistent calcium intake in treatment groups. Lumbar spine and FN BMD decreased significantly women consuming coconut oil. The LS BMD remained constant, while interestingly, FN BMD increased in the oil mixture group.
A small study compared six premenopausal (23-33 years old) to six postmenopausal (51-68 years old) women (Meydani and others 1991). All subjects consumed 2.4 g n-3 FA for 12 weeks. All women showed a significant reduction of interleukin-6 production (a pro-inflammatory cytokine that enhances bone resorption), where the postmenopausal group had a 2-fold reduction compared to the premenopausal women. Production of tumor necrosis factor, another pro-inflammatory cytokine, was significantly reduced in the postmenopausal women, but not in the premenopausal women. The sample sizes of the groups were very small and a larger study would be necessary to provide more confident conclusions. Due to inadequate knowledge of the effect of the dietary $n$-$6$:$n$-$3$ FA ratio on bone mineral measurements in a premenopausal population, it is impossible to draw any defined conclusions.

In a recent randomized, double-blind, balanced order, three-period crossover study, 23 male and female moderately hypercholesterolemic subjects (mean age=49.3 y) consumed either an average American diet ($9:1$ $n$-$6$:$n$-$3$ FA ratio), a high LA diet ($3.5:1$ $n$-$6$:$n$-$3$ FA ratio), and a high ALA diet ($1.6:1$ $n$-$6$:$n$-$3$ FA) (Griel and others 2007). Each subject consumed each diet for 6 weeks, with a 3-week washout period in between diets. The high ALA diet significantly lowered N-telopeptides of type I collagen compared to the control diet, whereas bone-specific alkaline phosphatase was unaffected in all diets.

These aforementioned studies with older adults suggest that the ratio of dietary $n$-$6$:$n$-$3$ FA may affect bone health; however, additional research is necessary to better understand this relationship in postmenopausal women. To our knowledge, this was the first study to evaluate the $n$-$6$:$n$-$3$ FA ratio and bone mineral measurements in young-adult women. These women had not yet reached the age of peak bone mass. Bone cell cultures from growing rats fed diets supplemented with $n$-$3$ FA showed positive effects on bone formation in rat fed the lowest $n$-$6$:$n$-$3$ FA ratio diet compared to other diets (Watkins and others 2000).

There were no strong significant associations found in this current study. The present findings do not support the beneficial effects of $n$-$3$ FA and a reduced $n$-$6$:$n$-$3$ FA ratio on bone health, as seen in the previous, though very limited, research. One limitation to this study was the small sample size of postmenopausal women. A second
limitation was that data were collected for studies in which the objectives did not relate to dietary intake of essential FA. In addition, the types of $n$-3 FA (i.e. plant or marine sources) in the diet may also be worthwhile to further analyze the relationship of dietary essential FA and bone mineralization in the subjects. Lastly, the larger studies from which the data was collected from were all cross-sectional. It would be valuable to conduct a longitudinal study to see whether bone mineral measurements, pro-inflammatory cytokines as well as dietary intake of $n$-6 FA and $n$-3 FA and the $n$-6:$n$-3 FA ratio changed over a defined period of time.

**Conclusion**

Few studies have investigated the effects of $n$-3 FA on bone health in humans. Appreciable associations between the dietary $n$-6:$n$-3 FA ratio with TB or site-specific BMC or BMD measurements were not found in this study. Significant, but weak associations, between LS BMC and estimated intake of $n$-6 FA and the $n$-6:$n$-3 FA ratio was seen in the young-adult women. Further research is necessary to gain a more sophisticated understanding of the relationship of dietary intake of omega-3 FA and the $n$-6:$n$-3 FA ratio with bone mineral measurements.
References


Chapter VIII

Summary and Future Directions

The dietary intake of omega-3 fatty acids is low in the average American diet, while dietary omega-6 fatty acid intake is high. This leads to an elevated omega-6:omega-3 fatty acid ratio which has been positively associated with chronic inflammation. Previous research has suggested that lowering the omega-6:omega-3 fatty acid can help attenuate inflammation in the body, potentially maintaining or lowering the risk of developing chronic inflammatory diseases such as osteoporosis. It has been estimated that the current omega-6:omega-3 fatty acid ratio is approximately 10:1, if not greater. Researchers have indicated that a 4-6:1 ratio should be targeted in order to decrease inflammation. In a sample population (n=202) recruited from Blacksburg, VA, there were no appreciable associations found between estimated omega-6:omega-3 fatty acid ratios and total body and site-specific bone mineral measurements. The sample was further divided into two subsamples based on age: young-adult women (n=136) and postmenopausal women (n=46). Once again, no appreciable associations were found in either subsample. However the population of these subsamples may have been too few (particularly the postmenopausal women), to have showed any significant results. In addition bone mineral measurements were used, while biomarkers for bone activity or inflammation were not.

A dietary approach to increase the intake of omega-3 fatty acids while lowering the essential fatty acid ratio is to consume functional food and beverage products that have been enriched with omega-3 fatty acids.

The majority of previously omega-3 fatty acid enriched products comprise of lipids from marine sources, which have been accompanied by the production metallic and fish off-flavors, due to the high susceptibility to lipid oxidation. The use of plant-derived omega-3 fatty acids may be an alternative, despite the fact that these specific omega-3 fatty acids are less effective in lowering inflammation compared to its longer-chain omega-3 fatty acids, found in the marine sources.

Flaxseed is the richest plant source of omega-3 fatty acids, where more than half its total fat content is comprised of alpha-linolenic acid. The concept of formulating a flaxseed-enriched milk-based beverage ("flaxmilk") is supported by the combination of
nutrients inherent in flaxseed and milk, potentially enhancing health benefits provided by flaxmilk compared to consuming the components separately. A hedonic sensory panel was conducted with untrained participants to taste flaxmilk and rate its palatability on a 9-point hedonic scale. The recruited sample evaluated flaxmilk and provided a mean hedonic score of 6.35 ± 1.53, surpassing the targeted a priori score of 6.0. Demographic information (including but not limited to gender, age, household income, level of education completed) was collected but no significant effects were seen on the hedonic scores between subsamples. However when participants were informed that flaxmilk may have a positive health benefit; they were more willing to consume it on a regular basis, and in larger volumes.

The functionality of flaxseed added into a chocolate milk foundation was explored and its physical, chemical and sensory characteristics were analyzed and compared to a commonly accepted beverage on the market (chocolate milk). Six attributes (color, thickness, chocolate flavor, malty flavor, sweetness and aftertaste) were evaluated for their intensities in the two beverages. Untrained panelists detected differences of all questioned attributes between beverages except for color. Flaxmilk was perceived to be thicker, possess a maltier flavor and more aftertaste compared to the chocolate milk control. Instrumental analysis of physical and chemical characteristics found significant differences in L, a, b values, viscosity, total moisture content, total protein content, total fat content and lipid profile between the beverages. Flaxmilk provides 0.41 g omega-3 fatty acids in 100 g portion, whereas the control contains 0 g omega-3 fatty acids. This demonstrates that the addition of flaxseed into the chocolate milk system has a major effect on its colloidal chemistry.

Very limited, yet promising research is available on the effect of dietary alpha-linolenic acid on the attenuation of inflammation. A dietary feeding trial using flaxmilk was not performed for this current dissertation. It would be valuable to investigate whether regular daily consumption of flaxmilk will have an effect on inflammation in the body (measured with inflammatory biomarkers such as pro-inflammatory cytokines) and whether the beverage remains palatable when consumed in larger quantities than 22 mL over a period of time. As a secondary effect of attenuated inflammation, bone mineral measurements would be collected as well, to gain a better understanding of
whether an increase of omega-3 fatty acids will attenuate bone loss, particularly in postmenopausal women.

Furthermore, there are still unknown characteristics of flaxmilk such as lipid oxidation stability, sediment formation through the shelf life period in flaxmilk as well as beneficial packaging options to maintain stability of flaxmilk. As seen in Chapter IV, replicate played an effect for many of the analytical measurements of product characteristics, and therefore more standardized methods may be necessary to obtain more consistant observations of flaxmilk and the control beverage over a 14-day shelf life period.

There is so much unexplored potential in the formulation of novel functional foods and beverages with alpha-linolenic acids, and whether these products are effective in attenuation inflammation when incorporated regularly into the diet. This is one avenue to increase the dietary omega-3 fatty acid intake, while simultaneously decreasing the essential fatty acid ratio. This chocolate flaxseed-enriched milk-based beverage is helping break ground into this currently unfamiliar research area.
Appendix A

Preliminary work to formulate final flaxmilk recipe
Materials
Finely milled flaxseed (BevGräd™) was purchased from Pizzey’s Milling USA (Gurnee, IL) for its specific use in beverage manufacturing.

Raw milk was obtained from the Virginia Tech dairy farm. The raw milk was heated to 54.4°C (130°F) and separated into cream and skim phases using a pilot plant separator (Elecrem separator, model IG, 6400 rpm, Bonanza Industries, Inc., Calgary, Alberta). The skim phase (<0.5% milkfat) was subsequently used for the formulation of flaxmilk while the cream phase was discarded.

Dry ingredients such as cocoa powder, granulated sugar, vanilla extract, malted milk mix (Nestlé Carnation, Switzerland) were purchased from local grocery stores. Stabilizers were obtained from Danisco (Copenhagen, Denmark), and k-carrageenan and guar gum powders were obtained from TIC Gums (Belcamp, MD).

General Processing Procedure
Various dry ingredients of the preliminary recipes were incorporated into the skim milk (<0.5% milkfat) foundation and mixed using a vat-pasteurizer (Model P50.8770 (?), The Creamery Pack, Chicago, IL). The mixture of ingredients was heated to 54.4°C (130°F) and homogenized (APV Gaulin, Inc Homogenizer, Everett, MA with Reliance Duty Master AC Motor, Cleveland, OH) in a 2-stage process at 1500/500 psi. The flaxmilk was then placed back into the vat-pasteurizer to be heated at 73.89°C (165°F) for 30 min to pasteurize the beverage. After pasteurization, the flaxmilk was cooled and stored at -15.6°C (4°F) in stainless steel containers to avoid excess exposure to light and air. Processing of flaxmilk was completed in the Dairy/Beverage Processing Pilot Plant (DBPP) in the Department of Food Science and Technology (FST) at Virginia Tech.

Recipe Development
Preliminary flaxmilk recipe formulations were first developed using bench-top scale trials followed by bench-top sensory evaluation by the researcher. After an acceptable recipe was developed, pilot plant production of the beverage was performed. After processing, sensory evaluation by recruited consumers would take place at various locations on the Virginia Tech campus.

The first processing session in the DBPP took place on November 15, 2005 with the following recipe. This recipe used a chocolate milk recipe as the foundation and incorporated the flaxseed.

702 g BevGräd™ (18%)
1.6 oz Cocoa powder
3900 g Skim milk
8 oz Granulated sugar
6.5 g Grindsted SSD-5891 Stabilizer (Danisco)
This mixture would provide approximately 9 g omega-3 fatty acids (n-3 FA) per 8 fluid ounces (fl oz). However the consistency was very thick and pudding-like. Needless to say, the consistency was undesired and a decrease in flaxseed was necessary.
After contact with Pizzey’s Milling to discuss the failed recipe, it was recommended to use 2.2% - 2.8% BevGräd™ by weight. To maximize the amount of n-3 FA in the flaxmilk, the 2.8% Bevgrad™ by weight was used from then on.

Bench-top formulations were performed between November 21-30, 2005, to produce beverages to provide approximately 1.4 g n-3 FA/8 fl oz.

a) 253 g skim milk
   7.0 g BevGräd™
   1g pectin
   2 Tbsp cocoa powder (Hershey)
   2 Tbsp granulated white sugar

b) 6g BevGräd™
   1 g pectin
   2 Tbsp lecithin liquid
   -1 tsp lecithin
   -4 c water
   1 Tbsp boiling water
   fill to 8 oz with skim milk
   2 Tbsp cocoa powder
   1 Tsp powder sugar

c) 239 g skim milk
   6.5 g BevGräd™

d) 7 g BevGräd™
   239 g skim milk
   -put in half to mix
   1 g pectin
   ¼ c hot water
   2 Tbsp lecithin liquid
   -add remainder of milk
   -heat to 145° F, before milk boils

e) 7 g BevGräd™
   ¼ c hot water
   1 c skim milk
   1 g pectin
   1 Tbsp lecithin granule
   1 Tbsp cocoa powder
   1 Tbsp + 1 tsp granulated sugar
   -heat & blend at low heat
   -thick foamy texture
The second processing session in the DBPP took place on November 30, 2005 with the following recipe. Two recipes were used to determine which source of chocolate flavor was preferred between Hershey’s cocoa powder and Nestle Quik Chocolate powder. The recipes below were used.

f) 248 g skim milk
   0.45 g lecithin granule
   0.75 g pectin
   6.95 g BevGrăd™
   1 Tbsp cocoa powder
   1 Tbsp granulated sugar
   -seperated after half hour

g) 253.05 g skim milk
   7 g lecithin granule
   1 g pectin
   7 g BevGrăd™
   1 ½ tsp cocoa powder
   1 ½ tsp granulated sugar
   -very foamy

h) 250.7 g skim milk
   4.5 g lecithin
   1 g pectin
   7 g BevGrăd™
   2 Tbsp hot water
   1 ½ cocoa powder
   1 ½ granulated sugar

These mixtures would provide approximately 1.4 g n-3 FA/8 fl oz. The Nestle Quik chocolate powder created a very gritty undesired texture and cocoa powder was chosen for the chocolate flavoring in future recipes.
The third processing session in the DBPP took place on December 6, 2005 with the following recipe.

- 3900 g skim milk
- 109.19 g BevGrâd™
- 1/10 lb cocoa powder
- ½ lb granulated sugar
- 3.5 g stabilizer (grindsted SSD-5891)

This recipe produced a satisfactory beverage in the opinion of the researcher, but still not optimal. This recipe would need to be evaluated to estimate overall acceptability by the general public.

The fourth processing session in the DBPP took place on April 11, 2006 with a very similar recipe to the third session.

- 3900 g skim milk
- 109.2 g BevGrâd™
- 1.6 oz cocoa powder
- 8 oz granulated sugar
- 6.5 g stabilizer (grindsted SSD-5891)

A control chocolate milk of the same recipe minus the BevGrâd™ was also processed for comparison in preliminary sensory panels. Preliminary sensory evaluation by 103 subjects recruited from the Foods and Nutrition course (HNFE 1004 at Virginia Tech) was performed on April 12 and April 14, 2006. Sensory evaluation took place in the Sensory Evaluation Laboratory, equipped with 8 sensory booths, located in the FST building.

The students were asked to taste the flaxmilk sample thoroughly and rate its overall palatability using a 9 point hedonic scale (1 = “dislike extremely”; 5 = “neither like nor dislike”; 9 = “like extremely”). This hedonic score would represent the overall acceptability of the flaxmilk beverage. A minimum mean score of “6” (“like slightly”) or higher was targeted for acceptability. If this level of acceptability was not achieved, modification of the formulation would occur to improve the product acceptability.

A mean hedonic score of 4.22 was reached which indicated that adjustments to the current flaxmilk recipe were necessary to increase product acceptability by consumers. Attribute testing results are shown below.
Flaxmilk

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Chocolate milk

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From these results, it was proposed to increase the chocolate flavor and sweetness levels while decreasing thickness, grittiness, malty flavor and aftertaste.

Adjustments to the current recipe included the addition of kappa-carrageenan powder (TIC PRETESTED® Colloid 517 “T” Powder) to keep chocolate particles dispersed in the colloid, the addition of guar gum (TIC PRETESTED® GuarNT Bland 200 HV) to add a creamy texture to the system, the addition of malted barley to complement the flaxseed’s natural malty/nutty flavor and the addition of vanilla extract to enhance the chocolate flavor present in the flaxmilk. The grindsted SSD-5891 stabilizer (Danisco) was removed from the recipe and replaced by the combination of kappa-carrageenan and guar gum powders.

With the idea to add chocolate flavored malted milk mix to the recipe to replace the cocoa powder and granulated sugar, benchtop formulations were performed on May 19, 2006, using various levels of malted milk mix. The amount of Bevgrad remained at 2.8% by weight to provide approximately 1.4 g n-3 FA/8 fl oz.

a) 11.7% malted milk
   231.50 g skim milk
   6.45 g BevGråd™
   27.10 malted milk mix

b) 9.4% malted milk mix
   237.80 g skim milk
   6.65 g BevGråd™
   22.30 g malted milk mix

c) 8.1% malted milk mix
   238.00 g skim milk
   6.60 g BevGråd™
   19.25 g malted milk mix

Benchtop sensory evaluation by the researcher occurred and the 11.7% malted milk mix recipe tasted the best, however slightly on the sweet side. Therefore, 11% malted milk mix was chosen as a whole number to be used as the amount of malted milk mix to be added into the recipe.

Benchtop formulations were performed on May 31, 2006, to produce beverages to provide approximately 1.4g n-3 FA/8oz using different amounts of kappa-carrageenan
powder. The literature accompanied with TIC PRETESTED® Colloid 517 “T” Powder suggested the typical usage levels were 0.1% - 0.4%.

a) 0.113% 517 “T”  
240.3 g skim milk  
6.7 g BevGräd™  
19.5 g malted milk mix  
0.30 g 517 “T”

b) 0.096% 517 “T”  
231.55 g skim milk  
21.8 g malted milk mix  
6.5 g BevGräd™  
0.25 g 517 “T”

c) 0.098% 517 “T”  
223.10 g skim milk  
26.10 g malted milk mix  
6.25 g BevGräd™  
0.25 g 517 “T”

From these formulations, it was estimated that 0.1% of the kappa-carrageenan recipe is the optimal amount needed to be added to the recipe to keep the chocolate and flaxseed particles dispersed in the flaxmilk colloidal dispersion.

The fifth procession session in the DBPP took place on June 2, 2006 with the following recipe

137.6 oz skim milk  
3.85 oz BevGräd™  
16.10 oz malted milk mix  
0.16 oz 517 “T”

During the incorporation of the dry ingredients into the milk, fish eyes were produced specifically when the kappa-carrageenan was added to the milk. Fish eyes are lumps that form when gums are added to a wet environment and remain undissolved as they clump together. To overcome this, a hand blender was used to help break up the clumps. In future processing, the mixing of all dry ingredients together first and gentle sprinkling into the milk was performed. There was also a slight separation at the top of the flaxmilk, possibly due to wheying off or excess water by syneresis. This separation at the top of the flaxmilk was resolved by a small addition of guar gum to act like a sponge and pick up excess water and keep it dispersed in the flaxmilk.

The ratio of kappa-carrageenan and guar gum powders was the next step to experiment with the recipe. The literature accompanied with TIC PRETESTED® GuarNT Bland 200 HV Powder suggested the typical usage levels began at 0.5%. Benchtop formulations of
various amounts of the gum powders were performed on June 9, 2006 followed by sensory evaluation by the researcher. All formulations were heated to 130°F, blended using a kitchen blender (to somewhat simulate the homogenizer treatment) and further heated to 165°F to simulate the pasteurization stage.

a) 0.1% 517 “T”
   109.80 g skim milk
   3.05 g BevGräd™
   12.80 g malted milk mix
   0.10 g 517 “T”

b) 0.1% 517 “T”
   122.95 g skim milk
   3.45 g BevGräd™
   14.40 g malted milk mix
   0.10 g 517 “T”

c) 0.1% 517 “T” & 0.5% GuarNT
   114.20 g skim milk
   3.20 g BevGräd™
   12.00 g malted milk mix
   0.10 g 517 “T”
   0.60 GuarNT
   -VERY THICK!

d) 0.1% 517 “T” & 0.1% GuarNT
   100 g skim milk
   2.8 g BevGräd™
   10.50 g malted milk mix
   0.10 g 517 “T”
   0.10 Guar NT

These formulations suggested the usage of approximately 0.1% GuarNT powder into the flaxmilk recipe to avoid the thick and gelled consistency as seen with the 0.5% GuarNT formulation. After discussing the visual sensory properties of the formulations with Dr. Frank Conforti (Professor in Dept. of HNFE, Virginia Tech), he suggested to use 0.05% GuarNT instead to avoid any more thickness in the flaxmilk.

The sixth processing session in the DBPP took place on June 13, 2006 with the following recipe

68.8 oz skim milk
1.93 oz BevGräd™
7.4 oz malted milk mix
0.068 oz 517 “T”
0.03 oz Guar NT (0.05% by weight)
After processing, the flaxmilk still had separation at the top of the flaxmilk, which prompted a slight increase of the GuarNT content. It is possible that the homogenization stage sheared the guar gum and became ineffective.

The seventh procession session in the DBPP took place on June 20, 2006 with the following recipe

- 69.76 oz skim milk
- 7.461 oz malted milk mix
- 1.952 oz BevGräd™
- 0.069 oz 517 “T”
- 0.063 oz Guar NT (0.8%)
- 1 Tbsp vanilla extract

This formulation had little separation at the top after 24 hr. Preliminary sensory evaluation by 10 graduate students in the HNFE and FST departments participated on June 26, 2006. Sensory evaluation took place on the bench top in Wallace Hall or in the FST building. Each student was asked to rate the beverage using the 9-point hedonic scale and also to rate the intensity of seven attributes (color intensity, thickness, vanilla flavor, chocolate flavor, malty flavor, sweetness and aftertaste) using a 9-point attribute diagnostic test as described previously.

The mean hedonic score was 4.8 and attribute testing results are below.

<table>
<thead>
<tr>
<th>color</th>
<th>thickness</th>
<th>vanilla flavor</th>
<th>chocolate flavor</th>
<th>malty flavor</th>
<th>sweetness</th>
<th>aftertaste</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>7.2</td>
<td>2.5</td>
<td>5.3</td>
<td>4.7</td>
<td>4.5</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Comparing these results with the results from the April sensory panels, it was devised to increase the sugar content with either granulated sugar or splenda to increase the sweetness level and possibly increase the vanilla extract content to enhance the chocolate flavor slightly.

The eighth processing session in the DBPP took place on July 12, 2006 with the following recipes.

a) CONTROL
- 1000 g skim milk
- 110 g malted milk mix (11%)
- 28 g BevGräd™ (2.8%)
- 1 g 517 “T” (0.1%)
- 0.91 g GuarNT (0.8%)
- 1 Tbsp Vanilla extract

b) SUGAR
- 1000 g skim milk
- 110 g malted milk mix (11%)
- 28 g BevGräd™ (2.8%)
- 1 g 517 “T” (0.1%)
Another round of preliminary sensory evaluation on these three formulations took place on July 13, 2006 with 10 subjects recruited from the Nutrition Across the Lifespan course (HNFE 2014 at Virginia Tech). Sensory evaluation took place in a classroom (219 War Memorial Hall). Each student was asked to rate the beverage using the 9-point hedonic scale and also to rate the intensity of six attributes (color intensity, thickness, chocolate flavor, malty flavor, sweetness and aftertaste) using a 9-point attribute diagnostic test as described previously. The following scoresheet was completed by the subjects for each sample. Samples were coded and presented in random order.

The results are presented below for the three formulations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hedonic score</th>
<th>Color</th>
<th>Thickness</th>
<th>Chocolate flavor</th>
<th>Malty flavor</th>
<th>Sweetness</th>
<th>Aftertaste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.89</td>
<td>6.2</td>
<td>5.9</td>
<td>4.9</td>
<td>4.6</td>
<td>4.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Sugar</td>
<td>4.6</td>
<td>6.6</td>
<td>7.2</td>
<td>4.2</td>
<td>5.8</td>
<td>4.65</td>
<td>5</td>
</tr>
<tr>
<td>Splenda</td>
<td>5</td>
<td>6.1</td>
<td>6.15</td>
<td>4.4</td>
<td>4.6</td>
<td>4.6</td>
<td>5.3</td>
</tr>
</tbody>
</table>

The Control recipe had the highest hedonic score; however the sample size was only 10. Therefore, an additional 15 graduate students and staff located in Wallace Hall were recruited to sample solely the Control sample and rate the beverage using the 9-point hedonic scale. Combining these results with the previous 10; a mean hedonic score of 6.0 was achieved in the sample size of 25 subjects. This encouraged the use of the control recipe as one step closer to the finalized recipe for flaxmilk.

The ninth processing session in the DBPP took place on July 24, 2006 with the following recipe. Five gallons of flaxmilk was processed in order to perform the larger scale production process.

Eight gallons of raw milk was obtained from the Virginia Tech dairy farm and separated using the pilot plant separator to yield approximately 5 gallons.

16.26 kg skim milk
1.79 kg malted milk mix (11%)
4.5528 g BevGrăd™ (2.8%)
16.26 g 517 "T"
14.78 g GuarNT
16 ¾ Tbsp vanilla extract

A second large sized sensory evaluation panel was held on July 26, 2006. A booth was set up in front of the Squires Student Union on the Virginia Tech campus to recruit as many subjects as possible to represent the general public. Subjects provided informed consent to participate in the sensory panel. The same informed consent form was used as before. Each subject sampled the flaxmilk and rated its overall palatability on a 9-point hedonic scale as previously described. A mean hedonic score of 6.35 was achieved in the sample size of 62 subjects. With the larger sample size, the mean hedonic score is accepted to represent “like slightly” or higher on the hedonic scale and the current recipe used will be used as the final recipe for future research.

**Final Recipe**

Achieving a mean hedonic score ≥ 6, the following recipe is accepted as the final recipe (reported by percentages) for flaxmilk.

**Beverage Formulation of Flaxmilk**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage in beverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td>87.17</td>
</tr>
<tr>
<td>Malt milk mix</td>
<td>9.60</td>
</tr>
<tr>
<td>Flaxseed</td>
<td>2.47</td>
</tr>
<tr>
<td>Vanilla extract</td>
<td>0.61</td>
</tr>
<tr>
<td>κ-carageenan</td>
<td>0.09</td>
</tr>
<tr>
<td>guar gum</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

*a The beverage formulation was made up to 100%.*
Appendix B
Institutional Review Board of Human Subject Research approval
DATE: April 7, 2006

MEMORANDUM

TO: Sharon M. Nickols-Richardson
    Clara Lau
    Susan E. Duncan

FROM: David M. Moore

SUBJECT: IRB Exempt Approval: "Acceptability of a Flaxseed-Enriched Milk-Based Beverage" , IRB # 06-240

I have reviewed your request to the IRB for exemption for the above referenced project. I concur that the research falls within the exempt status. Approval is granted effective as of April 7, 2006.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.

2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

cc: File
    Department Reviewer: Kathy Hosig
Appendix C
Forms for Hedonic Sensory Panel
Title of Project: Acceptability of a Novel Flaxseed-enriched Milk-Based Beverage
Investigators: Clara S. Lau; Sharon M. Nickols-Richardson; Susan E. Duncan

I. PURPOSE
You are invited to participate in a sensory evaluation panel about a novel milk-based beverage where the fatty acid profile has been altered. The purpose of this study is to see how people rate the acceptability and other attributes about this product. About 150 individuals who are at least 18 years of age will be in this study.

II. PROCEDURES
There will be 1 session that will take about 15 minutes of time. You will complete a survey that asks questions about you and any food allergies that you might have. Then, you will be presented with 1 sample of novel flaxseed-enriched milk-based beverage where the fatty acid profile has been altered. You will drink this beverage and rate it. Should you find the beverage unacceptable or offensive, you may spit it out.

Certain individuals are sensitive to some foods such as flaxseed, milk, and chocolate. If you are aware of any food or drug allergies, please tell the investigator now, and please list these allergies on your survey.

III. RISKS
There is no more than minimal risk with your participation in this study. If you have a known allergy to flaxseed, milk, and/or chocolate, please inform the investigator now.

IV. BENEFITS OF THIS PROJECT
Your participation in this study will give important information about the acceptability of novel milk-based beverage with an altered fatty acid profile.

V. EXTENT OF ANONYMITY AND CONFIDENTIALITY
The results of your performance as a panelist will be kept strictly confidential. Individual panelists will be referred to by a code number for analyses and in any publication of results.

VI. COMPENSATION
You will not be compensated for your participation in this study.

VII. FREEDOM TO WITHDRAW
It is essential to sensory evaluation projects that you complete each session in so far as possible. However, there may be conditions preventing your completion of all sessions. If after reading and becoming familiar with the sensory project, you decide not to participate as a panelist, you may withdraw at any time without penalty. There may be reasons for why the investigator decides that you should not be in the study. For example, if you indicate that you have a food allergy to flaxseed, milk, and/or chocolate, you will be asked to not participate.
VIII. APPROVAL OF RESEARCH
This research project has been approved by the Institutional Review Board for projects involving human subjects at Virginia Polytechnic Institute and State University and by the Department of Human Nutrition, Foods and Exercise and the Department of Food Science and Technology.

IX. SUBJECT'S RESPONSIBILITIES
I know of no reason that I cannot participate in this study which will require:
(1) completion of a survey;
(2) tasting and rating of novel milk-based beverage where the fatty acid profile has been altered.

__________________________________________________________________________
Signature/Date

Please provide address and phone number so investigator may reach you in case of emergency or schedule changes.

Address ________________________________________________________________

Phone _________________________________________________________________

------------------------------------------------------------------------- (tear off)------------------------------------------------------------------------

XI. SUBJECT'S PERMISSION
I have read the information about the conditions of this sensory evaluation project and give my voluntary consent for participation in this project. I may withdraw from this study at any time without penalty.

_________________________________________ Date

Investigator's Signature Date

Should you have any questions about this research or its conduct, you may contact:

Clara S. Lau, Graduate Research Assistant and Investigator
(540) 231-7387; clara@vt.edu

Sharon M. (Shelly) Nickols-Richardson, Investigator
(540) 231-5104; snrichar@vt.edu

Dr. David M. Moore, IRB Chair
(540) 231-4991
Panelist Demographic Survey

Panelist#: ______

Please circle one response or complete the question:

1. What is your gender?  Male  Female
2. What is your marital status?   Married  Single  Divorced  Separated  Other
3. What is your age:     18-25  26-35       36-45  46-55       over 55
4. What is your household income before taxes (if a student, estimate your parent’s income)?
   Under $20,000  $21-40,000       $41-70,000 $71-100,000  over $100,000
5. What is your level of education completed?     High School  College  Advanced degree
6. What is your employment status?
   Full time  Part time  Self-employed  Unemployed  Retired  Student  Homemaker
7. How many cups of milk do you consume each day? (8 ounces = 1 cup)
   1  2  3  4  more than 4
8. What type of milk do you normally consume?
   Skim (fat-free)  1%  2%  whole
9. Do you consume chocolate milk on a regular basis?   Yes  No
10. Do you consume any food products containing flaxseed on a daily basis?  Yes  No
    If yes, list products:
11. Do you enjoy beverages with a malty flavor?  Yes  No
    If yes, please list these beverages:
12. Do you enjoy foods with a malty flavor?  Yes  No
    If yes, please list these foods:
13. To your knowledge are you allergic to: (circle all that apply)
    Flaxseed  Milk  Dairy foods  Chocolate  Chocolate flavoring/Cocoa  Malted Barley
Panelist #_____

**Consumer Preference Test for Novel Milk-based beverage**

Please taste the sample thoroughly and rate its overall palatability on the scale below. You may expectorate the sample if you wish.

Dislike extremely □  Dislike very much □  Dislike moderately □  Dislike slightly □  Neither Like nor Dislike □  Like Slightly □  Like Moderately □  Like very much □  Like extremely □

**Comments?**

___________________________________________________________________
Flaxmilk Beverage Responses

Would you consume this beverage on a daily basis?  Yes  No
If yes, how much of this product would you consume on a daily basis?
____________________
If yes, how much would you consume at one time?
__________________________

Would you consume this beverage on a daily basis if you knew that it provided a positive health benefit?  Yes  No
If yes, how much of this product would you consume on a daily basis?
____________________
If yes, how much would you consume at one time?
__________________________

THANK YOU FOR YOUR PARTICIPATION!!
Appendix D
Forms for Attribute Intensity Sensory Panels
Used in pilot panel, prior to final flaxmilk recipe
Panelist #_____

Attribute Intensity Test for Novel Milk-based beverage
Please taste the sample thoroughly and rate its overall intensity of the specified attributes on the scale below (where 1 is none and 9 is very). You may expectorate the sample if you wish.

Sample #_____

How intense is the color of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none very

How intense is the thickness of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none very

How intense is the grittiness of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none very

How intense is the chocolate flavor of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none very

How intense is the malty flavor of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none very

How intense is the sweetness of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none very

How intense is the aftertaste of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none very

Comments?
___________________________________________________________________

THANK YOU FOR YOUR PARTICIPATION!! Please come by the kitchen to provide your Name and Student ID# in order to receive your 5 extra credit points for HNFE 1004.
Panelist #_____

Attribute Intensity Test for Novel Milk-based beverage
Please taste the sample thoroughly and rate its overall intensity of the specified attributes on the scale below (where 1 is none and 9 is very). You may expectorate the sample if you wish.

Sample #_____

How intense is the color of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none

How intense is the thickness of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none

How intense is the vanilla flavor of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none

How intense is the chocolate flavor of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none

How intense is the malty flavor of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none

How intense is the sweetness of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none

How intense is the aftertaste of this beverage?
1 2 3 4 5 6 7 8 9
☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none

Comments?
___________________________________________________________________
Used for panels using final flaxmilk recipe
Panelist #_____

Consumer Preference Test for Novel Milk-based beverage

Please taste the sample thoroughly and rate its overall intensity of the specified attributes on the scale below (where 1 is none and 9 is very). You may expectorate the sample if you wish.

Sample #_____

How intense is the color of this beverage?
1  2  3  4  5  6  7  8  9

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none          very

How intense is the thickness of this beverage?
1  2  3  4  5  6  7  8  9

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none          very

How intense is the chocolate flavor of this beverage?
1  2  3  4  5  6  7  8  9

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none          very

How intense is the malty flavor of this beverage?
1  2  3  4  5  6  7  8  9

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none          very

How intense is the sweetness of this beverage?
1  2  3  4  5  6  7  8  9

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none          very

How intense is the aftertaste of this beverage?
1  2  3  4  5  6  7  8  9

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐
none          very

Comments?
___________________________________________________________________

Please slide your tray and score sheet through the window and wait for the next tray.
Panelist #_____

Consumer Preference Test for Novel Milk-based beverage

Please taste the sample thoroughly and rate its overall intensity of the specified attributes on the scale below (where 1 is none and 9 is very). You may expectorate the sample if you wish.

Sample #_____

How intense is the color of this beverage?

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
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<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

none

How intense is the thickness of this beverage?

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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</tr>
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<tbody>
<tr>
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<td>□</td>
<td>□</td>
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</tr>
</tbody>
</table>

none

How intense is the chocolate flavor of this beverage?

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
<th>8</th>
<th>9</th>
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<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

none

How intense is the malty flavor of this beverage?

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
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<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

none

How intense is the sweetness of this beverage?

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

none

How intense is the aftertaste of this beverage?

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</tbody>
</table>

none

Comments?

THANK YOU FOR YOUR PARTICIPATION!!
Appendix E

Data from physical and chemical analysis of beverages
### L values

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Replicate</th>
<th>Day of storage</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaxmilk</td>
<td>1</td>
<td>1</td>
<td>45.79 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>44.65 ± 0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>45.07 ± 0.25</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>44.07 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>42.63 ± 0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>42.65 ± 0.10</td>
</tr>
<tr>
<td>Flaxmilk</td>
<td>2</td>
<td>1</td>
<td>45.17 ± 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>44.56 ± 0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>43.55 ± 0.63</td>
</tr>
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<td>Control</td>
<td>2</td>
<td>1</td>
<td>41.85 ± 0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>42.43 ± 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>40.32 ± 0.15</td>
</tr>
<tr>
<td>Flaxmilk</td>
<td>3</td>
<td>1</td>
<td>45.01 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>44.46 ± 0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>46.07 ± 0.13</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>1</td>
<td>44.71 ± 0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>41.81 ± 0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>42.16 ± 0.22</td>
</tr>
</tbody>
</table>

### a values

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Replicate</th>
<th>Day of storage</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaxmilk</td>
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Table E.1. Color of chocolate flaxseed-enriched and control dairy-based beverages on each test day (day 1, 7 and 14) during a 14-day storage period (4°C)

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<th>Control (mean ± SD)</th>
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<td>43.54 ± 1.38&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>42.29 ± 0.25&lt;sup&gt;bd&lt;/sup&gt;</td>
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<td>41.71 ± 1.07&lt;sup&gt;bd&lt;/sup&gt;</td>
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<td>7.81 ± 0.21&lt;sup&gt;bd&lt;/sup&gt;</td>
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<td>7.16 ± 0.56&lt;sup&gt;bd&lt;/sup&gt;</td>
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<td>7.45 ± 0.42&lt;sup&gt;bd&lt;/sup&gt;</td>
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<td>8.03 ± 0.22&lt;sup&gt;bd&lt;/sup&gt;</td>
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<sup>a</sup>,<sup>b</sup> Means ± standard deviation with different letters in the same row are significantly different at p<0.05
<sup>c</sup>,<sup>d</sup> Means ± standard deviation with different letters in the same column are significantly different at p<0.05
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### Total lipid content (%)

#### (top layer)

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#### (bottom layer)

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### Total moisture content (%)

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Figure E.1. Total moisture content (%) of chocolate flaxseed-enriched and control dairy-based beverages over a 14-day storage period (4°C). Significant differences were found in total moisture content between beverages on each day of storage.
## Total ash content (%)

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## Total protein content (mg/mL)

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Appendix F

Fatty acid profiles of standard and beverages
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Figure F.1. Fatty acid profile of linseed oil standard.
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Figure F.2. Fatty acid profile of flaxmilk on day 1 of storage.
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<th>15:0</th>
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Figure F.3. Fatty acid profile of flaxmilk on day 7 of storage.
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Figure F.4. Fatty acid profile of flaxmilk on day 14 of storage.
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Figure F.5. Fatty acid profile of the control chocolate milk beverage on day 1 of storage.
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</table>

Figure F.6. Fatty acid profile of the control chocolate milk beverage on day 14 of storage.
VITA

Clara Lau, daughter of Alfred and Judy Lau, was born in Vancouver, British Columbia. She has an older brother, Bernie. At the age of 5, she and her family relocated to the States. She attended school in Baltimore, MD up to the 9th grade when she and her parents moved to the city of Seoul, Korea and she attended Seoul Foreign High School. Two years later, she ended up in the San Francisco Bay Area, which is now home. She finished off her senior year at El Cerrito High in 1999, before heading to Davis, CA for college. Clara received her B.A. in Psychology and her B.S. in Nutritional Sciences from the University of California, Davis in 2003. She enrolled in the Human Nutrition, Foods and Exercise (HNFE) doctoral program in the fall of 2004. While at Virginia Tech, Clara was supported by funding from HNFE, the Multicultural Academic Opportunities Program, the American Association of Family & Consumer Sciences and the Livestock and Seed Program (USDA/AMS) through various graduate teaching assistantships, fellowships and scholarships. During her last year, Clara moved to Washington DC for an internship with the Livestock and Seed Program in AMS, working as a nutritionist while writing her dissertation. After graduating from Virginia Tech, Clara will head back to Washington DC to continue working as a nutritionist with the Livestock and Seed Program.