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Variations in carotenoids and retinol in milk and cheese from Jersey cows at an organic dairy compared to a conventional dairy over a pasture season

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VARIATIONS IN CAROTENOIDS AND RETINOL IN MILK AND CHEESE FROM JERSEY COWS AT AN ORGANIC DAIRY COMPARED TO A CONVENTIONAL DAIRY OVER A PASTURE SEASON

BY

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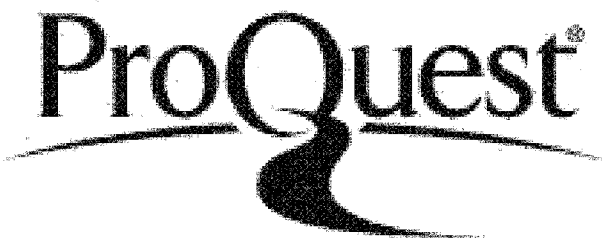

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DEDICATION

I dedicate this thesis to my family, Armand and Annette Beliveau, Sarah Beliveau, Rebekah and David Schmitz, and Steve and Melanie Arsenault. I could not have done this without your love, encouragement, and support throughout the process. Thank you for believing in my and pushing me to be greater than I could ever be on my own.

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ABSTRACT

VARIATIONS IN CAROTENOIDS AND RETINOL IN MILK AND CHEESE FROM JERSEY COWS AT AN ORGANIC DAIRY COMPARED TO A CONVENTIONAL DAIRY OVER A PASTURE SEASON

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Amy Rose Beliveau

University of New Hampshire, September, 2012

Carotenoids, widely distributed in nature, are considered to be potentially beneficial in the prevention of a variety of diseases including cardiovascular disease, certain cancers and eye diseases. As humans are unable to synthesize carotenoids, the diet is the only source of these beneficial components. Carotenoid concentration in cow's milk varies greatly as a result of feeding practice and season, but no research thus far has investigated these variations in Jersey cows over an entire pasture season. The objectives of this experiment were to 1) determine the differences in concentrations of carotenoids and retinol in milk from cows consuming different diets, 2) examine the changes that occurred over a pasture season, and 3) assess the relationship of these components between a fresh, mozzarella cheese and the milk from which it was made. Individual milk samples were collected biweekly, beginning in May and ending in November, from 18 Jersey cows, 9 at an organic dairy fed on pasture and supplemented with total mixed ration (TMR) and 9 at a conventional dairy fed exclusively TMR. High performance liquid chromatography (HPLC) was used to analyze carotenoid concentrations from each individual milk sample. Total carotenes, total xanthophylls, lutein, β-cryptoxanthin, α-

and β-carotene and 13*cis* β-carotene were significantly higher in milk from the cows fed on pasture compared to milk from the cows fed TMR. In milk from cows fed on pasture, total carotenes, total xanthophylls, lutein, β-cryptoxanthin, α- and β-carotene, and 13*cis* β-carotene varied significantly over the pasture season. In milk from cows fed TMR, only total xanthophylls, retinol and lutein changed significantly over time. Bulk milk samples were also collected biweekly at each dairy and made into Mozzarella cheese. HPLC was used to analyze carotenoid concentrations in the raw and pasteurized milk, the whey, and the cheese. Total carotenes in raw milk, pasteurized milk and whey were positively related to total carotenes in cheese, and total xanthophylls in pasteurized milk were positively related to total xanthophylls in cheese. Results indicate that even for Jersey cows, a breed known to have 'yellow' milk, pasture feeding will increase the carotenoid concentration in milk and its products and the concentration changes over time.

CHAPTER I

CAROTENOIDS

Definition and Classification of Phytonutrients:

Phytonutrients are bioactive nonnutrient plant compounds, or chemicals, thought to promote human health. The *phyto* is a derivative of a Greek word meaning plant. Over 5000 individual phytonutrients have been identified in fruits, vegetables, and grains, while many still remain unknown. Phytonutrients are classified as carotenoids, phenolics, alkaloids, N-containing compounds, and organosulfur compounds. Though the evidence for physiological effect is more conclusive regarding nutrients, phytonutrients are newer to the realm of nutritional sciences and there is still much to be discovered. This does not, however, mean that they are necessarily any less important to human health. Carotenoids are among the most studied phytonutrients, because they are nature's most widespread pigments and because of their provitamin and antioxidant roles.

Carotenoids, widely distributed in nature, are fat-soluble colored pigments ranging from light yellow through orange to deep red. The different colors produced by these pigments are found in every form of life. They are biosynthesized by all photosynthetic bacteria, cyanobacteria, algae, higher plants and by some non-photosynthetic bacteria, fungi, and yeasts (1). Animals, however, are unable to synthesize carotenoids de nova. The diet is their only source of these compounds.

Over 600 different carotenoids have been isolated and characterized, a number that is increasing still, occurring in plants, microorganisms, and animals. In addition to their role in aesthetics, carotenoids, or foods rich in these phytonutrients, are considered to be potentially beneficial in the prevention of a variety of major diseases including cardiovascular disease, certain cancers and eye diseases (2). These effects are attributed to a small portion of the hundreds of carotenoids in nature, given that so few are found in human blood and tissue, and only two in the retina and lens of the eye (2). As many as 50 of these are present in the human diet and can be absorbed and metabolized by the human body. However, only six carotenoids represent more than 95% of the total blood carotenoids in humans: β-carotene, β-cryptoxanthin, α-carotene, lycopene, lutein and zeaxanthin (3). These six, all important in human nutrition due to their biological activities, are the most widely studies of the carotenoids.

Carotenoid Structure

Carotenoids are a class of hydrocarbons with a skeleton of eight isoprene units (Figure 1). These isoprene units are joined in a head-to-tail pattern, except at the center, which gives symmetry to the molecule. Thus, the two central methyl groups are in a 1,6-positional relationship and the remaining non-terminal methyl groups are in a 1,5-positional relationship (1). The majority of carotenoids have a 40-carbon polyene chain, considered the "backbone" of the molecule. Some carotenoids have a structure consisting of fewer than 40 carbon atoms. These compounds are referred to as apocarotenoids when carbon atoms have been lost from the ends of the molecule or as norcarotenoids when carbon atoms have been lost formally from within the chain (4). Every carotenoid chain is

terminated with cyclic or acyclic end groups and may or may not contain an oxygen-containing functional group. There are seven different possible end groups.

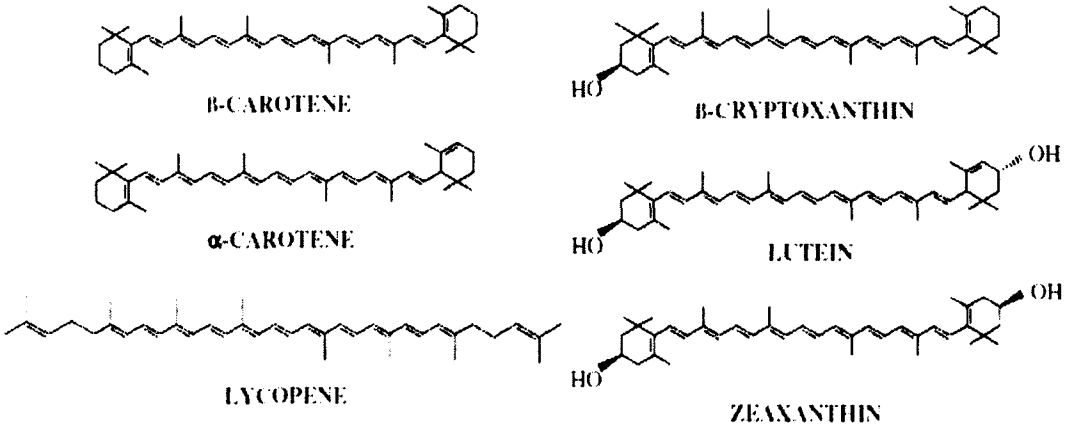


Figure 1. The structures of the predominant carotenoids found in human plasma (2)

Based on their chemical structure, carotenoids are classified under two groups: carotenes (hydrocarbons) and xanthophylls (oxygenated derivatives of the hydrocarbons) (1). Included in the carotene group are β-carotene, α-carotene, and lycopene, and among the xanthophylls are lutein, zeaxanthin, and β-cryptoxanthin. Carotenoids can also be divided into provitamin A and non-provitamin A compounds based on their structure, particularly the presence of a beta-ionone moiety, which is necessary for conversion to retinol. Those with provitamin A activity include β-carotene, α-carotene, and β-cryptoxanthin.

The characteristic feature of the carotenoid structure is the long system of alternating double and single bonds in the polyene backbone, forming the central part of the molecule. This constitutes a conjugated system in which the π-electrons are effectively delocalization over the entire length of the polyene chain. It is this feature that gives the carotenoids as a group their distinctive molecular shape, chemical reactivity, and light-absorbing properties, and may account for their antioxidant properties in vivo (1, 4, 5).

The overall molecular geometry (size, shape, presence of functional groups) is vital for ensuring that the carotenoid fits into cellular and subcellular structures in the correct location and orientation, allowing it to function efficiently. Specific interactions with other molecules in the immediate vicinity are also crucial for correct functioning (4).

Table 1. Classification of carotenoids[1]

Basis	Sub-group	Characteristics	Examples
Structure	Carotenes	Constituting carbon and hydrogen	α-carotene, β-carotene, β-cryptoxanthin
	Xanthophylls	Constituting carbon, hydrogen, and oxygen	Lutein, zeaxanthin, violaxanthin, fucoxanthin
Cyclization	Acyclic	End group not closed	Lycopene
	Alicyclic		
	Monocyclic	One end group open, one closed	γ-carotene
	Bicyclic	Both closed	β-carotene
Structural alteration	Allenic	Continuous double bond	Neoxanthin
	Acetylenic	Presence of a triple bond	Dehydro apocarotenoid
	Apocarotenoid	Less than 40 carbon atoms	Bixin
	Higher carotenoid	More than 40 carbon atoms	Crocetin
Function	Primary	Required for photosynthetic process	β-carotene, lutein, zeaxanthin, violaxanthin, antheraxanthin, neoxanthin
	Secondary	Presence not directly related to plant survival	α-carotene, capsanthin, bixin, lycopene, astaxanthin

[1]Adapted from *Chemistry and Biotechnology of Carotenoids* (1)

As a group, carotenoids are hydrophobic molecules with little to no solubility in water. They are thus generally restricted to hydrophobic areas within cells, such as the inner core of membranes, except when they are associated with proteins that allow them access to an aqueous environment. The nature of the specific end groups in carotenoids also influences the polarity of carotenoids, affecting their interactions with biological membranes and other molecules (1, 4). Carotenes, for example, are more hydrophobic than xanthophylls, which have polar end groups that allow them to interact with molecules in the aqueous phase more easily.

Because of isomerism around carbon double bonds, carotenoids can have different configurations, which are distinctly different molecular structures that can be isolated as separate compounds. Each double bond in the polyene chain of a carotenoid can exist in two configurations, designated *cis* or *trans*. There can also be rotation about any of the carbon single bonds. Thus, in principle, a carotenoid with a defined *cis/trans* configuration can adopt a very large number of geometrical isomers, resulting in many theoretical shapes. However, only a few are really encountered in nature because carotenoids typically exist in a particular preferred, low-energy conformation. The most stable form of the conjugated polyene chain is a linear, extended conformation, first because the conjugated system is greatly stabilized when the double bonds are coplanar, and second because the presence of a *cis* double bond creates greater steric hindrance between nearby hydrogen atoms and/or methyl groups (4, 5). Carotenoids in the all-*trans* configuration have an extended conjugated double-bond system and are linear, rigid molecules. The *cis*-isomers are no longer simple linear molecules, and as their overall shape differs substantially from that of the all-*trans* form, their ability to fit into subcellular structures may be greatly altered. The *cis*-isomers are much less likely to aggregate and are, therefore, usually more readily solubilized, absorbed, and transported than the all-*trans*-isomers. However, because the *cis*-isomers are generally less stable thermodynamically than the *trans* form, most carotenoids occur in nature predominantly or entirely in the all-*trans* form (4, 5).

The shape and size of the end groups are also important factors in their function. Acyclic carotenoids such as lycopene are long, linear molecules with flexible end groups.

Cyclization shortens the overall length of the molecules and increases the effective bulk of the end groups and the space they occupy (4).

Though traditionally thought of as plant pigments, carotenoids have a wide distribution and occur extensively also in animals and microorganisms. Their striking colors are familiar, but their less obvious roles make them essential components in oxygenic photosynthetic organisms. Without them, photosynthesis and life in an oxygen atmosphere would be impossible. The natural functions and actions of carotenoids are determined by their physical and chemical properties, depending on their molecular structure. The following sections will discuss the various functions of carotenoids, including potential biological actions in humans, and well established biological actions in plants and humans.

Carotenoid Function in Plants

Carotenoids are vital constituents of the photosynthetic apparatus of plants and other organisms. They have unique photosynthetic properties, and understanding these properties and how photosynthetic organisms use them can help increase understanding of some of the beneficial health effects of carotenoids. The major function of carotenoids in plants is in photosynthesis, where they play a crucial role in the photosystem assembly, are involved in light harvesting, and provide protection from excess light.

Light harvesting

The photosynthetic unit is divided into two parts, the light harvesting antenna and the reaction center. The conversion of light energy into chemical potential takes place in the

photosynthetic reaction centers. However, the reaction center pigments are responsible for the absorption of only a tiny fraction of the light. More than 99 percent is supplied to the reaction center by singlet-singlet energy transfer from the antenna systems (6). Antenna pigments vary according to organisms and the conditions in which they live, but in many the carotenoid polyenes are responsible for a large fraction of the light absorbed and used.

In carotenoids, the transition is a $\pi \rightarrow \pi^*$ transition, in which one of the bonding π-electrons of the conjugated double-bond system is promoted to a previously unoccupied π^* antibonding orbital. The π-electrons are highly delocalized and the excited state is of comparatively low energy, so the energy required to bring about the transition is relatively small, corresponding to light in the visible region in the wavelength range of ~ 400-550 nm (4). This gives rise to the intense colors of carotenoids, and enables them to absorb light in the gap of chlorophyll.

$Car \rightarrow {}^1Car$

${}^1Car + Chl \rightarrow Car + {}^1Chl$

The absorption of light energy by an organic molecule results in a higher-energy excited state of that molecule. In carotenoids, there are two low-lying excited singlet states, S_2 and S_1. The characteristic strong absorption of light in the visible region is attributed to a strongly allowed transition from the ground state S_0 to the second singlet excited state, S_2. The energy levels of this excited state, and of the somewhat lower first excited state S_1 that can be formed by internal conversion from S_2 to S_1, are slightly above those of chlorophyll. Thus, singlet-singlet energy transfer can take place from excited carotenoid

to generate the excited singlet state of chlorophyll, which then relay the excitation energy to the reaction center and are active in photosynthesis (4, 7).

In summary, carotenoids are vital to the light-harvesting complexes in photosynthesis because they are able to absorb light energy rapidly and efficiently transfer that energy to the chlorophylls, allowing photosynthesis to harvest energy over a wider range of wavelengths than would be possible with chlorophyll alone (7).

Photoprotection

Not only do carotenoid pigments act as energy donors in light-harvesting as described above, they also play an important role in photoprotection of plant tissues. Within the light-harvesting system (antenna system) of plants, they are able to prevent photooxidative damage. Carotenoids protect against this by dissipating excess energy via quenching of the triplet state chlorophyll, as well as the singlet state oxygen that is produced as a result of the triplet state chlorophyll (1, 5, 8). This role is also associated with their role as antioxidants in human health.

Oxygen is relatively stable in its ground state, a triplet state ($^3O_2$) with two unpaired electrons possessing the same spin quantum number and located in different antibonding (π) orbitals. However, electron reactions that occur in the presence of oxygen can result in the production of reactive intermediates, which can be very damaging in the cell (9). Singlet oxygen ($^1O_2$) can also be generated by an input of energy, especially under certain conditions like exposure to high light intensities or drought. With the spin restriction removed, the oxidizing ability of the oxygen is greatly increased (9). Singlet oxygen is produced by light absorption by photosensitizers and, in plants, particularly by the

chlorophylls and their precursors (9). Singlet oxygen is a highly reactive species which can react with and destroy lipid bilayer membranes in cells, as well as other vital components of an organism. Thus, singlet oxygen formation in photosynthesis is potentially very injurious (6).

Chlorophyll is the main light-absorbing pigment in the light-harvesting complex, the inner antenna, and in the reaction centers. It is very efficient in absorbing light. However, if the energy is not efficiently used, the spins of the electrons in the excited state can rephase and give rise to a lower energy state: the chlorophyll triplet state. With an even longer lifetime, chlorophyll in the triplet state can react with $^3O_2$ to produce the very reactive $^1O_2$ if no efficient quenchers are around (9).

$^3Chl + {}^3O2 \rightarrow Chl + {}^1O2$

While chlorophyll is absolutely necessary in photosynthesis, it also carries this potential danger of being a singlet oxygen producer (photosensitizer). Fortunately, carotenoids function in two ways to help prevent oxidative damage in photosynthetic organisms. They can either act to quench chlorophyll triplet states at a rate which prevents singlet oxygen formation via a triplet-triplet energy transfer process (A), as long the carotenoids are in close proximity, or they can quench singlet oxygen itself through energy transfer or chemical reaction (B).

(A) $^3Chl + Car \rightarrow Chl + {}^3Car$

(B) $^1O_2 + Car \rightarrow {}^3O_2 + {}^3Car$

The triplet state of carotenoids that is formed from either reaction can then dissipate the excess energy directly as heat, returning harmlessly to the ground state (6, 9).

These important roles of carotenoids in photosynthesis are made possible by the fact that many naturally occurring carotenoids have excited singlet states higher in energy than excited states of chlorophyll, and triplet states lower in energy than excited states of molecular oxygen. This allows light harvesting and photoprotection to be thermodynamically favorable (10).

Carotenoid Function in Humans

Absorption and transport

There are several steps involved in the absorption of dietary carotenoids: 1) the food matrix is mechanically and enzymatically disrupted to release the carotenoids which are then incorporated into lipid droplets of the gastric emulsions, 2) carotenoids are transferred from the lipid droplets to mixed micelles produced by the action of bile salts, biliary phospholipids, dietary lipids, and their hydrolysis products in the small intestine, 3) carotenoids are taken up by intestinal mucosal cells and packed into chylomicrons, and 4) carotenoids, or their metabolic products, are transported to the lymphatic and/or portal circulation. Each one of these steps can be influenced by various and multiple factors, however, which makes assessing the effects of each factor on overall carotenoid bioavailability difficult (11).

Bioavailability is defined as the fraction of an ingested nutrient that becomes available to the body for utilization in function and/or storage. The factors that can affect

bioavailability include, species of carotenoid, molecular linkage, amount consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors, and interactions (11). Food processing, including heat, mechanical and enzymatic treatments, help to disrupt the food matrix in which the carotenoids are stored, which promotes their release and dispersion during digestion. Carotenoids are also lipid soluble, and so are better absorbed in the intestines when ingested with dietary lipids (1).

Provitamin A Activity

An excellent source of vitamin A is its dietary precursor carotenoids, which, depending on their structure, can be converted endogenously to retinol. Carotenoids are hypothesized to exert a variety of positive health effects which will be discussed, but so far, provitamin A activity is the best-established, and only clearly proven function of carotenoids (1, 12).

Vitamin A includes the compounds retinal, retinol and its esters. Provitamin A refers to the carotenoids that are able to be converted to retinol. Around 50 carotenoids possess at least one β-ionone end group, which makes them capable of yielding vitamin A (13). Of the carotenoids present in the human diet and detectable in human plasma, the carotenes β-carotene, α-carotene, and the xanthophyll β-cryptoxanthin have provitamin A activity. (1, 12) However, all-*trans*-β-carotene is the most suitable and important precursor for vitamin A. It is unique, compared to other carotenoids, because it has a β-ionone structure as the terminal ring system on each end of the polyene chain. Because of its symmetrical

chemical structure, it is the only carotene capable of theoretically yielding two molecules of all-*trans*-retinal (13).

There are two major oxidative pathways for the conversion of carotenoids to vitamin A in mammals (14). The first is through cleavage at the central 15,15' double bond via β-carotene 15,15'-dioxygenase and monooxygenase (BCMO1) to yield two molecules of all-*trans*-retinal (central cleavage), and the other is through a stepwise cleavage via β-carotene-9,10'-oxygenase to yield a sequential set of β-apocarotenals that ultimately convert to one molecule of all-*trans*-retinal and other smaller fragments (excentric cleavage) (12, 15, 16)

All-*trans*-β-carotene can follow either pathway, but it yields two molecules of the all-*trans*-retinal through the first, upon oxidative cleavage of the central 15,15' carbon-carbon bond, catalyzed by an intestinal β-carotene 15,15'-dioxygenase and monooxygenase (BCMO1) (1). This is the first step in the pathway for the biosynthesis of vitamin A (all-*trans*-retinol and retinyl esters) (13) as illustrated in Figure 2.

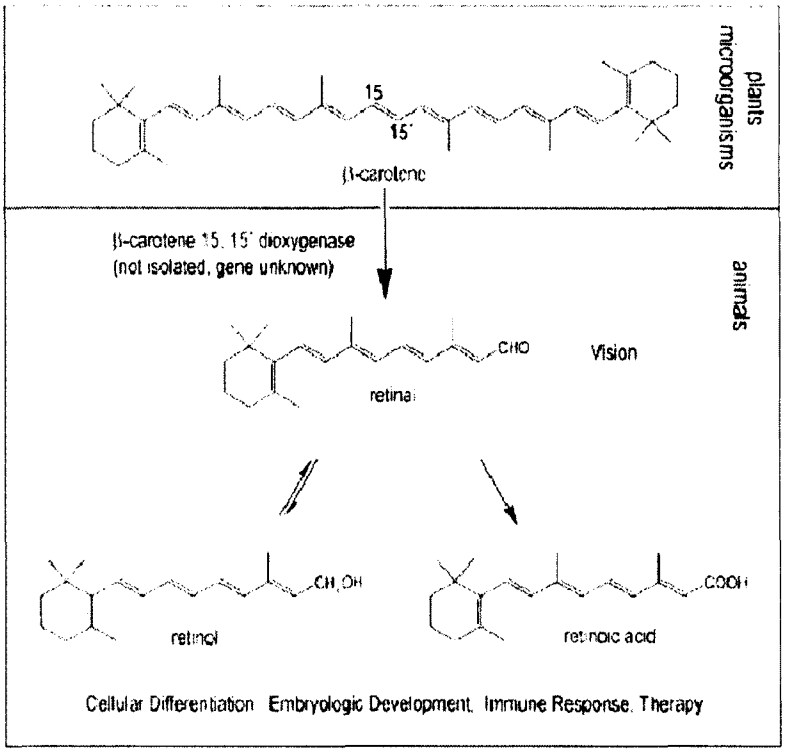


Figure 2. Diagram of the main steps in retinoid formation (17)

After consumption of carotenoids with provitamin A activity, both intact carotenoids and their metabolite retinol are found in circulation. In humans, conversion of β-carotene into vitamin A takes place predominantly in the small intestine (18). However, it has been recently demonstrated that the responsible β-carotene-cleaving enzyme BCMO1 is also present in tissues other than intestine, including the liver and the retinal pigment epithelial cells (13). Although clearly not all provitamin A dietary carotenoids are ultimately converted to vitamin A, they are an important source of this vital nutrient.

Role of vitamin A in human health

Vitamin A is of crucial importance for many physiological processes. Research suggests that it plays an essential role in the promotion of general growth and development, in cell

differentiation and maintenance of various epithelia, in immune function, in embryonic development, reproduction and postnatal growth (13, 18, 19). It may also play a role in processes such as glycoprotein synthesis, carcinogenesis, and growth hormone production (12). In addition to these, one of the most researched and most predominant roles of vitamin A in human health is in vision. Scientist began to see the ocular manifestations of a vitamin A deficiency soon after the vitamin was discovered in the early part of the 20th century. The vigorous scientific investigations that ensued provided an explanation for vitamin A's role in the visual process. This was the first clear demonstration of a molecular role for a vitamin. Those early studies also clearly associated vitamin A with maintaining the integrity of the body's epithelial tissues and immune system, as noted (20).

Negative impact of vitamin A deficiency

The prominent signs of the vitamin A deficiency are night blindness and xerophthalmia, which consists of progressively more severe changes in the conjunctiva and cornea of the eye (21). Other signs include loss of appetite, hyperkeratosis, increased susceptibility to infections, metaplasia and keratinization of the epithelial cells of the respiratory tract and other organs, and an abnormal perception of color. An acute vitamin A deficiency can result in blindness, although this is extremely rare in the United States.

Vitamin A deficiency also results in histopathological changes in respiratory, gut, and other epithelial surfaces, as well as in the immune system (20). Since vitamin A regulates the expression of many genes, and thus plays a major role in controlling cellular differentiation and maturation in rapidly generated and differentiating tissues, a

deficiency compromises the physical and biological integrity of epithelial tissue. As epithelial tissue is the first barrier to infection, the immune system is negatively impacted (20). Animal studies have also shown that maternal deficiency in pregnancy leads to placental dysfunction, fetal loss, and congenital malformations (20, 22, 23).

Current vitamin A intake

According to the Food and Nutrition Board, National Institute of Medicine, in the *Dietary Reference Intakes: Recommended Intakes for Individuals*, children 1-3 years should be consuming 300 μg/d of vitamin A, children 4-8 years should be consuming 400 μg/d, males and females 9-13 years 600 μg/d, males > 13 years 900 μg/d, and females > 13 years 700 μg/d (24). According to the National Health and Nutrition Examination Survey (2007-2008) however, the mean amount of retinol consumed by males and females 2 years and over is 440 μg/d (25). This does not account for the vitamin A activity of carotenoids consumed. In order to understand how that contributes to the vitamin A intake of the US population, it is important to understand the carotenoid conversion factors.

The ratio of the amount of provitamin A carotenoid given in an oral dose to the amount of vitamin A derived from this dose is defined as the carotenoid-to-vitamin-A conversion factor or carotenoid equivalent to vitamin A (18). Because of the provitamin activity of carotenoids, retinol intake is often reported as retinol activity equivalent (RAE), which takes intake of both carotenoids and retinol into account. The recently accepted conversion factor for β-carotene is 12, and for β-cryptoxanthin and α-carotene it is 24. This means that 12 micrograms of β-carotene and 24 micrograms of β-cryptoxanthin or

α-carotene, respectively, theoretically exert the activity of 1 microgram of vitamin A (12). The estimated efficiency factor for conversion of dietary β-carotene to vitamin A was previously 6:1, rather than this newer value of 12:1, suggesting that the bioconversion of β-carotene to vitamin A may not be as efficient as previously hypothesized (18). There may therefore be an even greater need for increased consumption of provitamin A carotenoids. With this in mind, NHANES 2007-2008 also reported that the average amount of RAE consumed by males and females ≥ 2 years still does not meet the recommendation.

An added benefit of consuming these carotenoids is that the conversion of β-carotene to retinol is regulated through feedback mechanisms, meaning that only the required amount is metabolized to retinol, and so the toxicity of pharmacological doses of vitamin A is not a concern with the consumption of higher levels of dietary β-carotene (13).

Given that the current intake of vitamin A, or REA, is below the recommended daily allowance, increasing consumption of carotenoids with provitamin A activity could help increase vitamin A status. Although provitamin A carotenoids are primarily found in foods such as fruits and vegetables, consumption of animal products is relatively high in the US. Increasing the carotenoid content of those animal products, such as milk and cheese, would only serve to increase vitamin A status by way of foods already being consumed.

Role of Carotenoids in Disease Prevention

In addition to provitamin A activity, carotenoids are hypothesized to have further biological activity that may contribute to beneficial health effects. Epidemiological evidence has shown a positive link between higher dietary intake and tissue concentrations of carotenoids and lower risk of chronic disease (26). Beta-carotene and lycopene specifically have been shown to be inversely related to risk of certain cancers and cardiovascular disease, whereas lutein and zeaxanthin may play more of a role in disorders of the eye (26). It has been suggested that the antioxidant properties of carotenoids are the primary mechanism by which these beneficial effects occur. More recently, research is also showing that their effects may be mediated through other mechanisms such as gap junction communication, cell growth and regulation, modulating gene expression, and immune response (26). Carotenoids such as α- and β-carotene, as well as β-cryptoxanthin can also be converted to vitamin A and thus contribute to its related roles in development and disease prevention. The following section will discuss the evidence thus far on the relation of carotenoids to cancer, cardiovascular disease, and disorders of the eye, as well as the possible biological mechanisms by which these carotenoids produce these positive health effects.

Cancer

The role of carotenoids in cancer prevention has been extensively reviewed (26-37). Observational epidemiological research has shown that a high intake of fruit and vegetables rich in carotenoids, primarily β-carotene and lycopene, or high blood concentrations of these carotenoids, are associated with a reduced risk of cancer at

several common sites (27). Although epidemiological studies cannot prove causal relations, they are still very useful in pointing to potential protective effects of foods, or food components like carotenoids, in disease prevention.

While association seems inconsistent for breast and prostate cancer, it appears to be consistent for lung and stomach cancer, across various research designs and statistical analyses between the studies (28, 29), strongly suggesting an effect on cancer prevention. Although the research primarily looks at fruit and vegetable intake, it has been suggested by some studies that the carotenoids are the chemoprotective agents providing the beneficial effects.

Over the last 30 years, evidence has accumulated in favor of cancer-preventing effects of β-carotene in particular. Overwhelming observational evidence has supported the association between consumption of carotenoid-rich foods and lower cancer risk. As reviewed by Hercberg (2005) over 125 case-control or cohort studies relevant to the association between β-carotene – either dietary intake or tissue concentrations – and cancer have been conducted, with various measures in diverse populations. Associations have been seen in men and women, various racial groups, and current smokers, former smokers, and nonsmokers (36). Epidemiologically, the evidence is quite strong. Several examples of the most recent studies are described below.

In a cohort of 27,084 male smokers living in southwestern Finland and ages 50-69 years, consumption of fruits and vegetables was associated with lower lung cancer risk during a 14 year follow-up period (38). Lower risks of lung cancer were observed for the highest

versus the lowest quintiles of lycopene, lutein + zeaxanthin, β-cryptoxanthin, total carotenoids, serum β-carotene, and serum retinol.

Michaud et al., (2000) examined the relation between lung cancer risk and intakes of α-carotene, β-carotene, lutein, lycopene, and β-cryptoxanthin in 2 large US cohorts (39). The first included 46,924 men with a 10 year follow-up period. The second included 77,283 women with a 12 year follow-up period. Results showed that α-carotene and lycopene intakes were significantly associated with a lower risk of lung cancer. An inverse, though not statistically significant, association with β-carotene, lutein, and β-cryptoxanthin intakes and lung cancer risk was also found. Lung cancer risk was significantly lower in subjects who consumed a diet high in a variety of carotenoids.

In the Netherlands Cohort Study on Diet and Cancer, carotenoid intake of 58,279 men, ages 55-69 years, were measured at baseline. With a 6.2 year follow-up period, protective effects on lung cancer incidence were found for intake of lutein + zeaxanthin, and β-cryptoxanthin (40).

Data from the Health Professionals Follow-Up Study, with a 12 year follow-up period and 47,356 participants, showed that frequent tomato or lycopene intake was associated with a reduced risk of prostate cancer. The associations persisted even after controlling for fruit and vegetable consumption (41).

Recently, serum concentrations of α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein + zeaxanthin, retinol, α-tocopherol and γ-tocopherol were measured in a sample of women in the Women's Health Initiative clinical trials at baseline and at years 1, 3, and 6 and in a sample of women in the observational study at baseline and at year 3 (42). Risk

of invasive breast cancer was inversely associated with baseline serum α-carotene concentrations. Analysis of repeated measurements also indicated that α-carotene and β-carotene were inversely associated with breast cancer.

With so much evidence in the observational research, several large intervention trials have been conducted with the hopes of confirming the role of carotenoids in cancer prevention. Two randomized, double-blind, placebo-controlled trials showed adverse effects of β-carotene supplementation. The Beta-Carotene and Retinol Efficiency Trial (CARET) tested the combination of 30 mg of β-carotene ad 25,000 IU of retinol taken daily against a placebo in 18,314 men and women at high risk of developing lung cancer (43). CARET participants receiving the treatment had no chemoprotective benefit and had an adverse effect on the incidence of lung cancer and mortality. Around the same time, results from a study conducted in Finland on 29,133 male smokers, ages 50-69 years, showed a higher incidence of lung cancer and mortality among the men who received β-carotene (20 mg/day) than among those who did not (44). Beta-carotene had little or no effect on the incidence of cancer other than lung cancer, however.

Conversely, results of a prospective, randomized trial conducted in Linxian County, China on 29,584 adults, ages 40-69 years, over a 6 year follow-up period showed a significantly lower mortality among those who received supplementation with β-carotene, vitamin E and selenium (45), mainly because of lower cancer rates.

It has been suggested that the seeming contradiction between the observational studies and these randomized trials can be explained by the fact that the doses used in the clinical trials were much higher than the highest levels found in ordinary dietary intake, levels

that have been associated with the decreased risk of cancer. The positive effects seen in the study conducted in Linxian County, China may be a result of the fact that the population has a much lower baseline micronutrient status because of poor life conditions (46). Three other randomized, double-blind, placebo-controlled trials showed no differences in incidence of cancer or total mortality among those supplemented with β-carotene, at similar levels as the previously mentioned trials, and the placebo (46-48).

There are several ways in which the anticancer effects of carotenoids may be explained (2). Their antioxidant function could prevent free radical-induced damage to cellular DNA and other molecules. This mechanism is well researched, and most likely related to the positive effects of carotenoids in reduced cancer risk.

Carotenoids are considered to be the most potent singlet oxygen quenchers (1, 49, 50). They can react with any of the radical species that the biological system is likely to encounter including hydrogen peroxide, singlet oxygen, nitrogen oxides, super oxide anion, among others. They are also effective deactivators of electronically excited sensitizer molecules which are involved in the generation of radicals and singlet oxygen (49). Antioxidant activity may increase as the number of conjugated double bonds increases in the carotenoid molecule. Lycopene, with eleven conjugated and two non-conjugated double bonds, is the most efficient of the natural carotenoids. Beta-carotene also acts as a chain-breaking antioxidant to terminate lipid oxidation, and β-carotene and lutein have been shown to decrease the cellular release of lactate dehydrogenase to protect cells from lipid peroxidation and membrane damage (1).

In addition, the anticancer effects of carotenoids may be explained by the immunomodulatory effects on a number of transcription systems, which would enhance immune surveillance in tumorigenesis. Carotenoids and/or their metabolites impact cell signaling pathways, influence the expression of certain genes, and may inhibit certain regulatory enzymes (49). They also have been shown to enhance cell to cell communication, particularly gap junction communication (51). Gap junction communication is implicated in the regulation of cell growth, differentiation and apoptosis, and an alteration of any kind in the gap junction communication of cells can lead to unrestrained cell proliferation (49). The regulation of gap junction communication is complex and the mechanisms involving carotenoid activity are not fully understood. Research suggests, however, that carotenoids can help in upregulation of gap junctions by increasing the expression of connexin-43, a protein subunit present in cell membranes, thus enhancing cell to cell communication (52). Beta-carotene is one of the most effective stimulators of gap junction communication.

Cardiovascular disease

Though the research is still not entirely conclusive, many epidemiologic studies have shown an association between increased consumption of carotenoids and reduced risk of cardiovascular disease. A review conducted in 2006 on carotenoids and cardiovascular health looked at cross-sectional, case control, and prospective studies to date that showed a relationship between various carotenoids and cardiovascular disease (53). In several of the studies related to dietary intake, higher intakes of α- and β-carotene, lutein and zeaxanthin were inversely associated with risk of cardiovascular disease, but the most significant results were with β-carotene (54-56). A cross-sectional study examining

serum levels of α- and β-carotene and lycopene also showed that high levels of these carotenoids may reduce risk of cardiovascular disease incidence and mortality (57).

Until the past decade, research on the subject was primarily observational and did show a correlation but not a casual relationship (54). More recently some experimental studies have been conducted, but the topic is still complex and in need of further research. Several of the trials showed no effect of the supplementation on cardiovascular disease risk (46-48), and one showed that supplemental β-carotene was actually associated with an increased risk of certain types of cardiovascular disease (43), As with the evidence showing an increased risk of lung cancer with carotenoid supplementation, these results indicate that the benefits of carotenoids may disappear at higher doses and when not consumed in food. Overall, the evidence seems to point towards a reduction in the risk for developing cardiovascular disease when the diet is high in carotenoids.

The beneficial effects of carotenoids in relation to cardiovascular disease may be as a result of their antioxidant mechanisms as well as a feedback mechanism that inhibits HMG-CoA reductase, a rate-controlling enzyme that produces cholesterol (1, 2). One contributor to the development of cardiovascular disease is the oxidation of low-density lipoproteins (LDL). Oxidized LDL is readily taken up by foam cells in the vascular endothelium where it contributes to the development of atherosclerotic lesions. As LDL is a major transporter of β-carotene and lycopene through circulation, they have the capacity to trap peroxyl radicals and quench singlet oxygen within LDL. Research has also shown that a 10 μM concentration of either β-carotene or lycopene inhibits cholesterol synthesis in macrophage cell lines (1).

Eye health

Research suggests that lutein and zeaxanthin may reduce the risk of developing cataract, a clouding of the lens in the eye, and age-related macular degeneration (AMD), a degeneration of the retina and the retinal pigment epithelium in the macular region (58, 59). These are the two most common eye diseases in older people, and for many in the US, treatment is not available. Increasing dietary carotenoids to help lower risk of developing these conditions would thus be beneficial. There is also a possibility that these carotenoids may slow progression once these conditions are present (60). Many researchers have examined the effect of lutein and zeaxanthin intake– both in whole food and supplemental form – as well as concentrations in the body and relationship to AMD.

Lutein and zeaxanthin supplementation increases plasma concentrations of these carotenoids (61-63), and has been shown to contribute to macular pigment status (64), the level of these carotenoids in the macula and lens of the eye. It has also been shown to lower the odds for pigmentary abnormalities, a sign of early age-related maculopathy (65). Significant associations have been found between lutein and zeaxanthin concentrations in ocular tissues, serum, and plasma, and a possible reduced risk of AMD (65-67).

The evidence for a protective effect of lutein and zeaxanthin on eye disease is strongest in clinical studies, although epidemiologic data also supports this role (68). Dietary intake of these xanthophylls has recently been associated with lower prevalence of neovascular AMD and reduced long-term incidences of AMD when compared to individuals consuming low levels (68-70). Several prospective studies examining the association

between carotenoids intakes and cataracts in both men and women have shown that those with the highest intake of lutein and zeaxanthin had a decreased risk of cataracts compared with those in the lowest quintile (71-73). In a study examining the relation between plasma concentrations of lutein and zeaxanthin and age-related macular degeneration in elderly men and women, risk of age-related macular degeneration was significantly higher in people with the lower plasma concentrations of zeaxanthin (74).

Lutein and zeaxanthin are referred to as macular pigment (MP) as they are the only carotenoids present within the entire retina and macula of the human eye. These carotenoids may help improve vision throughout life by directly affecting the optics on the eye (75). They protect the eye from photo-oxidative damage and lipid peroxidation by acting as antioxidants, and shield the eye from potentially harmful short-wave radiation by acting as blue light filters (67). Because these carotenoids absorb blue light, they may reduce photooxidative damage that would otherwise occur in the retina when exposed to light in those wavelengths. As cataracts and macular degeneration can be caused by blue-light mediated free radical damage to the retina, these mechanisms are most likely responsible for the beneficial role of these carotenoids in improved visual function. In addition to reducing the effects of light-induced oxidative damage, it appears that lutein and zeaxanthin also protect the retina by reducing inflammatory response (67, 68).

Although the evidence thus far is predominantly epidemiological, the research suggests that diets high in carotenoids may result in a decreased risk for several of the most common chronic diseases in the US. It is therefore valuable to investigate ways in which the carotenoid content in foods such as milk and milk products might be increased, with the possibility that dietary carotenoids could increase as a result.

CHAPTER II

MILK

Milk Consumption in the US

Cow's milk and milk products contain many nutrients beneficial to human health, including protein, calcium, potassium, and magnesium. Due to fortification, milk is also a good source of vitamins D and A. Milk is one of the primary contributors to the calcium and Vitamin D intake by Americans. This is beneficial because evidence shows that intake of milk and milk products is linked to improved bone health, especially in children and adolescents. Evidence also indicates that intake of milk and milk products is associated with a reduced risk of cardiovascular disease and type 2 diabetes and with lower blood pressure in adults (76).

Recently, however, factors such as high cost and an increase in soda consumption have led to a decrease in milk consumption in the US (77). According to the *Dietary Guidelines for Americans 2010*, the recommendation for milk and milk products is 3 cups/day for adolescents and adults ages 9 years or older, 2 ½ cups/day for children ages 4 to 8 years, and 2 cups for children ages 2-3 years (76). The current intake of milk and milk products is less than the recommended amounts for most adults, children and adolescents ages 4 to 18 years, and many children ages 2 to 3 years (76). The National Health and Nutrition Examination Survey (2005-2006) reported that the average intake of milk was ~3/4 cup for individuals 2 years of age and older (78). Consequently, dietary

intakes of several nutrients – calcium, vitamin D, magnesium and potassium – are low enough to be of public health concern for both adults and children. The US Department of Agriculture and US Department of Health and Human Services suggest that Americans choose foods that provide more of these nutrients, including increasing intake of milk and milk products (76).

One important way that increased milk consumption can be promoted is by increasing the amount and concentration of beneficial nutrients in the milk. Because interest in, and desire for, foods with health promoting properties is increasing, potential opportunities to increase these beneficial components in milk, including carotenoids such as α- and β-carotene, β-cryptoxanthin, lutein and zeaxanthin, as well as vitamins A and E, should be thoroughly examined.

Carotenoids cannot be synthesized in animals and must therefore be consumed in the diet (79, 80). Beta-carotene is the most abundant carotenoid in cow's milk. However, trace amounts of lutein and other carotenoids have been identified, as well as α-tocopherol and retinol. Recently, research has shown these nutrients to play a beneficial role in various health related areas such as cancer, cardiovascular disease, glucose metabolism, macular degeneration and cataract formation, and immune function (37, 53, 66, 81-83).

With the potential to increase the concentration of these beneficial components in milk, not only could milk consumption begin to increase in the US, but the milk already being consumed could be of even higher nutrient quality.

Milk Synthesis and Composition

Milk is a complex dispersion consisting of fat, protein, carbohydrates, mineral salts, and water (Table 2). It is unique in its composition, in that it is simultaneously a true solution, a colloidal dispersion, and a dilute emulsion. In a true solution, ions or tiny molecules are dissolved in a liquid. In milk, the lactose, minerals and whey proteins are dissolved in water. A colloidal dispersion is a two-phase system in which particles with a diameter between 1 and 1000 nanometers are evenly dispersed in a dispersion medium. In milk, the casein micelles are evenly dispersed in water. Lastly, an emulsion is a mixture of two or more liquids that are normally immiscible. Milk fat is excreted in an emulsified form and fluid milk is a dilute emulsion of fat globules in water. Milk is often homogenized to make this dispersion stable. Table 2 also shows the current standard values for components of interest in milk, including retinol and carotenoids, according to the USDA National Nutrient Database (84).

Table 2. Nutrient composition of whole milk[1]

Nutrient	Units	Value per 100.0 g
Proximates		
Water	g	87.91
Protein	g	3.21
Total lipid (fat)	g	3.31
Ash	g	0.68
Sugars, total	g	4.88
Components of interest		
Retinol	µg	46.00
β-carotene	µg	7.00
α-carotene	µg	0.00*
β-cryptoxanthin	µg	0.00*
Lutein + zeaxanthin	µg	0.00*
α-tocopherol	mg	0.07

[1]Adapted from USDA National Nutrient Database for Standard References, Release 24 (2011)

*Database shows values of 0.00; components are listed as a reference

Milk Production in the Ruminant

Daily milk production by a dairy animal is the results of a combination of six biological elements: the number of secretory cells produced up to that day of the lactation, the number of these cells that have died by apoptosis, and the rate of secretion per cell of each of the four major constituents (fat, protein, water and lactose) on that day (85).

Precursors for milk synthesis are derived from the bloodstream. The primary substrates extracted from the blood by the mammary gland during lactation include glucose, amino acids, fatty acids, and minerals, as well as any vitamins and carotenoids present in the milk. Because of bacterial fermentation of carbohydrates in ruminants, the volatile fatty acids acetate and β-hydroxybutyrate (BHBA) are also critical substrates.

Lactose is the most common carbohydrate found in milk. It is a disaccharide made up of one molecule of glucose and one molecule of galactose combined in a 1:4 carbon linkage as a β-galactoside. Since glucose can be converted to galactose within the mammary gland via the enzyme uridine diphosphoryl galactose-4-epimerase, glucose is the direct precursor for lactose and the formation of lactose has priority over glucose supply. In relation to milk production, water enters the mammary cells in an osmotic response to lactose and the soluble ions Na+ and K+ in cells. Thus, lactose production in the mammary gland is the major driver of milk production and total milk yield (85).

Milk proteins are synthesized from amino acids derived either from the bloodstream – all the essential amino acids and many nonessential – or from amino acids synthesized by the secretory cells of the mammary gland. Concentrations of amino acids available to the

mammary gland largely determine protein production. Availability of the necessary energy required to drive metabolic processes also contributes to protein production (85).

The mammary specific proteins include casein micelle subunits – α-casein, β-casein, κ-casein, and γ-casein – and the whey proteins. Casein proteins account for 80 percent of the milk specific proteins. They are hydrophobic, and under the usual ionic conditions in milk, most of the casein proteins are strongly associated with one another to form the familiar casein micelles. They are not only a source of amino acids but the colloidal structure of the micelle allows the transport of large amounts of calcium and phosphorus, in a stable form (86). The whey or milk serum proteins include β-lactoglobulin, α-lactalbumin, immunoglobulins, serum albumin, lactoferrin, and transferrin. Some of these are mammary specific proteins, but some (e.g., immunoglobulin and albumin) are derived from the blood. Beta-lactoglobulin is the major whey protein in the milk of cows, accounting for more than 50 percent of the whey protein (86).

Production of fat in milk is mainly a function of the concentrations of fatty acids available to the mammary gland, with some contribution from acetate and BHBA from the blood (85). Fatty acids for production of triglycerides in milk are derived from three sources, 1) the bloodstream (i.e., dietary lipids) via hydrolysis of chylomicra, 2) mobilization from body stores, and 3) de novo synthesis within the mammary cells from nonglucose sources (85). Of these major milk constituents, fat content is the most variable.

Carotenoids in Milk

Carotenoids in cow's milk consist primarily of all-*trans*-β-carotene and, to a lesser extent, lutein, zeaxanthin, and β-cryptoxanthin (87). As carotenoids cannot be synthesized in animals, the cows' diet is the primary source of these phytonutrients in the milk they produce.

The carotenoid concentration of the milk is determined in part by the nature and amount of dietary supply through forage intake as well as the ruminal digestive process. As the carotenoid levels in milk are relatively low, the efficiency of this transfer seems to be strongly limited. It is likely that the different steps of carotenoid transfer from diet to milk (i.e., rumen digestion, intestinal absorption and tissue metabolism) could influence the carotenoid availability to the mammary gland for secretion in milk (87).

The first event in the cow's digestive process of carotenoids is degradation of the vegetable matrix that releases carotenoids into the rumen liquid phase. Absorption of carotenoids (primarily β-carotene) in the small intestine is believed to be controlled by passive diffusion (88). Beta-carotene is predominantly transported by high density lipoproteins. Carotenoid availability for secretion in milk is governed by its transport into lymph and plasma, its metabolism within tissues – the carotenoids with provitamin A activity can either be converted to vitamin A (mainly in the liver, but also in the intestine and even in the mammary gland) or can remain as the carotenoid – and its storage in adipose tissues.

Data on carotenoid flux, transport and metabolism within and among tissues such as intestinal wall, liver, adipose tissues and mammary gland are currently insufficient to yet

fully understand regulation of milk carotenoid concentration and composition (87). The process of final transportation of the carotenoid from plasma lipoproteins into milk fat is also not yet understood.

Variation in Milk Composition

The basic driving forces for manipulating the composition of milk are improving the manufacturing and processing of milk and dairy products, altering the nutritional value of milk to conform to dietary guidelines set forth by the governmental agencies, and using milk as a delivery system for nutraceuticals with known benefits to human health (89). With the publication of the first *Dietary Guidelines for Americans* in 1980 which emphasized reduction in total fat, saturated fat, and cholesterol, and identified animal products such as milk as the main source of these components, interest in manipulating milk composition dramatically increased. In addition to altering the fatty acid composition in milk to a more favorable profile for human health, other beneficial nutrients and phytonutrients can also be increase in milk to make it more healthful to the consumer.

Research has shown that many factors have the potential to impact the composition of milk. Broadly, these fall under cow nutrition and management, cow genetics, and/or dairy manufacturing technologies (90). More specifically they include individual variability and heritability, breed, stage of lactation and parity, season, geographic location, and especially feeding practices (91-94). Of the main milk components – lactose, protein, fat – fat is the most variable, and with it many of the fat soluble components like vitamins A and E and carotenoids.

Individual variability and heritability

Plasma and milk concentrations of carotenoids are highly variable among individual cows, as is retinol, but to a lesser degree (87, 88, 95, 96). The variability is due to the genetics of individual cows, their health status, rearing history, individual management, etc. It can also reflect differences in the number of active secretory cells, apoptosis rates, and secretion rate of individual cows for each milk component (85). Pollett et al., (2004) showed that variation associated with individual cows contributed the most to the variations of milk yield, fat, protein, lactose, water and total solids. Jensen et al., (1999) showed that in Holstein dairy cows with similar milk yield and milk fat concentration, retinol and β-carotene concentrations in both plasma and milk varied (88). Morris et al., (2002) concluded that "both genetic and phenotypic correlations between plasma and milk fat carotenoid concentrations are high, indicating that selecting bulls in terms of plasma carotenoids could control carotenoid concentration in the milk of their daughters" (95). Information on differences between individual cows and their capacity to transfer these vitamins from feed to blood and then into milk is very limited (88).

Breed

Milk carotenoid variability between breeds has been seen since the early 20th century, when Baumann et al., (1934) examined the β-carotene and retinol content of butter fat from milk produced by five different breeds of cow (Ayrshire, Guernsey, Holstein, Jersey, Brown Swiss) (97). The results of his research showed that the carotenoid content was highest in Guernsey butter and lowest in Holstein and Ayrshire butter, while Brown Swiss and Jersey butters were intermediary. The vitamin A, on the other hand, was

highest in Holstein butter and lowest in Guernsey butter, while the vitamin A content in the butter from other breeds were in between. This study was also one of the first to show that the diet of the cows influenced the concentrations of both β-carotene and vitamin A. Since then others have confirmed his work (88, 96, 98) showing that Jerseys and Guernseys tend to have higher β-carotene and lower vitamin A contents in milk fat compared to other breeds.

Stage of lactation

Week of lactation and calendar month of production influence variations in milk yield, and many of the major milk constituents such as fat, protein, lactose, water and total solids (85). The concentrations of fat and protein in milk tend to decline after calving, reaching their lowest when cows are 40-60 days post partum. This decline is primarily due to dilution, however, as milk yield increases with increasing production of lactose by the mammary gland, and milk fat production tends to peak at the same time as milk yield (Figure 3). Beyond 40-60 days post partum, the concentration of fat in milk increases until the end of lactation, although milk total fat yield is actually declining (90).

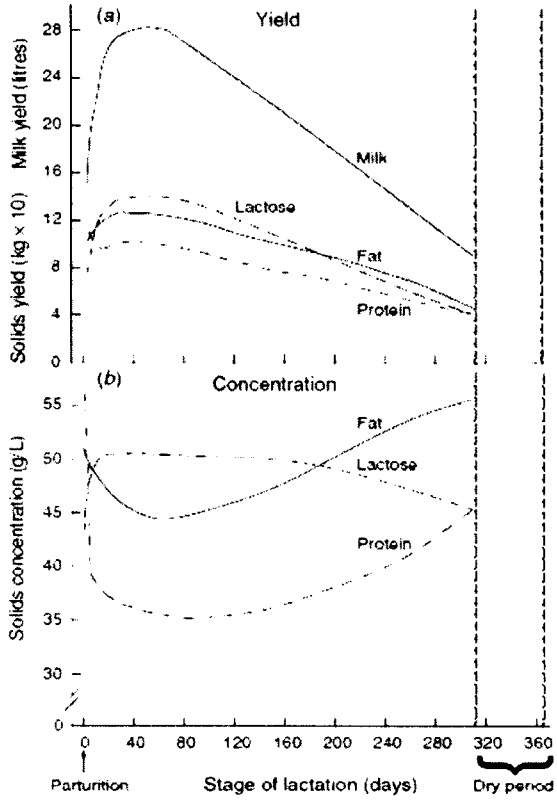


Figure 3. Relationships between yields of milk fat, protein and lactose and their concentrations in milk over a lactation of 310 days (90)

On the other hand, changes in β-carotene and retinol concentrations during early and mid lactation are poorly documented, and the effect of stage of lactation has not been clearly established (87). Beta-carotene and retinol concentrations are much higher in colostrum than in milk, and these concentrations decrease rapidly during the first week post-partum. Some research has reported (88) that β-carotene concentration in milk fat increases between day 40 and 305 of lactation, reaching a peak around day 200, whereas retinol varied only slightly. It is possible that an increase in β-carotene concentration in milk over the lactation cycle may be due to the decrease in overall milk yield over the time, thus decreasing the dilution effect (87, 88). In addition, due to seasonal variations in

dietary β-carotene concentration, the effect of stage of lactation is often partially confounded by the nature of the diet and/or climatic changes etc. (87).

Parity

Variation due to parity seems to be lower than variations related to other non-dietary factors (87). In 1983, Larsen et al., (99) showed that in early lactation, β-carotene concentrations in milk decreased between lactations 1 and 2 from 1.4 to 1.2 μg/g fat, then increased with each successive lactation cycle. Variations were inversely related to milk yield, and positively related to milk fat content. Later in 1985, Ascarelli et al., (100) showed no effect of lactation number on plasma carotenoid concentrations in multiparous cows.

Dietary factors

The concentrations of vitamin A and the carotenoid β-carotene in milk is highly dependent on the concentration of β-carotene in the diet (87, 98, 101-103) A change in milk composition is only realized, however, when the components in the diet of the cow are absorbed and transported to the mammary gland, and ultimately secreted into milk as the desired component (89). As the amount of carotenoids found in milk are quite low when compared to the amounts found in various feeds, it is clear that there are many key steps between their consumption and secretion in milk that are yet to be understood. The nature of the diet, however, seems to have the greatest influence over variations in the concentrations of fat and fat-soluble vitamins and carotenoids. Grass based diets, especially pasture, usually lead to a higher milk β-carotene concentration than diets rich

in concentrates[1] or corn silage, although the values vary depending on the nutrient profile of the concentrate (87).

Nature of forage

Pasture feeding has been associated with high β-carotene concentrations in milk for a long time (97). However, not all grasses are the same in their composition or their effects. Winkelman et al., (1999) for example, showed that fresh grass has higher levels of carotenoids than either hay or grass silage (104). In another comparison of seven diets (105), milk concentrations and yield of β-carotene were directly related to β-carotene in plasma and to the amount of β-carotene ingested by cows, and they were higher with pasture-based diets as well as ryegrass silage than they were with hay-based diets, concentrate-rich diets, and corn silage based diets. Others have conducted research examining the nature of different forages (different species, immature versus mature grasses, consumption of grasses morning versus evening, drying and preservation methods etc.) and how they affect carotenoid concentrations in milk (98, 105, 106). Because grazing management affects both the amount and nature of grass ingested by animals, it is a potential factor for variation in milk composition. Unfortunately, data on effect of grazing management on milk composition remain scarce (87).

Seasonal variations

Seasonal variations in carotenoid and retinol composition of bulk milk products are mainly due to the nature of the forage fed, as that is the production factor that is most

[1]Concentrates are feeds containing a high density of nutrients; usually low in crude fiber content and high in total digestible nutrients

closely related to season. Except in countries where cows graze throughout the year (i.e. New Zealand), milk fat is generally rich in carotenoids and retinol when it is produced during the summer months from grazing animals than during the winter months when animals are fed preserved forages (88). In countries where cows are on pasture all year, carotenoid and retinol concentrations are higher in winter versus summer milk, unlike in most other countries (87). This may be due, at least in part, to a lower bioavailability of those components during the summer months (87). Stage of lactation may also play a role in the seasonal effect, especially in production systems characterized by seasonal calvings. However these factors play a role in seasonal variations of milk composition, many researchers have seen the concentrations of major milk constituents, as well as fat soluble vitamins and carotenoids vary as a function of season (94, 96, 103, 107-110). Some research has also shown geographic location to play a role in milk composition (109, 110) although this also may be attributed to feeding, breed or calving period depending on the production region; and the regional effects may also interact with seasonal effects (107).

With so much opportunity for variation in milk content of carotenoids and retinol, components of nutritional interest due to their potentially beneficial health effects, understanding how different factors impact their presence in milk is vital. With much of the fluid milk produced in the US being used for milk products such as cheese, it is also valuable to understand how the concentration of these nutrients carries over through the cheese making process.

CHAPTER III

CHEESE

Cheese Consumption in the US

In contrast to the declining consumption of fluid milk in the US, cheese consumption is rising. Most of the cheeses consumed in the US fit into two broad categories, 'American' types (Cheddar, Colby, Monterey, and Jack) and 'other-than-American' types (primarily Italian varieties, such as Mozzarella, Parmesan, Provolone, Ricotta, Swiss and others). Estimates, according to the US Department of Agriculture, of the annual per capita use of American and other-than-American cheeses taken together have trended steadily upward over the last few decades (111). As shown in Figure 4, not only has total cheese consumption trended upward, but most notable is the growth in the consumption of other-than-American cheese.

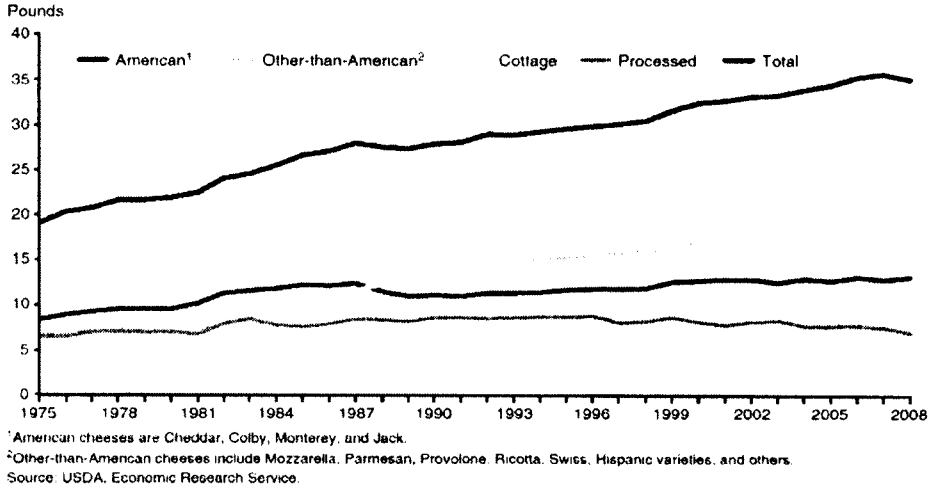


Figure 4. Total per capita cheese consumption, 1975-2008 (111)

In 2009, US total cheese availability (a proxy for consumption) was at 32.8 pounds per person, almost tripling since 1970, when it was 11.4 pounds per person (111).

According to the *Dairy Products 2010 Summary*, 4.42 billion pounds of Italian cheese (as part of the other-than-American category) were produced, a 5.8 percent increase from 2009 production. These cheeses accounted for 42.4 percent of the total cheese in 2010, and of the Italian production, Mozzarella accounted for nearly 80 percent (112). In 2009, Mozzarella edged out Cheddar as America's favorite cheese, with the two cheeses together accounting for 63 percent of cheese availability (113).

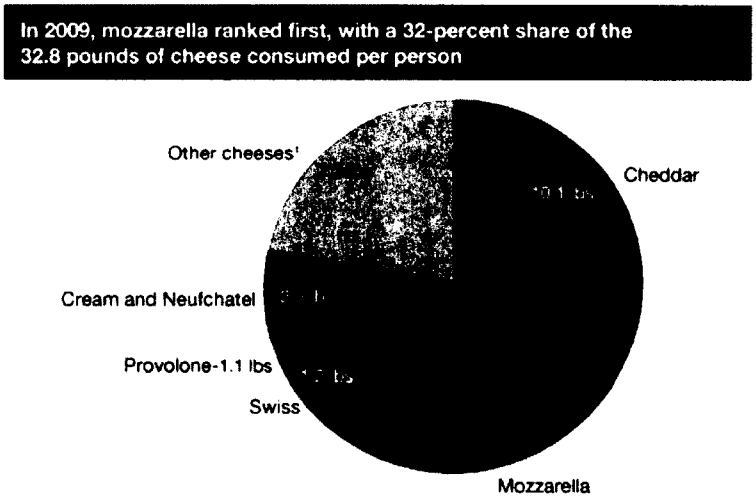


Figure 5. US cheese availability (consumption) in 2009 (113)

As consumer demand for cheese products has risen over time, dairy operations in the US have continued to allocate more of the milk and/or milk components to cheese production. Cheese sales are a key economic element of the dairy industry, constituting about 65 percent (82 billion pounds) of the milk used for manufactured products in 2010

(111). Consumer demand for cheese products is an important aspect of the dairy industry, and for the industry to continue its growth trend, continued increased cheese consumption is key.

Cheese sales and consumption are influenced by many factors, one of which is an emphasis on the nutritional benefits of cheese. Cheese is one of the three dairy products recommended for good health and nutrition according to the *Dietary Guidelines for Americans 2010* (76). Increasing its content of certain beneficial components, such as carotenoids, could be an effective way to increase the consumption of those nutrients as cheese consumption is already so high. In addition, it may promote increased consumption, which would benefit the dairy industry economically.

Cheese Production

To understand what the research has shown thus far about carotenoids in cheese, it is first necessary to understand the composition of cheese and the process by which it is made.

Cheesemaking is a process of 'selective concentration' of components in milk. Some components become more concentrated in the form of cheese curd, and others are separated out in the form of liquid whey. To illustrate this, Table 3 shows the selective concentration that takes place during the making of cheddar cheese from cow's milk (114). Exact values will differ slightly between cheese types, but generally follow these values.

Table 3. The selective concentration of milk components during manufacture of cheddar cheese[1]

Components	lbs (per 100 lbs) in Milk	% Lost to Whey	% Concentrated in Cheese
Water	87.000	95.5	4.50
Lactose	5.100	96.0	4.00
Fat	4.000	7.50	92.5
Casein	2.500	4.00	96.0
Whey protein	0.700	92.9	7.10
Salts	0.700	50.0	50.0
Calcium	0.120	40.0	60.0
Phosphorus	0.010	45.0	55.0
Sodium	0.006	96.0	4.00

[1]Adapted from *American Farmstead Cheese* (114)

Components that are polar or charged in nature such as lactose, whey proteins, sodium and free calcium ions generally remain dispersed in the water phase during cheesemaking. Thus, the moisture content in the cheese directly influences the levels of these components in the cheese. Conversely, casein and fat become more concentrated during cheesemaking and they directly impact final cheese yield.

Ideally, most of the fat is retained in the cheese, but this varies depending on the cheese type and the skill of the cheesemaker. Gentle treatment of the curd generally results in a greater retention of the fat. Calcium and phosphorus are also concentrated in the cheese, but their concentrations are largely dependent on the acidity throughout the process.

Steps of cheesemaking

The basic steps of cheesemaking vary slightly between cheese types, but follow this general outline: acidification, coagulation, cutting or breaking the curd, heating and holding the curd, draining the whey, pressing the curd, salting the curd, and in the case of Mozzarella cheese, stretching the curd.

Depending on the type of cheese being made, milk is pasteurized according to the current U.S. standards of identity for cheese. Mozzarella cheese, along with any other cheese that is aged for less than 60 days after production, must be made from pasteurized milk.

Acidification

The starter culture added to milk at the start of cheesemaking, consisting of harmless bacteria that occur naturally in raw milk, performs several functions. Primarily, it initiates the production of lactic acid through fermentation of lactose. This is important because it directly influences the pH, moisture content, and mineral content, three vital aspects of cheesemaking. The starter culture also suppresses the proliferation of undesirable microorganisms. The starter culture helps to suppress competing microbial activity by producing antimicrobial proteins, known as bacteriocins, and by the production of lactic acid which also inhibits many other microorganisms. In aged cheeses, the starter culture also plays an enzymatic role in ripening, and some produce carbon dioxide and specific flavor and aroma compounds, depending on the cheese type.

During the time following the addition of the starter culture, the bacteria become acclimated, beginning to produce the lactic acid and reproducing throughout the milk. Monitoring the acidity throughout cheesemaking is of critical importance. Two tests are commonly used to measure acidity: pH and titratable acidity (TA). The pH is a measurement of the concentration of hydrogen ions contained within the water phase, whereas the TA is a measurement of total acidity of the solution.

Coagulation

Coagulation is the next step in cheesemaking. In conjunction with the acidification, it creates the necessary environment for the protein and fat in milk to separate from the water and water soluble components. Because the casein micelles in milk are polar at the surface they attract a layer of water molecules around them. This layer of weakly bound water acts as a cushion that prevents the micelles from sticking together as they collide, allowing them to stay uniformly dispersed in the water phase of the milk.

During coagulation, the casein micelles are altered and become nonpolar at the surface. As a result, they lose their peripheral layer of water. With no cushion, the micelles begin to stick together as they move through the water. The net charge repulsion between micelles is also eliminated as the pH decreases through acidification. As they collide they form growing chains that branch out in different directions, and these chains interlock with one another to form a spongelike, three-dimensional casein matrix throughout the entire water phase of the milk. Fat droplets, around ten times larger than the casein micelles, become entrapped in the matrix.

Most cheeses are made by rennet coagulation. Rennet refers to enzyme preparations derived from the abomasum of ruminants that are used to coagulate milk. The term is often used generically, however, to describe any enzymatic agent used to coagulate milk. Today, milk coagulants can be obtained from a number of animal, plant and microbial sources.

As coagulants are quite potent, very small amounts are used to set large amounts of milk. To obtain a uniform distribution of the enzymes in the milk, necessary for uniform

coagulation, the coagulant is diluted in cold water (according to the manufacturer's instruction). The diluted coagulant is then added to the milk immediately, because the enzymes become unstable in the diluted state. The milk must also be brought to an optimal temperature before the addition of rennet. If the temperature is too low or too high, the rennet enzymes become inactivated and the milk will not coagulate. The right acidic environment is also crucial for optimal enzyme activity.

Rennet coagulation is a two-stage process, the enzymatic phase and the nonenzymatic phase. During the enzymatic phase, the coagulant enzyme attacks the casein micelles and snips off the polar end of κ-casein molecules. The κ-casein is primarily concentrated at the surface of the casein micelle, where it forms a 'hairy' protective polar covering that enables the micelle to remain dispersed in water. When these are degraded by the enzyme, the micelle surface is exposed. This enzymatic phase typically occurs during the first ten minutes following addition of rennet to milk. After a large proportion of the κ-casein has been removed, the casein micelles begin to collide and stick together, during the nonenzymatic phase, to form chains and eventually a three-dimensional casein matrix. The length of both the acidification and coagulation are very important because the rate of acid production during both affects the acid production throughout the remainder of the cheesemaking process.

Coagulation behavior is profoundly influenced by milk chemistry. For example, very high casein content in milk can lead to a rapid coagulation and a firm curd, whereas very low casein content can result in a weaker curd. Low concentration of ionic calcium also increases coagulation time and weakens curd. Milk pH affects both the enzymatic and nonenzymatic phases of coagulation, accelerating coagulation time as it decreases. A pH

of 7.0 or higher (normal pH ~ 6.5-6.7), often the case for milk from cows in late lactation or with mastitis, inactivates the rennet. Proteolysis in the milk before renneting causes damage to the casein micelles, which leads to poor coagulation properties. This can occur as a result of improper cleaning and sanitation practice by the cheesemaker, and also occurs in milk from animals that are late in lactation or that have mastitis. Coagulation behavior is also influenced by movement or physical disruption.

Cutting or breaking the curd

When milk coagulates, the water and water soluble components remain within the gel. When the curd is cut, it contracts and expels the liquid whey from the gel over time; the greater the surface area, the greater the whey expulsion. This process is called syneresis. The purpose of cutting the curd is thus to increase the surface area and enhance syneresis. The optimal size of the cut varies depending on the type of cheese being made and the desired properties of the final curd.

For rennet coagulated cheeses, cutting takes place when the curd is firm enough to cut cleanly into uniformly sized pieces. If the curd is too weak at cutting, fat and casein may be lost to the whey and the final moisture content and cheese yield decrease. If it is too firm, it can become tough and rubbery, resisting syneresis and resulting in higher moisture content cheese.

Heating and holding the curd

The size of the cut varies depending on the type of cheese being made, but each discrete piece should be the same size to promote uniform acidification, whey expulsion and temperature throughout. After cutting, the curd particles are fragile and can easily shatter

which allows fat globules to be lost into the whey. The curd is thus left undisturbed for a period of time before heating and stirring. During this time the curd firms up and develops a thin film on its surfaces through a process called *healing*. After healing, the curds may be heated, depending on the cheese type. This must occur at a specific rate, to a specific final target temperature, where it is held for a specific period of time. During heating, the curd must also be stirred at a certain rate.

The primary function of heating and holding is to further promote syneresis. The curd particle size, the stirring rate, the time-temperature profile, and the acidity profile together determine the amount of whey that is expelled. Another important function of this step is to regulate the loss of calcium phosphate from the curd to the whey. If the pH is too high during heating, most of the calcium phosphate remains in the casein matrix, rather than dissolving in the water phase of the curd and being lost in the whey. If the pH is too low during heating, calcium phosphate shifts to the soluble form as hydrogen ions from lactic acid are absorbed by the casein matrix and the calcium phosphate is released into the whey.

Draining the whey and pressing the curd

Draining the whey initiates the permanent separation of whey from the curds. This allows the curd particles to settle, coalesce and fuse together into a large curd mass, ultimately forming the body of the cheese. The curd is then compacted to expel more whey, to hasten the knitting of the curd, and to impart a desirable shape and texture to the curd for ripened cheeses. This could occur solely under the force of gravity, or light pressure may be applied as the curd settles and compacts. Temperature during pressing is also very

important. Cooling too quickly during pressing can result in excess surface curd fusion which acts as a barrier to the whey drainage. Pressing when the temperature is too high can result in excess loss of fat into the whey giving a greasy, seamy finish.

Salting the curd

Salt is almost always applied to the cheese near or at the end of the cheesemaking process. Salt has a number of different functions in cheese, both immediate and during ripening (in aged cheeses). Immediately, the salt begins to dissolve into the water phase of the curd and moisture is drawn osmotically to the surface, where it accumulates as the salt diffuses inward, ultimately resulting in a lower moisture cheese. As the whey is lost as a result of salting, lactose is also removed. This is important because excessive lactose in the cheese can lead to abnormally low pH and abnormal fermentations during ripening. Salt may be applied to the cheese in different ways depending on the cheese type.

Special applications

Special applications include plasticization and stretching of the curd during the making of pasta filata cheeses. Pasta filata refers to a type of Italian cheese having a pliable, homogenous, fibrous structure (114). These cheeses are distinguished by a unique kneading treatment of the fresh curd in hot water while it is in the plastic stage. The individual curd particles begin to flow together and the mass is pulled apart and folded back over, then molded into its desired shape. This thermomechanical treatment gives the finished cheese its characteristic fibrous structure and melting and stretching properties (115). Among the several types of pasta filata cheese (Caciocavallo, Provolone,

Provolette, Pizza Cheese, Provole, Scamorze, and Provatura) Mozzarella is a prominent member. Although it originated in Italy, the US has become its principle producer.

Factors affecting cheese quality

With such a high consumption of cheese, and Mozzarella cheese in particular, it is important to know the accurate nutritional composition of this product, and to understand factors that impact it. The gross composition of milk used to make cheese, especially the concentrations of lactose, protein and fat, influences several aspects of cheese production, including rennet coagulation, gel strength, curd syneresis, cheese composition, yield and quality (116).

Late-lactation milk, for example, generally results in poor rennet coagulability, impaired curd syneresis, higher moisture cheese, and lower fat recovery (116). However, Guinee et al., (2007) found that with good farm management practices, such as maintenance of milk yield greater than 6 kg/cow/day and supplementation of a concentrate-based diet with pasture and/or silage, milk produced by spring-calved cows close to the end of lactation had good rennet coagulation and Mozzarella cheesemaking properties (117).

In addition to these cheesemaking properties, also valuable for cheesemakers to know is the final composition of the cheese they are producing. The macronutrient content of cheese is well known, but its composition in micronutrients and phytonutrient like retinol and carotenoids is much less documented (118). As milk varies in these components of interest, it is likely that cheese composition will vary in accordance with the milk from which it is made. These changes may be related to the conditions of milk production (feeding practice, farm management etc.) or the cheesemaking process. It is not fully

understood which of these factors is more influential in the final composition of the cheese. There is evidence indicating that the both the conditions of milk production *and* the cheesemaking process may impact the resulting carotenoid content.

Carotenoids in Cheese

Carotenoids and retinol are sensitive to a number of different physical and chemical factors including air, oxidizing agents, and ultraviolet light. Their degradation is initiated and/or accelerated by these things, especially at increasing temperature, and it is often catalyzed by mineral ions. Retinol can also be quite unstable at a pH of 4.5 or lower (87).

Consequently, the processing of milk to produce cheese, which involves both heating and acidification, as well as the packaging and storage environment (i.e. light, air, temperature) immediately following production, may result in degradation of these components and impact their content in the cheese (87). In addition, some may be lost in the cheesemaking process because of the selective transfer of constituents from the milk to the cheese. Carotenoids and retinol are fat soluble, so they mainly behave as milk fat in the cheesemaking process, however, not all fat in milk is retained in the cheese produced, and a small proportion of the retinol and carotenoids have been shown to be associated with whey proteins. Thus, a certain amount of these components could be lost to the whey during the process (87).

The major cause of carotenoid degradation during processing and storage seems to be oxidation, be it enzymatic or non-enzymatic in nature, as carotenoids are highly unsaturated compounds and are prone to isomerization and oxidation (1). Some studies have shown that thermal processing of milk (i.e. pasteurization) and exposure to light

result in isomerization of retinol from the all-*trans* form to the *cis* forms (87). Much less certain is how thermal processing affects carotenoid levels/stability.

Thus far, the limited research investigating the transfer of carotenoids from milk to cheese has been conducted on aged cheeses. Lucas et al., (2006) examined the rate of transfer of retinol, β-carotene, and xanthophylls from milk fat to cheese fat in five French farmhouse cheese varieties (Abondance, Tomme de Savoie, Cantalet, Salers, and Rocamadour) (118). For each of the cheese types, there was a significant linear correlation ($p < 0.001$) between the levels of retinol, β-carotene, and xanthophylls in cheese and in milk fat. Beta-carotene seemed to be the most stable. Its variability within the cheese fat depended almost exclusively on the level present in the milk fat ($R^2 = 0.96$). Variations in the xanthophylls and retinol were to a lesser extent explained by the milk fat composition ($R^2 = 0.72$ and $R^2 = 0.54$, respectively). Additionally, the results of this study showed only 5 percent of the β-carotene lost in the whey during the cheesemaking process, while 34 percent of the retinol and 36 percent of the xanthophylls were lost in the whey (118). The rate of retinol and carotenoid loss did not vary by the cheese type/cheesemaking technology. It is uncertain if these results would apply to a fresh cheese such as Mozzarella, or if the rate of loss is controllable throughout the cheesemaking process.

Lucas et al., (2006) conducted another study in France in which herd characteristics and feeding practices associated with differences in cheese composition were identified (119). Cheeses (Abondance, Tomme de Savoie, and Cantelet) were sampled from 54 farms at 6 time points each year over a two year period and were made with milk from Abondance, Montbeliarde, Holstein, and 'other breeds and breed mixes'. Overall, the results showed

that the cheeses made with milk from the cows on pasture had higher levels of carotenoids, retinol and tocopherols than the cheeses made with milk from the cows fed higher levels of concentrates (119). Thus, the cheese composition depended greatly on the milk composition, and consequently on the conditions of milk production.

In another study conducted by Hulshof et al., (2006) the effects of both season and processing on retinol and carotenoid concentration in milk and milk products was examined (120). The results showed that these nutrients were higher when the cows were fed pasture during the summer and early autumn months, and that less than 50% of these nutrients were retained in the Gouda cheese after ripening, relative to the raw milk. Other studies have shown little or no change in the concentration of retinol and carotenoids during the ripening or storage of cheese (87). Thus far, no research has investigated the potential changes in levels of retinol and carotenoids in Mozzarella cheese.

Carotenoids and Color

The majority of the research on Mozzarella cheese has focused on the serum phase (liquid phase) of the cheese as well as the physico-chemical parameters that influence structure and function (121). Research is lacking in the area of carotenoids related to this method of cheesemaking, and in the relation of carotenoid content with color.

Objective assessment of the color of food products (meat and meat products, egg yolk, fruits and vegetables, sweets and chocolate, and coffee, etc.) has been shown throughout the literature (122). Milk and milk products have also been shown to vary in color.

Agabriel et al., (2007) measured the color of bulk milk samples (5x: February, March, May, July, and September) from over 200 farms around France (103). Milk samples

during the grazing period (May, July, and September) had higher yellow and color indices than milk obtained during February and March. The β-carotene content of milk explained 30 and 43 percent of the variations in the yellow and color indices, respectively.

Noziere et al., (2006) measured the color of plasma and milk from 32 cows (Holsteins and Montbeliarde) under different feeding systems (98). Their results showed that the color indices of both plasma and milk were higher with grass silage than with hay diets. The plasma color index was higher in Holsteins than in Montbeliarde cows, and the milk color index was lower in Holsteins than in Montbeliarde cows. The concentrations of β-carotene explained 58 and 40 percent of the variability in color index in plasma and milk respectively.

There is also some evidence of variations in color (both from objective measurements and from sensorial panels) of aged/ripened cheeses depending on the diet of the cow (123). Numerous studies conducted by Verdier-Metz and colleagues examining the effects of diet on cheese (Saint-Nectaire and Cantel cheeses) sensory characteristics have shown milk from pasture-based diets to produce cheese that is more yellow than milk from cows fed grass silage, and cheese from grass silage diets to be more yellow than cheese from cows fed hay (123,124). When a Saint-Nectaire-type cheese was made with milk from cows of 3 different breeds (Holstein, Montbeliarde and Tarentaise) all fed hay but of differing types, even the type of hay impacted the resulting cheese color (125).

Carpino et al., (2004) examined the color of Ragusano cheese made with milk from Friesian cows under different feeding treatments, pasture and total mixed ration (126).

Measurements were taken after 4 and 7 months of aging. Results showed that the color of the cheeses produced from milk of cows consuming fresh native pasture plants was much more yellow than cheeses from cows fed total mixed ration, indicating that compounds from pasture plants transferred from the diet to the cheese and that there may be color compounds that are unique to the plants of the region where cheese is produced.

No research has been conducted thus far looking at milk and Mozzarella cheese over so many time points throughout the pasture season, and using exclusively Jersey cows. Jersey cows have been shown to produce more β-carotene than others, as well as fluid milk and milk fat with more intense yellow color (104) making them an interesting breed to investigate. This study is important and valuable for the consumer, because of the potential for health benefits, and for the dairy industry, as interest in pasture-based systems is increasing. It is also valuable to the local farmer and artisan cheesemaker to understand the variations that occur in the composition, and color, of milk and cheese.

CHAPTER IV

OBJECTIVES & HYPOTHESES

Objectives

First, to investigate the changes in concentration and yield of carotenoids, retinol, fat and protein in milk from Jersey cows under two different feeding systems over the pasture season (May-November). Cows at the Organic Dairy Research Facility were fed pasture supplemented with a total mixed ration (Appendix A) and cows at the Fairchild Dairy Teaching and Research Center were fed a total mixed ration (Appendix B).

Second, to examine the carotenoid concentration in a fresh, pasta filata type cheese in relation to the carotenoid concentration in the milk from which it was made. In addition, we will use an objective color analysis to determine the relationship of cheese color to carotenoid content.

Hypotheses

1. Milk from Jersey cows at the Organic Dairy Research Facility and the Fairchild Dairy Teaching and Research Center will differ significantly by the second month of pasture and will continue to differ significantly through the end of the pasture feeding.

2. The carotenoid concentration in milk will vary significantly over the pasture season within the Organic Dairy Research Farm.

3. The carotenoid concentration in fresh, pasta filata type cheese (Mozzarella) made from Jersey milk will correlate with the carotenoid values of the milk over the season, and will be reflected in the quantitative color analysis.

4. Yield of protein and curd will be lower in milk from Jersey cows fed on pasture compared to milk from cows fed a total mixed ration.

CHAPTER V

MATERIALS & METHODS

Milk Sourcing

Sourcing raw milk for the study was initiated in the fall of 2010. Meetings were held with managers at the UNH Organic Dairy Research Facility (ODRF) at the Burley-Demeritt farm in Lee, NH and at the UNH Fairchild Dairy Teaching and Research Center (Fairchild Dairy) on the University of New Hampshire campus in Durham, NH. The projects goals were discussed with the Master's Thesis committee and plans were made for collection of the raw milk from individual cows as well as from the bulk tank.

The cows at the ODRF, all Jersey cows, were fed a total mixed ration (Appendix A) during the winter months and were fed on pasture, supplemented with the total mixed ration, during the warmer months. In 2011 they began feeding on pasture on May 17 and ceased on October 19. The cows at the Fairchild Dairy are fed a total mixed ration (Appendix B) year round. Milk from a total of 18 Jersey cows was collected; nine cows at the ODRF and nine from the Fairchild Dairy Teaching and Research Center. The total milk yield from each of the cows on study was recorded, for each of the milkings when a sample was collected.

Collection of Milk

Individual Samples

Individual samples of milk were collected during the morning milking of nine Jersey cows at the ODRF and the nine Jersey cows at the Fairchild Dairy. Morning milkings occurred at 4:00 am and evening milkings occurred at 5:00 pm each day. Samples were collected biweekly beginning May 11, 2011 and ending November 10, 2011. Collections at the ODRF took place on Wednesday mornings and collections at the Fairchild Dairy took place on Thursday mornings. Small portions of the milk from each cow were redirected into sample cups throughout the whole milking process so that the resulting sample accurately represented milk from the entire milking, rather than just the initial, middle or end milk. Each sample was shaken and poured into 50 ml tubes and then stored in a dark refrigerator. A 15 ml aliquot of each of those individual samples was poured into separate tubes, labeled according to the date and cow number and frozen at -20°C for carotenoid analysis.

Milk samples from individual cows used to analyze fat and protein percent were collected during both milkings. The milk represented the entire day's milk, with a ratio of morning and evening milk relative to the yield at each milking (i.e., if 10 kg of milk were produced in the morning and 5 kg in the evening, the sample tube would contain a 2:1 ratio of morning to evening milk). Forty-five ml of milk from each individual cow were set aside in 50 ml sample tubes. An 18 mg tablet of preservative (8 mg Bronopol and 0.30 mg Natamycin; Broad Spectrum Microtabs II) was added to each tube to inhibit bacteria, yeast and mold, and reduce lipolysis in the milk. Milk was sent on the same day of

collection to Dairy One Cooperative Inc., (Ithaca, New York), for analysis of milk fat and milk protein. This company is recognized and approved by the Association of Official Analytical Chemists (AOAC) Research Institute and the FDA as an independent reference laboratory, and is one of the largest milk analysis laboratory networks in the United States.

Bulk Samples

Nine to ten liters of milk were collected in a Nalgene carboy (always disinfected, washed, and sanitized with bleach and water before and after every milk collection) during the same morning milking of nine Jersey cows at the ODRF and 9-10 liters during the morning milking of nine Jersey cows at the Fairchild Dairy. At both dairies, the nine Jersey cows in the study were the first to be milked on the mornings of collection, so that their milk was directed into an empty bulk tank. The 9-10 liter samples were taken from the bulk tank before the remainder of the cows were milked, so that the bulk sample represented the milk from the nine cows combined, not the entire herd. A 50 ml aliquot of the raw bulk milk was poured into a sample tube and set aside, out of which a 15 ml aliquot was frozen at -20°C for carotenoid analysis. Eight liters were also removed from the bulk sample and refrigerated at 4°C for production of Mozzarella cheese on the same day of collection.

Production of Cheese

On each of the days that milk was collected, eight liters of the milk from the bulk tank were used to make Mozzarella, a fresh cheese. All data were recorded during cheese production and all instruments were sterilized in boiling water prior to use. The whey

volume, curd yield, temperature, pH and titratable acidity were monitored and recorded throughout. The pH and titratable acidity of the raw milk were tested using the pH meter and mini titration system (Hanna Instruments, HI 84429), respectively. A modified HACCP protocol was followed to ensure quality control and sanitation throughout the cheesemaking process.

Pasteurization

The eight liters of milk were pasteurized in a bench-top batch pasteurizer (Pressure Vac low temperature pasteurizer, Schleuterfor Co., Janesville, WI) to 68°C-70°C, with the temperature monitored to ensure safety. Once the target temperature was reached, milk was cooled to 32°C and poured into sanitized top pan of a double boiler. A 50 ml aliquot of the pasteurized milk was set aside, 15 ml of which was frozen for carotenoid analysis.

Ripening and coagulation

A starter culture (thermophilic, direct set, C201) was added to the pasteurized milk at 32°C and then milk was stirred (1-2 minutes) with a stainless steel slotted spoon. The milk was covered and let set inside the double boiler, with water in the bottom pan around 38°C to keep milk at 32°C for 30 minutes. After the 30 minute ripening period, a 50 ml aliquot of the ripened milk was removed and set aside to be tested for titratable acidity. Next, a ½ teaspoon of liquid rennet (animal rennet; New England Cheesemaking Supply Company, Inc., Deerfield, MA) was diluted with ¼ cup of cool water and the diluted rennet solution was added to the ripened milk. It was stirred gently with a stainless steel slotted spoon in an up and down motion for 1 minute, and then the top was stirred for several more minutes. The pan was then covered and set inside the double

boiler on the bench with water at 38°C in the bottom to keep milk at 32°C for 75 minutes. Temperature was monitored throughout coagulation process. At 75 min, firmness of the curd was checked; three gloved fingers were inserted into the curd at a 45°angle and lifted gently. If the curd did not break in a clean line it was left for another 5 minutes and checked again. Once set, the curd was cut with a cheese cutting knife (submerged in boiling water prior to use) into ¼ -inch cubes. The cubes were set undisturbed for 20 minutes, allowing the whey to further drain from the curd. After the 20 minutes, the pH was tested.

Heating the curd

The curds were then slowly heated to 38°C, by no more than 2° every 5 minutes, while gently and constantly being stirred. Temperature was elevated by removing two cups of water from the bottom pan of the double boiler and replacing it with two cups of boiling water every few minutes. As the temperature rose, the spoon was used to break up any pieces of curd that were larger than the ¼ -inch cubes. Once at 38°C, the curds were set, covered and undisturbed within the double boiler on the counter for 30 minutes. The temperature remained at 38°C ± 1° throughout. During this time, cheese cloth was cut and used to line the strainer inside of a large pan. At the end of the 30 minutes, the pH of the curd and whey were measured and recorded.

Draining the whey

The whey was drained from curds through the strainer (lined with cheese cloth) within the pan and measured. A 15 ml aliquot of the whey was removed and frozen at -20°C for carotenoid analysis. The remainder of the whey was then disposed of. The curd within the

cheese cloth in strainer was allowed to continue draining into the pan at room temperature until the pH of the whey reached 6.0; usually around 3 hours. The curd was placed in the refrigerator at 4°C overnight.

Salting and Stretching

On day two of cheese production, the curd was removed from the refrigerator and brought to room temperature. Five ml of whey was squeezed from the curd into a small beaker with a funnel lined in muslin once at room temperature. A small piece of the curd was also removed and the pH tested. Once the pH of both the curd and whey reached about 5.2 the curd was cut into 1x1 in cubes and placed in a sterilized stainless steel bowl. A full pan of water was boiled and used to sterilize instruments and to continually refill the bottom pan of the double boiler during stretching. Bottled water was also boiled and 12 cups poured into the top pan of the double boiler. This water was used to stretch the curd when it was around 65°C. To the water, 4 tbsp of cheese salt (non-iodized) was added and mixed until dissolved. The bottom pan of the double boiler was filled with boiling water as well, to keep stretching water at about 65°C. The double boiler was placed inside the sink. With nylon liners and vinyl gloves on, about 1/6 of the cut curds were placed into the stretching salt water. The pieces were stirred around and pressed together once they were soft enough to stick together. When curds stretched without breaking, they were kneaded together into a shiny ball, then immediately placed in cold water. This process was completed with portions of the remaining curd until six balls were stretched and placed in the cold water. After every two balls were stretched, 1 tbsp of salt was added to the stretching water. The temperature of the stretching water always remained above 60°C. If it began to decrease, some of the boiling water was added to the

bottom pan of the double boiler. The cheese was weighted to record total yield. One of the balls was cut into three portions and each one vacuum sealed separately, labeled, and frozen at -20°C for carotenoid analysis. The remaining five balls were also vacuum sealed and refrigerated.

Milk Analysis

Fat, true protein, lactose, total solids, and solids not fat (SNF) were measured by Dairy One Cooperative Inc. and titratable acidity (TA) and pH were measured using the titrator and pH meter, respectively.

Carotenoid Extraction and Analysis

Components measured were lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and 13-*cis* β-carotene, retinol and α-tocopherol. Although α-tocopherol was not a focus of the current research, it was included in the analysis and results as a reference of its variation over time and between dairies, as this information may be useful for future studies. The components of interest were analyzed using a Hewlett Packard/Agilent Technologies 1100 series High Performance Liquid Chromatography (HPLC) system with a photodiode array detector (Agilent Technologies, Palo Alto, CA). A 5 μm, 200 Å polymeric C30 reverse-phase column (Pronto-SIL, MAC-MOD Analytical Inc., Chadds Ford, PA) was used to separate the analytes. The HPLC mobile phase solvent A consists of methanol/*tert*-butyl methyl ether/water (83:15:2, vol/vol/vol, with 1.5% ammonium acetate in the water). Organic solvent B consists of methanol/*tert*-butyl methyl ether/water (8:90:2, vol/vol/vol, with 1% ammonium acetate in the water). The gradient procedure at a flow rate of 1 ml/min begins at 100% Solvent A for 2 minutes to 70%

Solvent A over a 6 minute linear gradient and held at 70% A for 3 minutes, then a 10 minute linear gradient to 5% Solvent A, a 4 minute hold at 5% Solvent A and, finally, a 2 minute linear gradient back to 100% Solvent A. The system is held at 100% Solvent A for 10 minutes for equilibrium back to initial conditions (127). Carotenoids, retinol, and α-tocopherol were detected at 452, 325 and 290 nm, respectively, and were identified by comparing retention times and spectral analysis with those of pure standards (> 95%). Concentrations of compounds were calculated by using an external standard curve and were then adjusted by percentage recovery of the added internal standard.

Frozen milk samples were thawed to room temperature. Two 2 milliliter aliquots of milk were removed from each for analysis. The remainder of each milk sample was frozen at -20°C. Milk was extracted for carotenoids according to the method of Qin et al (127). In a labeled 16 x 100 glass culture tube (Fisher #14-961-29), 2 ml of milk were mixed with 100 ml 12% pyrogallol (Fisher #A263) in ethanol (3 g pyrogallol in 25 ml Etoh), 3 ml potassium hydroxide (Fisher #P250) in water (1:1, wt/wt,100 mg KOH in 100 ml DiH2O), and 3 ml Etoh (Acros 200 proof, Fisher #61509-0020). This tube was capped and vortexed for 30 seconds, sonicated for 1 minute, and incubated at 37°C for 2 hours. The tube was removed from heating block and cooled for 5 minutes, at which point 100 μl of internal standard (ISTD) and 4 ml of hexane (Optima, Fisher #H303-4) were added. The internal standard was ethyl-8'-apo-β-caroten-8'-oate (Carotenature, Lupsingen, Switzerland). The tube was vortexed for 30 seconds, sonicated for 1 minute, and centrifuged at 2100 RPM for 5 min. With a glass Pasteur pipette, the upper layer of hexane was removed and put into a clean culture tube. This extraction process was repeated and the hexane layers combined. To the hexane extracts, 3 ml of ethanol and 3

ml DiH2O were added. The tube was vortexed for 30 seconds and centrifuged at 2100 RPM for 5 minutes. The upper hexane layer was again removed and put into a clean culture tube. This extract was dried under nitrogen. Once dry, 100 µl of ethanol was added to and the tube vortexed for 30 seconds. The re-suspended sample was transferred to amber HPLC vial (11 mm, 2.0 ml, Restek, Fisher #24385) with glass insert (200 µl flat bottom – Restek, Fisher #24385) and crimp sealed (11 mm PTFE, Restek, Fisher #14-930-15E) for analysis on HPLC auto sampler, where 20 µl of the sample were injected. Each milk sample was extracted and analyzed in duplicate. Results from each analysis were recorded and used to compare composition of the milk throughout the year from the Jersey cows on pasture at the ODRF vs. the Jersey cows fed total mixed ration at the Fairchild Dairy.

Cheese Analysis

Carotenoid Extraction and Analysis

High Performance Liquid Chromatography (HPLC) was used to separate and quantify the carotenoid and vitamin content of the Mozzarella cheese, following the same methods used for milk. Frozen cheese samples were thawed to room temperature. Methods used for milk extractions were also followed for cheese using 1g of cheese (cut into 2mm x 2mm pieces) from each sample. Extraction and analysis of each was performed in duplicate. Extractions on the first five cheese samples were run using 3g of cheese and values corrected for following analysis. The density of Mozzarella cheese (0.954 g/ml) was accounted for when reporting concentrations of carotenoids and nutrients in cheese.

Result from each analysis were recorded and used to compare carotenoid composition of the cheese throughout the year made with milk from the Jersey cows on pasture at the ODRF vs. milk from the Jersey cows fed total mixed ration at the Fairchild Dairy.

Color Analysis

Mozzarella cheese samples were frozen immediately after production at -20°C and stored until analysis. A commercial brand Mozzarella cheese (Bella Gioioso) was purchased and frozen overnight at -20°C. Samples and standard were thawed together in a 20°C water bath and sliced to 1 cm thickness. Color comparison between cheeses was measured using the L*a*b* color space also referred to as CIELAB. L*a*b* values were measured with a spectrophotometer (Konica Minolta CM-600d). The standard commercial brand white Mozzarella cheese was used as the target for comparison. Reported were the ΔE*ab values which express the color difference in a single numerical value for simplicity (128). Measurements were taken in triplicate and averages were compared to the standard to obtain ΔE*ab values.

Statistical Analysis

Raw data was entered in Microsoft® Office Excel 2007 (Microsoft Corporation, Redmond, WA.). At each time-point, a cow's mean concentrations of retinol, α-tocopherol, β-carotene, α-carotene, 13*cis* β-carotene, lutein, zeaxanthin, and β-cryptoxanthin were calculated from two samples. A cow's total carotene at each time-point was calculated by summing the mean concentrations of β-carotene, α-carotene, and 13*cis* β-carotene. Likewise a cow's total xanthophyll concentration at each time-point was calculated by summing the mean concentrations of lutein, zeaxanthin and β-

cryptoxanthin. Given the number of time-points in which a viable milk sample was not available (i.e., empty cells in the database due to cows drying off or sick) the data was collapsed across weeks to maximize the sample and achieve sufficient statistical power. First, weeks 13 and 14 were removed from the database because less than half of the cows provided milk for the nutrient analyses. Second, mean component concentrations were calculated using weeks 1 and 2, weeks 3 and 4, weeks 5 and 6, and so on to weeks 11 and 12, yielding six time-points. The sample table below elucidates some of the aforementioned procedures using arbitrary data for an unidentified component from a single cow between baseline and collection six. As shown in the sample table: the column labeled A lists the first six collection points of the study; columns B and C show the raw component concentration from milk sample 1 and sample 2, respectively; column D shows the calculated mean of columns B and C; column E shows the collapsing of each two collections to a single time-point (e.g., weeks one and two are collapsed to create time-point one); and column F provides the calculated mean at each time point (e.g., mean component concentration for time-point 1 was calculated by taking the mean of week one and two in column D) .

A	B	C	D	E	F
	Component (ng/μl)				
Collection	Sample 1	Sample 2	Mean component	Time-point	Time-point Mean
1	0.025	0.045	0.035	1	0.0350
2	0.035	0.035	0.035		
3	0.045	0.075	0.060	2	0.0675
4	0.055	0.095	0.075		
5	0.065	0.085	0.075	3	0.0650
6	0.075	0.035	0.055		

To account for the yield variation among cows and over time, a weighted concentration was calculated for each value by multiplying the mean concentration of each component by the yield volume. In reference to the table above, the values in column D would be multiplied by the corresponding week's milk volume.

Lastly, overall means for each cow were calculated for all nutrients as well as yield (weight and volume), fat content (percentage and weight), and protein content (percentage and weight), by averaging the values collected between baseline and collection 14 regardless of any missing data-points.

SPSS® version 19.0 (SPSS Inc. Chicago, IL) was used to perform descriptive and inferential analyses. Pearson product-moment correlations were performed for all continuous variables at time-point 1 and for overall mean values from the entire experimental period. Pearson correlations were also performed on mean values (experimental groups combined) from raw bulk milk, pasteurized bulk milk, whey, and cheese at all time-points. A one-way repeated measures Analysis of Variance (ANOVA) was performed to assess within group changes from baseline for vitamin and carotenoid concentrations, fat and protein percents, and milk yield in cows at the ODRF cows, and in cows at the Fairchild Dairy. A two-way repeated measures ANOVA was performed to assess within group changes over time and between group differences at each time-point. Lastly, regressions analysis was performed to determine if group, milk yield, and fat percentage could significantly predict vitamin and/or carotenoid concentration. The reported means, standard error of the means (SEM) and p-values are unadjusted for the number of comparisons.

CHAPTER VI

RESULTS

Milk Production and Composition

The mean values for milk production and composition are given in Table 4. Values for the means and standard error of the mean (SEM) were calculated from 7 cows at each dairy and across 6 monthly time-points, beginning in May. The predominant carotenoid found in the milk was β-carotene, although lutein, α-carotene, zeaxanthin, β-cryptoxanthin, and 13*cis* β-carotene were detected as well. As expected, concentrations of the carotenoids were significantly higher in milk from the Organic Dairy (OD) ($p < 0.05$) except for β-cryptoxanthin which was significantly higher in milk from the Fairchild Dairy (FD) ($p < 0.05$). Retinol was significantly higher in milk from the FD as well, whereas α-tocopherol was not significantly different between groups.

Table 4. Milk production and composition[1]

	OD Farm		FD Farm		Effect		
	Mean	SEM	Mean	SEM	Time	Group	Time*Group
Production							
Milk yield	8.774	0.795	11.208	0.562	< 0.001	0.017	0.014
Fat %	4.789	0.158	5.761	0.144	0.002	0.002	0.162
Concentration							
Carotenes	0.277	0.455	0.119	0.150	< 0.001	<0.001	< 0.001
Xanthophylls	0.017	0.029	0.011	0.025	0.001	0.019	< 0.001
Retinol	0.208	0.378	0.276	0.229	< 0.001	0.019	< 0.001
α-tocopherol	0.317	0.205	0.342	0.597	0.001	0.640	< 0.001
Lutein	0.011	0.018	0.003	0.010	< 0.001	<0.001	0.001
Zeaxanthin	0.002	0.004	0.001	0.002	0.546	0.001	0.086
Lutein + Zeaxanthin	0.013	0.022	0.004	0.012	< 0.001	<0.001	< 0.001
β-cryptoxanthin	0.004	0.007	0.007	0.013	< 0.001	0.014	< 0.001
α-carotene	0.013	0.021	0.007	0.005	< 0.001	<0.001	< 0.001
β-carotene	0.262	0.436	0.112	0.145	< 0.001	<0.001	< 0.001
13*cis* β-carotene	0.001	0.002	0.000	0.001	< 0.001	<0.001	< 0.001

[1]Values are means and SEM were calculated using 7 cows from each group across 6 time-points
OD = Organic Dairy, FD = Fairchild Dairy
All yields are reported in kg
All concentrations are reported in ng/μl
F-value and P-value were calculated by two-way repeated measures ANOVA

Mean milk yield (expressed as kg/morning milking), fat percent, and concentrations (in ng/μl) of carotenoids, retinol and α-tocopherol in milk from cows at the OD and FD at all six time-points are reported in Table 5 and Table 6, respectively. Table 4 shows that milk yield, fat percent, and each carotenoid, except zeaxanthin, in milk from both groups combined significantly changed over time ($p < 0.01$). However, since the changes over time in this table reflect milk from both groups combined, it does not show how these values changed over time at each dairy separately. Tables 5 and 6 thus show the changes in these values over time in milk from the OD and FD, respectively. Table 5 shows that each component being measured, except the zeaxanthin, significantly changed over time ($p < 0.01$) in the milk from the OD. However, only milk yield, total xanthophylls, retinol, α-tocopherol, lutein, and lutein + zeaxanthin combined significantly changed over time (p

< 0.05) in milk from the FD, as shown in table 6. Fat percent, total carotenes, zeaxanthin, β-cryptoxanthin, α- and β-carotene, and 13*cis* β-carotene in milk did not significantly change over time. Figure 6 illustrates the variations in concentrations of carotenoids and nutrients over time.

Table 5. Organic Dairy mean milk yield, nutrient and carotenoid changes over time[1]

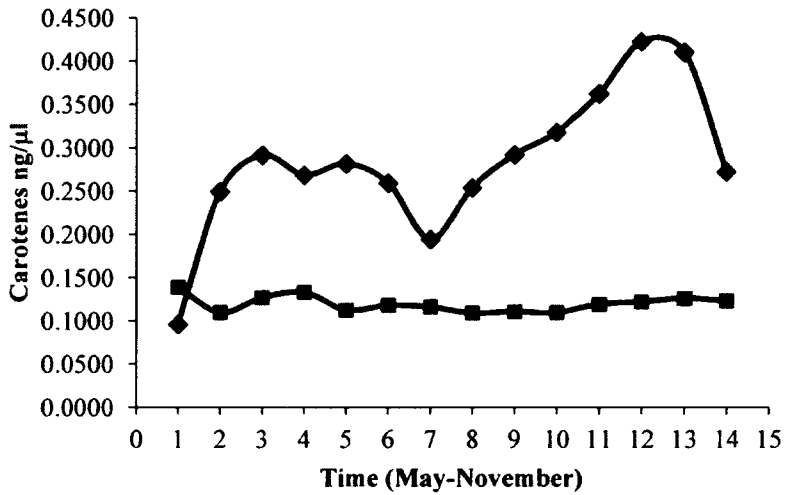
Time-point	1		2		3		4		5		6				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	F	*P*-value	Partial Eta[2]
Yield kg	11.13	0.881	8.84	0.729	9.41	0.885	7.69	1.022	7.66	0.784	7.99	0.574	19.595	< 0.001	0.797
Fat %	4.48	0.173	4.61	0.232	4.45	0.237	4.90	0.240	4.84	0.163	5.42	0.317	4.676	0.004	0.483
Carotenes	0.1728	0.300	0.2905	0.789	0.2704	0.370	0.2240	0.372	0.3049	0.558	0.3923	0.889	16.818	< 0.001	0.771
Xanthophylls	0.0153	0.038	0.0151	0.041	0.0180	0.037	0.0139	0.029	0.0172	0.036	0.0212	0.039	6.534	0.001	0.567
Retinol	0.2642	0.436	0.2085	0.558	0.2297	0.356	0.1760	0.368	0.1991	0.300	0.1871	0.242	4.307	0.006	0.463
α-tocopherol	0.3071	0.395	0.2635	0.454	0.3336	0.334	0.3494	0.196	0.3253	0.146	0.3240	0.324	3.856	0.010	0.435
Lutein	0.0107	0.033	0.0085	0.022	0.0116	0.027	0.0092	0.020	0.0104	0.025	0.0128	0.021	3.837	0.010	0.434
Zeaxanthin	0.0019	0.072	0.0019	0.006	0.0023	0.005	0.0017	0.004	0.0020	0.005	0.0023	0.005	1.313	0.290	0.208
Lutein + Zeaxanthin	0.0127	0.035	0.0104	0.034	0.0139	0.033	0.0110	0.023	0.0124	0.029	0.0152	0.025	6.310	0.001	0.558
β-cryptoxanthin	0.0027	0.006	0.0047	0.017	0.0041	0.005	0.0029	0.006	0.0048	0.008	0.0060	0.014	10.921	< 0.001	0.686
α-carotene	0.0087	0.010	0.0127	0.028	0.0127	0.017	0.0122	0.014	0.0156	0.037	0.0185	0.047	8.933	< 0.001	0.641
β-carotene	0.1640	0.291	0.2773	0.761	0.2570	0.353	0.2109	0.364	0.2879	0.527	0.3721	0.847	16.954	< 0.001	0.772
13*cis* β-carotene	0.0001	0.001	0.0006	0.003	0.0006	0.001	0.0009	0.004	0.0013	0.003	0.0016	0.005	12.858	< 0.001	0.720

[1]Values are means for 7 cows per group over 6 time-points (each time-point corresponds to one month of collections)
All concentrations are reported in ng/μl
F-value and P-value were calculated by one-way ANOVA

Table 6. Fairchild Dairy mean milk yield, nutrient and carotenoid changes over time[1]

Time-point	1		2		3		4		5		6				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	F	*P*-value	Partial Eta²
Yield kg	13.13	0.881	10.85	0.729	12.23	0.885	11.17	1.022	11.03	0.784	8.84	0.574	12.494	< 0.001	0.641
Fat %	5.71	0.173	5.85	0.232	5.71	0.237	5.76	0.240	5.50	0.163	6.04	0.317	1.799	0.139	0.204
Carotenes	0.1243	0.300	0.1286	0.789	0.1152	0.370	0.1125	0.372	0.1142	0.558	0.1207	0.889	1.296	0.288	0.156
Xanthophylls	0.0133	0.038	0.0120	0.041	0.0103	0.037	0.0098	0.029	0.0109	0.036	0.0095	0.039	3.220	0.017	0.315
Retinol	0.4122	0.436	0.3178	0.558	0.2707	0.356	0.2065	0.368	0.2133	0.300	0.2288	0.242	71.572	< 0.001	0.911
α-tocopherol	0.4044	0.395	0.3198	0.454	0.3613	0.334	0.3312	0.196	0.3132	0.146	0.3089	0.324	7.666	< 0.001	0.523
Lutein	0.0055	0.033	0.0029	0.022	0.0027	0.027	0.0029	0.020	0.0035	0.025	0.0031	0.021	4.210	0.004	0.376
Zeaxanthin	0.0010	0.072	0.0011	0.006	0.0006	0.005	0.0008	0.004	0.0009	0.005	0.0008	0.005	1.334	0.273	0.160
Lutein + Zeaxanthin	0.0064	0.035	0.0040	0.033	0.0033	0.033	0.0037	0.023	0.0044	0.029	0.0039	0.025	3.758	0.008	0.349
β-cryptoxanthin	0.0069	0.006	0.0080	0.017	0.0069	0.005	0.0061	0.006	0.0065	0.008	0.0056	0.014	2.335	0.062	0.250
α-carotene	0.0072	0.010	0.0070	0.028	0.0063	0.017	0.0066	0.014	0.0061	0.037	0.0066	0.047	1.224	0.319	0.149
β-carotene	0.1170	0.291	0.1213	0.761	0.1087	0.353	0.1057	0.364	0.1078	0.527	0.1137	0.847	1.525	0.207	0.179
13*cis* β-carotene	0.0002	0.001	0.0003	0.003	0.0002	0.001	0.0002	0.004	0.0002	0.003	0.0003	0.005	1.226	0.318	0.149

[1]Values are means for 7 cows per group over 6 time-points (each time-point corresponds to one month of collections)
All concentrations are reported in ng/μl
F-value and P-value were calculated by one-way ANOVA


Carotenes ng/µl
0.4500
0.4000
0.3500
0.3000
0.2500
0.2000
0.1500
0.1000
0.0500
0.0000
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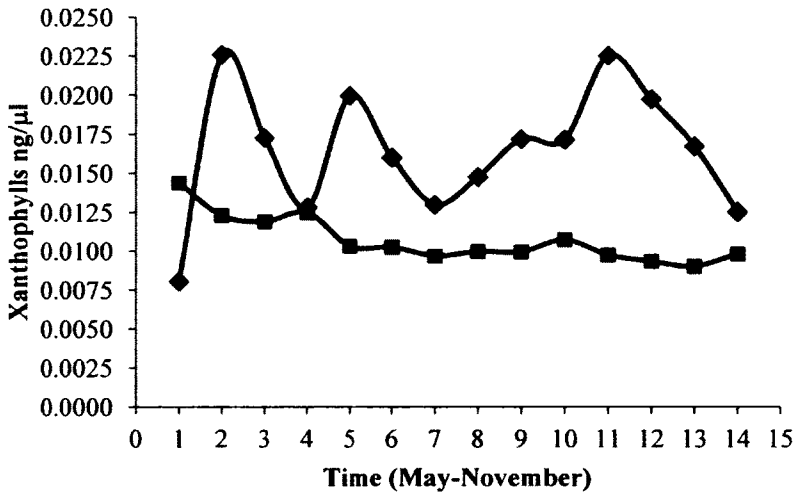
Time (May-November)


Xanthophylls ng/µl
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0.0225
0.0200
0.0175
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0.0125
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0.0075
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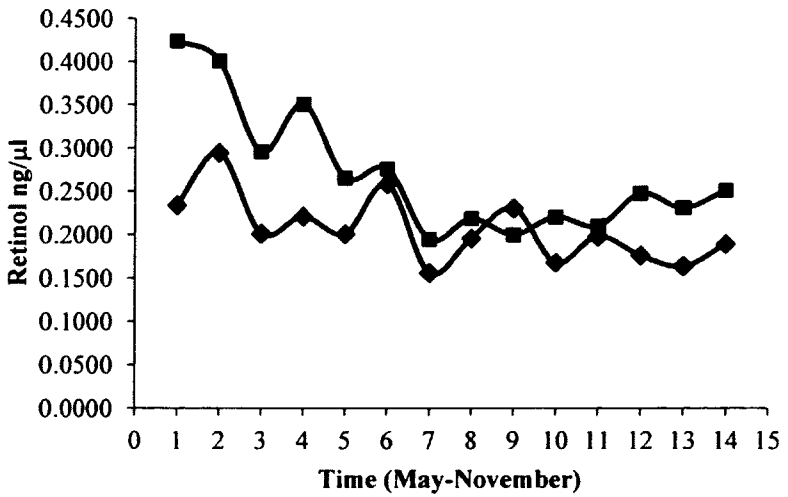
0.0025
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (May-November)


Retinol ng/µl
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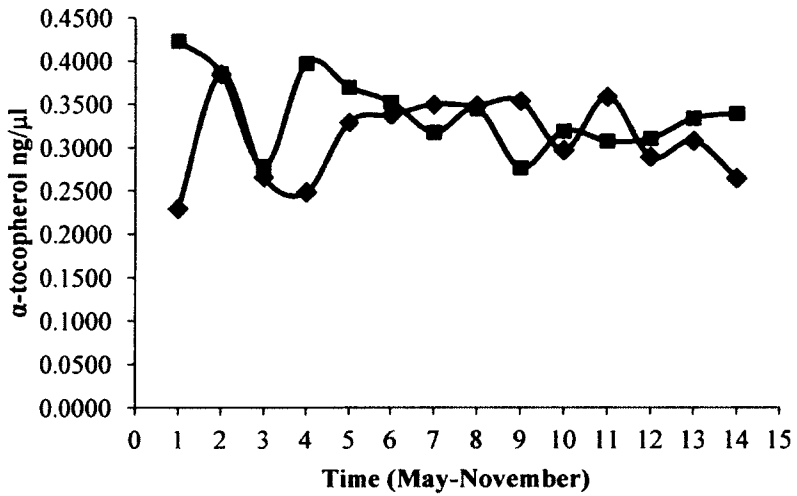
0.4000
0.3500
0.3000
0.2500
0.2000
0.1500
0.1000
0.0500
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (May-November)


α-tocopherol ng/µl
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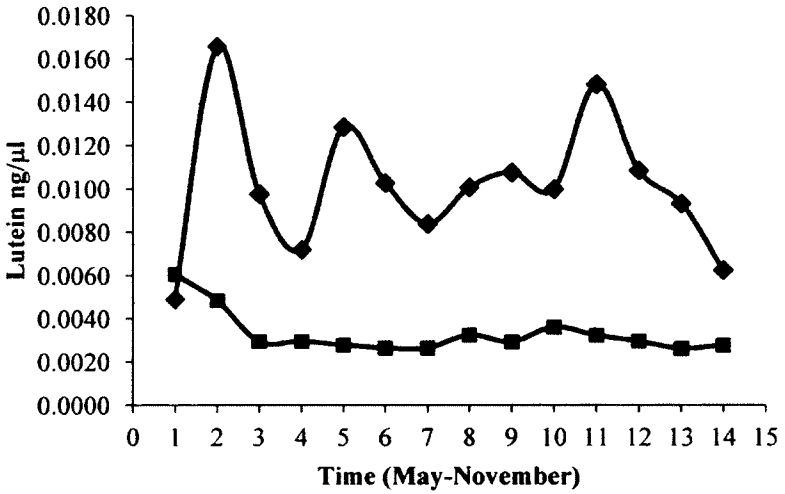
0.4000
0.3500
0.3000
0.2500
0.2000
0.1500
0.1000
0.0500
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (May-November)


Lutein ng/µl
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0.0160
0.0140
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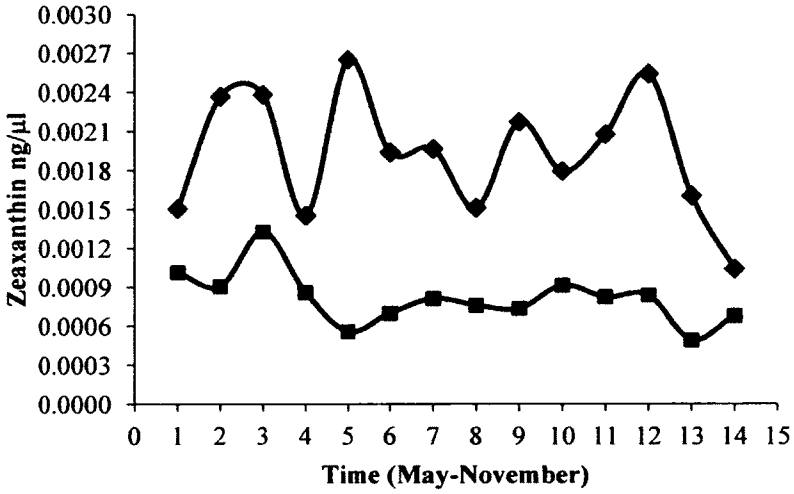
0.0100
0.0080
0.0060
0.0040
0.0020
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (May-November)


Zeaxanthin ng/µl
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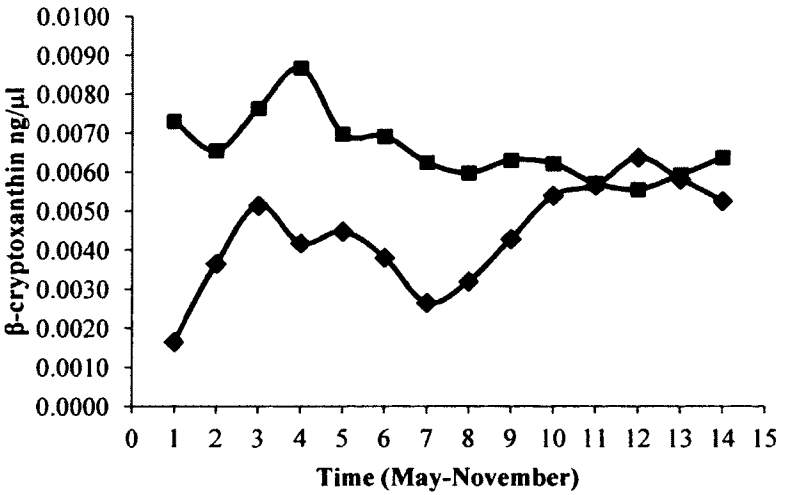
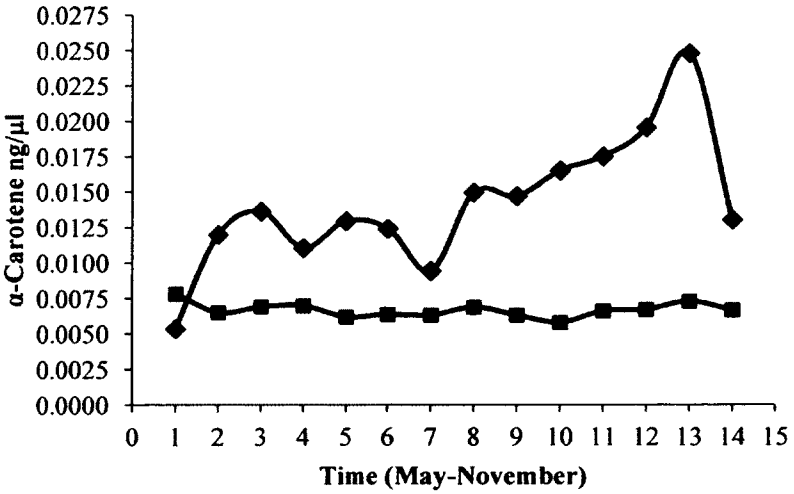
0.0024
0.0021
0.0018
0.0015
0.0012
0.0009
0.0006
0.0003
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (May-November)


β-cryptoxanthin ng/µl
0.0100
0.0090
0.0080
0.0070
0.0060
0.0050
0.0040
0.0030
0.0020
0.0010
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (May-November)


α-Carotene ng/µl
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0.0250
0.0225
0.0200
0.0175
0.0150
0.0125
0.0100
0.0075
0.0050
0.0025
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (May-November)

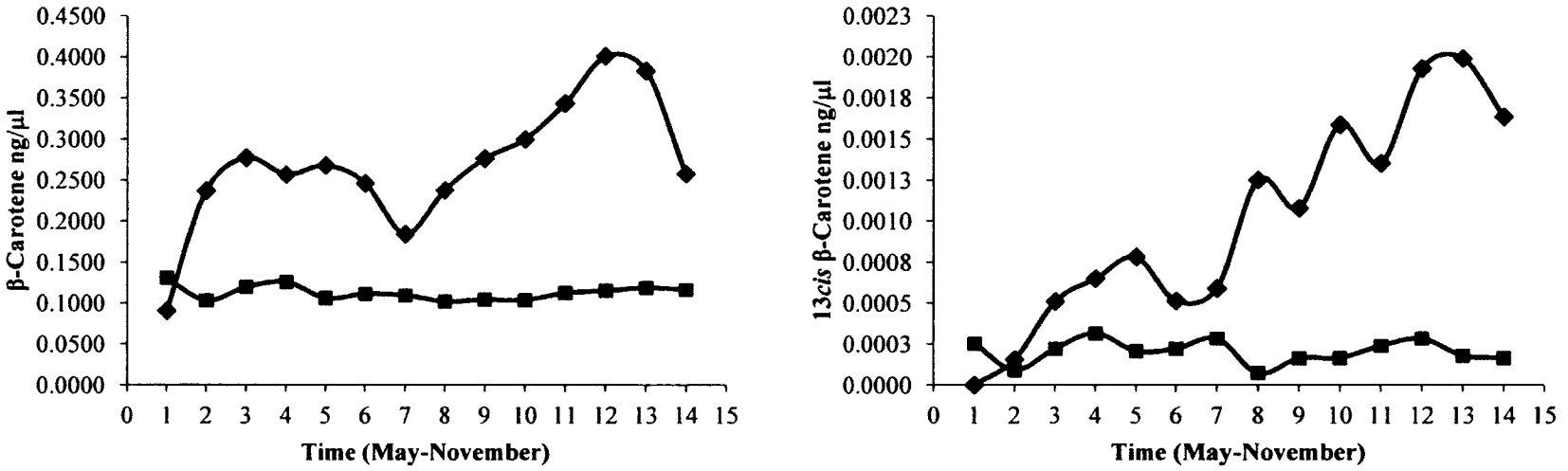


Figure 6. Changes in mean carotenoid and nutrient concentrations (ng/µl) at each biweekly collection point May-November between Organic Dairy (◆) and Fairchild Dairy (■); the first collection took place before the pasture season (May 18 – October 19) at the OD, and the last one after the cows at the OD had come off of pasture

In milk from cows fed pasture at the OD, there was an increase in total carotene concentration in milk between time-point 1 and time-point 6 (before they came off pasture) from 0.1728 to 0.3923 ng/μl (Table 5), respectively, as well as in the amount secreted from 2.09 to 3.139 mg/morning milking (Appendix C). There was also an increase in the β-carotene concentration in milk from the OD from 0.164 to 0.3721 ng/μl and in amount secreted from 1.986 to 2.979 mg/morning milking. Total xanthophyll concentration in milk from the OD increased from 0.0153 to 0.0212 ng/μl, and amount secreted from 0.168 to 0.17 mg/morning milking. Conversely, retinol concentration decreased from time-point 1 to time-point 6 from 0.2642 to 0.1871 ng/μl as well as in amount secreted from 2.854 to 1.489 mg/morning milking.

In milk from cows at the FD, total xanthophyll concentration in milk decreased from time-point 1 to time-point 6 from 0.0133 to 0.0095 ng/μl, as well as in the amount secreted from 0.246 to 0.114 mg/morning milking. Retinol concentration also decreased from 0.4122 to 0.2288 ng/μl, and amount secreted decreased from 5.465 to 2.022 mg/morning milking. Lutein concentration decreased from 0.0055 to 0.0031, and amount secreted decreased from 0.12 to 0.046 mg/morning milking. The concentrations of the remainder of the carotenoids in milk from the FD did not significantly vary over time (Table 6).

Values for overall milk yield, fat corrected milk yield, energy corrected milk yield, as well as nutrient and carotenoid yields can be found in Appendix C. Means were calculated from 9 cows at each dairy across 7 monthly time-points (May – November). Mean fat yield and milk fat at the OD were 401.7g/morning milking and

48.9g/kg/morning milking, respectively, while at the FD they were 594.4g/morning milking and 57.6g/kg/morning milking. Mean protein yield and milk protein at the OD were 294.3g/morning milking and 35.87g/kg/morning milking, respectively, while at the FD they were 415.1g/morning milking and 40.4g/kg/morning milking. Although overall milk yield at the FD was higher than at the OD (Appendix C), mean yields of each of the carotenoids, except β-cryptoxanthin, were still higher in milk from the OD. As expected, mean yields for retinol, α-tocopherol and β-cryptoxanthin were higher in milk from the FD.

Fat corrected yield and energy corrected yield were also calculated using the following equations (129):

Fat corrected yield = (0.4*yield kg) + (15*fat kg)

Energy corrected yield = (0.148*yield kg) + (5.873*fat kg) + (3.519*protein kg)

These values help to determine the amount of fat and/or energy provided by the milk, as variations in milk fat and milk protein content are not reflected in a simple milk yield value. Mean fat corrected milk yield at the OD and FD were 9.37 kg/morning milking and 13.07 kg/morning milking, respectively. Mean energy corrected milk yield at the OD and FD were 10.19 kg/morning milking and 14.27 kg/morning milking, respectively.

Although overall morning milk production was lower from cows at the OD than cows at the FD, almost all of the carotenoid yields/morning milking were higher at the OD. Those higher at the OD were total carotenes (OD = 2.391 mg, FD = 1.375 mg), lutein (OD = 0.089 mg, FD = 0.057 mg), α-carotene (OD = 0.118 mg, FD = 0.078 mg), β-carotene

(OD = 2.264 mg, FD = 1.293 mg) and 13*cis* β-carotene (OD = 0.008 mg, FD = 0.003 mg).

Milk Correlations

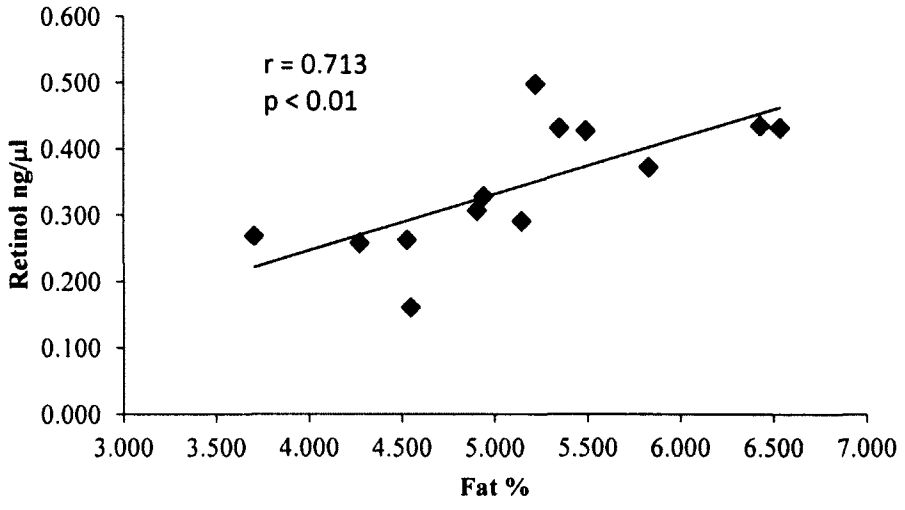
Pearson product-moment correlations were performed for all continuous variables at time-point 1 and for overall mean values, May through November. Figure 7 shows key significant ($p < 0.05$) correlations at time-point 1 including fat percent, retinol, and carotenoids. Figure 8 shows key significant ($p < 0.05$) correlations for overall means including milk yield, fat percent, retinol and carotenoids. Figures for the remainder of the significant correlations can be found in Appendix D and Appendix E. Tables 7 and 8 show all of the correlation values between milk yield, fat percent, and concentrations of each of the nutrients and carotenoids examined.

Fat percent was positively correlated with retinol and β-cryptoxanthin at time-point 1 (Figure 7). Each of these values was significantly higher in milk from the FD. Retinol and β-cryptoxanthin were also positively correlated, as were lutein and zeaxanthin. Beta-carotene was positively correlated with total xanthophylls, lutein, zeaxanthin, and α-carotene. For mean values over the whole time-period, fat percent was positively correlated with concentrations of retinol and β-cryptoxanthin, but negatively correlated with concentrations of lutein, zeaxanthin, and 13*cis* β-carotene (Figure 8). The latter three carotenoids were significantly higher in milk from the OD, while fat percent was significantly lower. Overall milk yield was also negatively correlated with total carotene concentration. Retinol and β-cryptoxanthin were positively correlated, as were lutein and zeaxanthin. Beta-carotene was positively correlated with lutein and α-carotene.

Table 7. Correlations between yield, carotenoids and nutrients[1]

	Fat %	Carotenes	Xanthophylls	Retinol	α-tocopherol	Lutein	Zeaxanthin	Lutein + Zeaxanthin	β-cryptoxanthin	α-carotene	β-carotene	13*cis* β-carotene
Milk yield	0.349	-0.387	-0.102	0.438	-0.341	-0.284	-0.506	-0.341	0.481	-0.144	-0.396	0.130
Fat %		-0.155	0.002	0.713**	0.663*	-0.437	-0.29	-0.437	0.854**	-0.130	-0.157	0.505
Carotenes			0.781**	0.308	0.503	0.762**	0.646*	0.778**	0.131	0.941**	1.000**	0.339
Xanthophylls				0.539*	0.695**	0.877**	0.657*	0.880**	0.393	0.726**	0.781**	0.370
Retinol					0.845**	0.143	0.168	0.153	0.834**	0.405	0.302	0.301
α-tocopherol						0.378	0.185	0.362	0.754**	0.612*	0.495	0.514
Lutein							0.683**	0.992**	-0.081	0.634*	0.782**	0.221
Zeaxanthin								0.769**	-0.111	0.497	0.652*	0.048
Lutein + Zeaxanthin									-0.09	0.640*	0.782**	0.202
β-cryptoxanthin										0.284	0.122	0.383
α-carotene											0.935**	0.325
β-carotene												0.336

[1]Mean values from 14 cows at time-point 1 (baseline)
**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)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Retinol ng/µl
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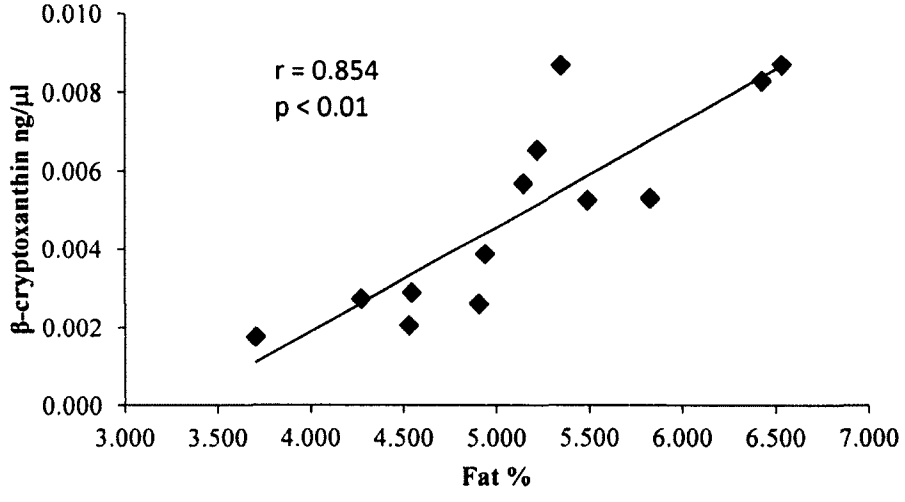
0.500
0.400
0.300
0.200
0.100
0.000
3.000 3.500 4.000 4.500 5.000 5.500 6.000 6.500 7.000
Fat %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β-cryptoxanthin ng/µl
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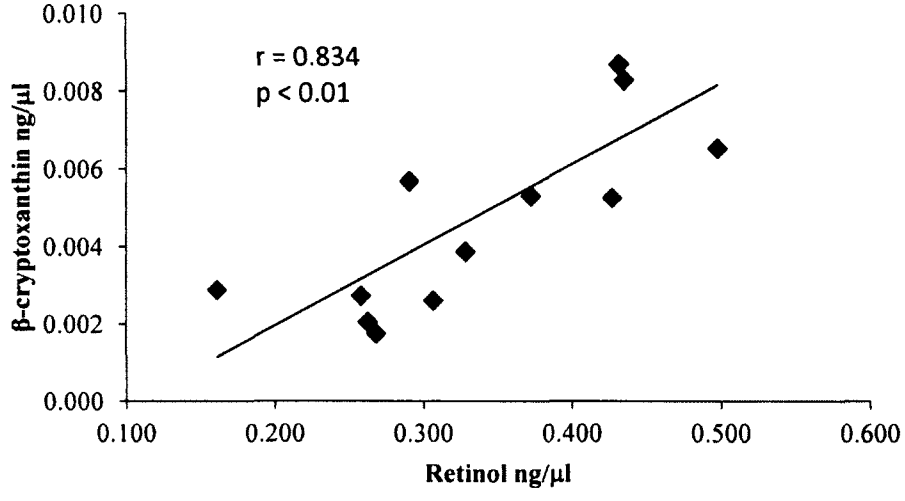
0.008
0.006
0.004
0.002
0.000
3.000 3.500 4.000 4.500 5.000 5.500 6.000 6.500 7.000
Fat %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β-cryptoxanthin ng/µl
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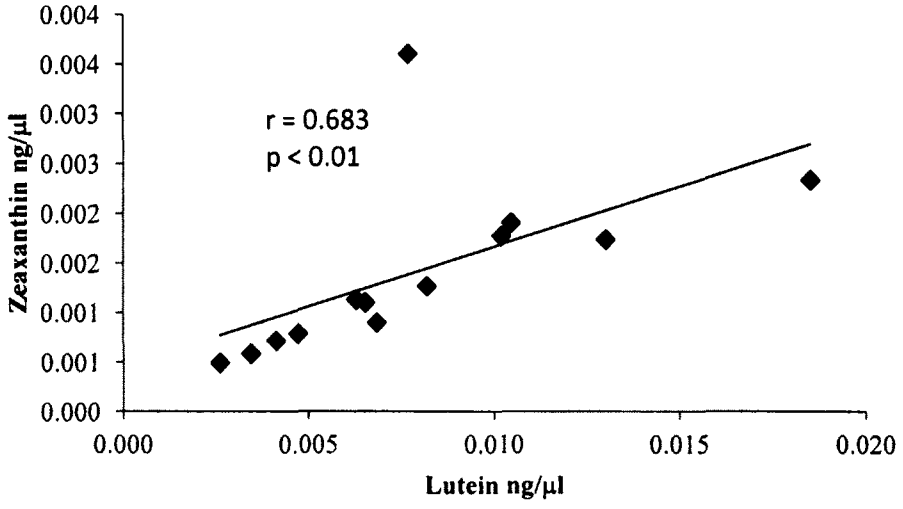
0.008
0.006
0.004
0.002
0.000
0.100 0.200 0.300 0.400 0.500 0.600
Retinol ng/µl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Zeaxanthin ng/µl
Lutein ng/µl
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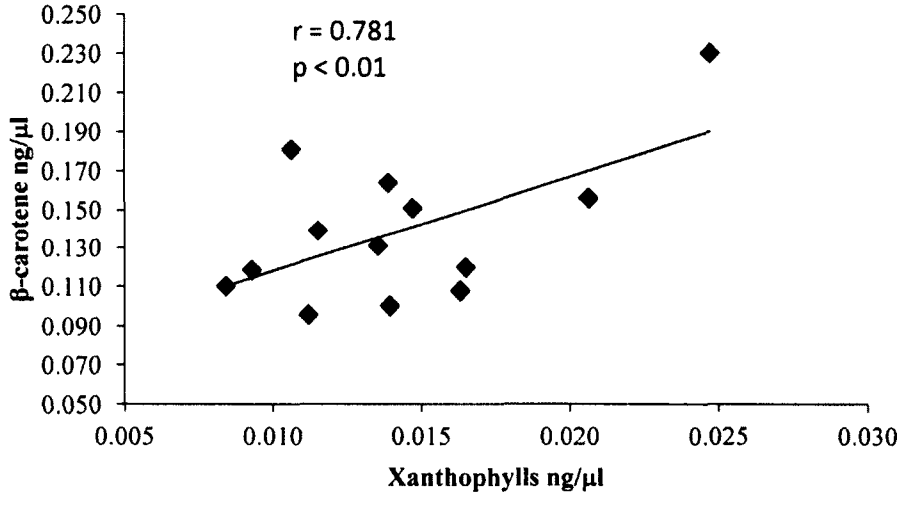
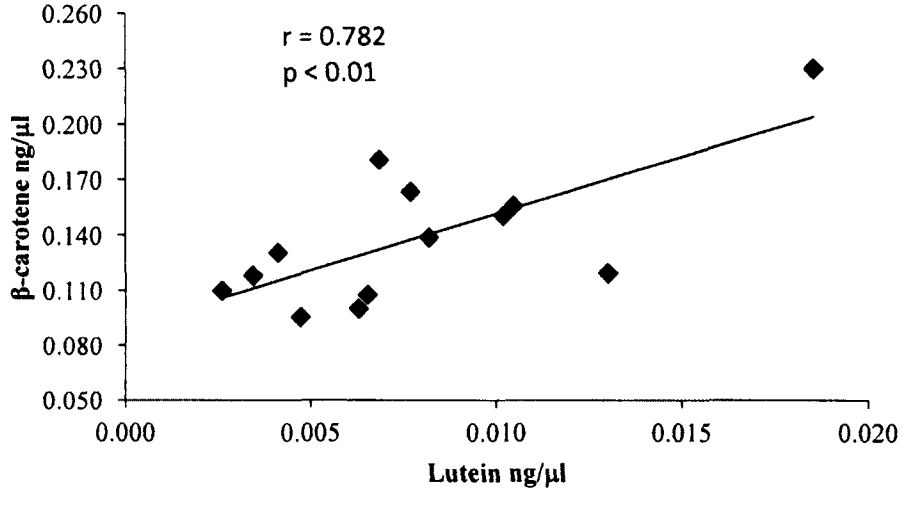
0.004
0.003
0.003
0.002
0.002
0.001
0.001
0.000
0.000
0.005
0.010
0.015
0.020


r = 0.781
p < 0.01
β-carotene ng/µl
Xanthophylls ng/µl
0.250
0.230
0.210
0.190
0.170
0.150
0.130
0.110
0.090
0.070
0.050
0.005
0.010
0.015
0.020
0.025
0.030


r = 0.782
p < 0.01
β-carotene ng/µl
Lutein ng/µl
0.260
0.230
0.200
0.170
0.140
0.110
0.080
0.050
0.000
0.005
0.010
0.015
0.020

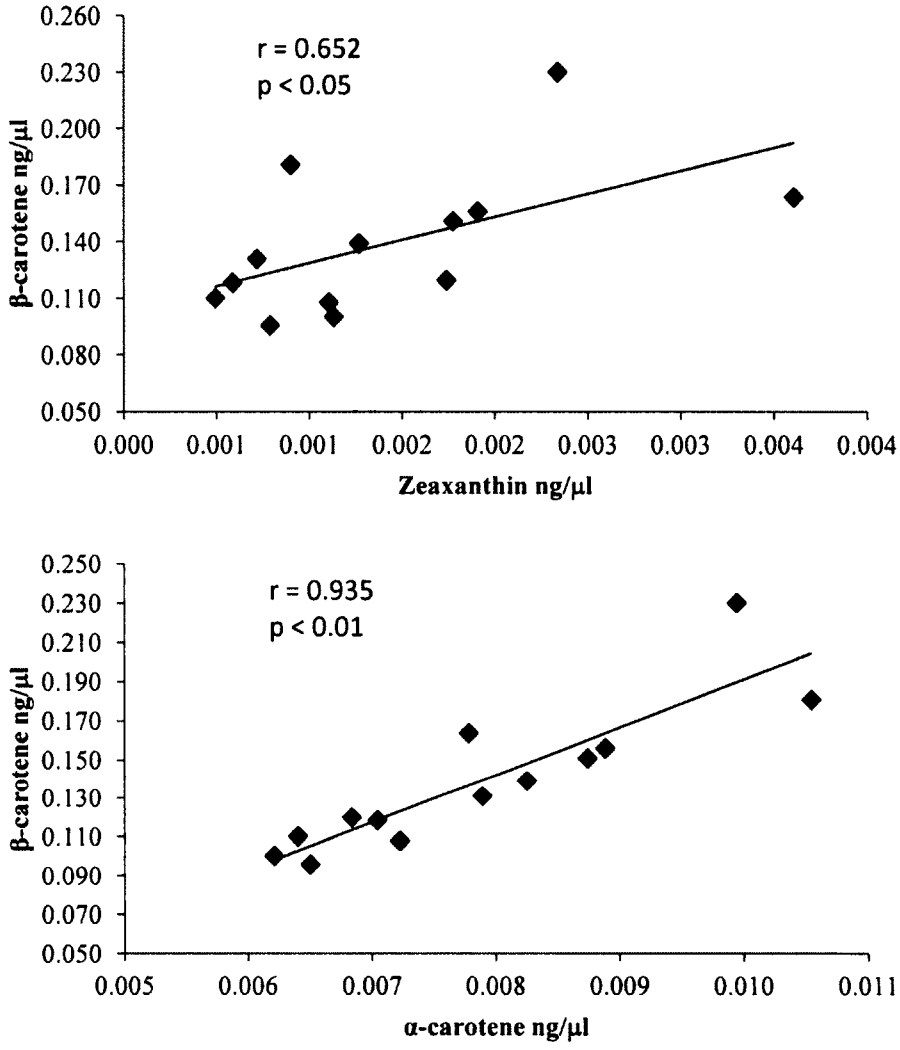
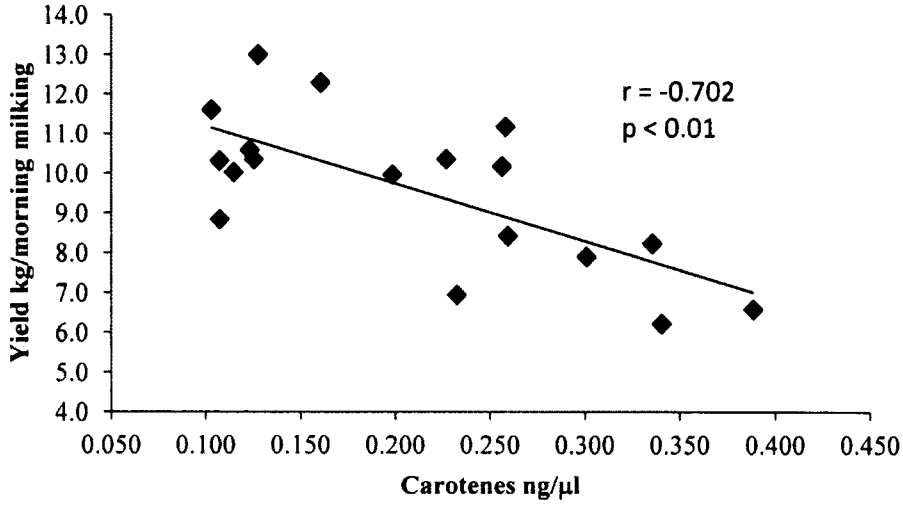


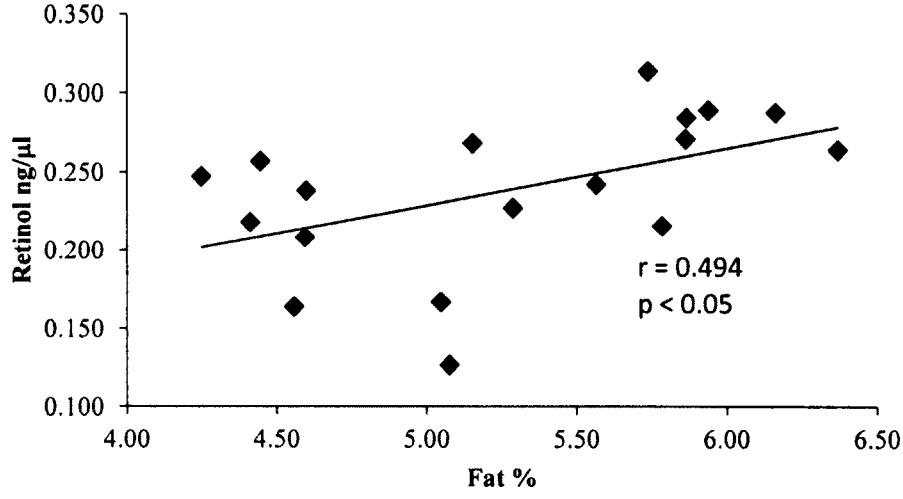
Figure 7. Relation between concentrations of fat percent, vitamins and carotenoids; values are from 14 cows at time-point 1

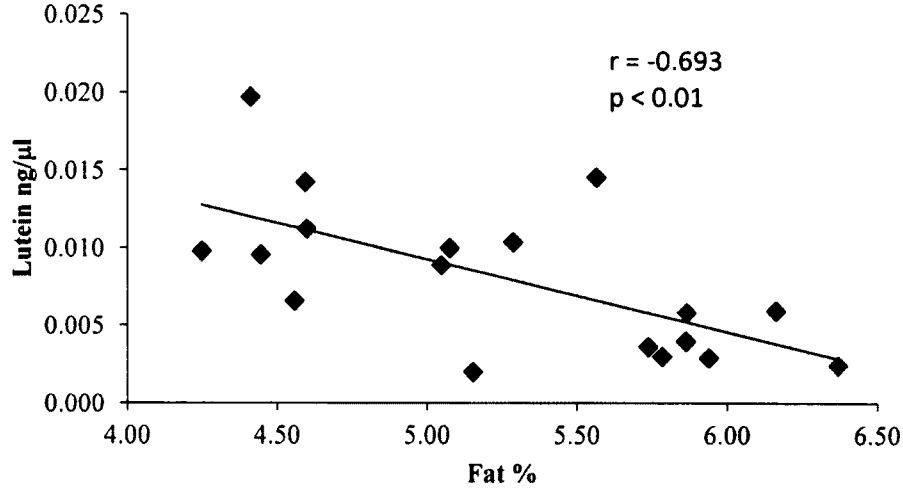
Table 8. Correlations between yield, carotenoids, and nutrients[1]

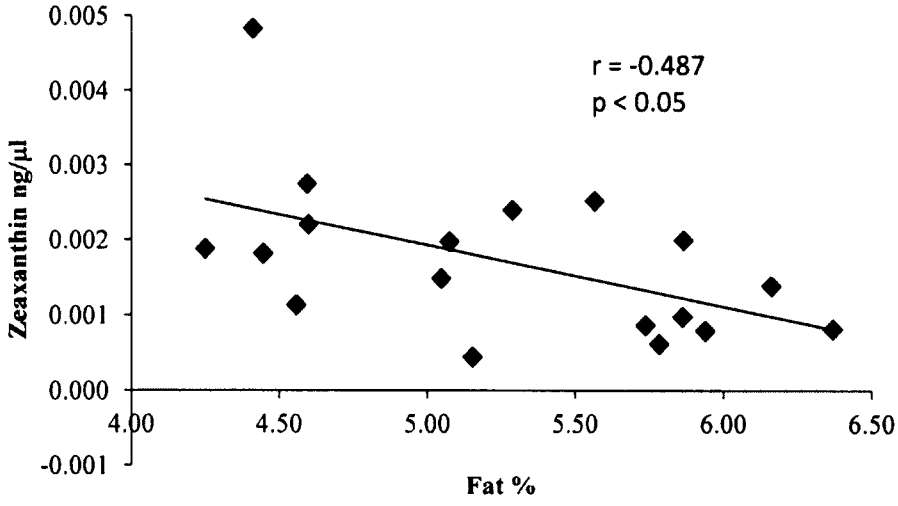
	Fat %	Fat yield	Protein %	Protein yield	Fat corrected yield	Energy corrected yield	Carotenes	Xanthophylls	Retinol	α-tocopherol	Lutein	Zeaxanthin	β-cryptoxanthin	α-carotene	β-carotene	13*cis* β-carotene
Milk yield	0.359	0.900**	0.260	0.928**	0.946**	0.943**	-0.702**	-0.200	0.247	0.139	-0.405	-0.272	0.398	-0.722**	-0.700**	-0.430
Fat %		0.723**	0.841**	0.623**	0.639**	0.641**	-0.406	-0.360	0.494*	0.162	-0.639**	-0.487*	0.584*	-0.461	-0.421	-0.483*
Fat yield			0.580*	0.971**	0.993**	0.992**	-0.724*	-0.333	0.439	0.210	-0.611**	-0.440	0.564*	-0.763**	-0.726**	-0.562*
Protein %				0.598**	0.502*	0.529*	-0.432	-0.135	0.425	0.155	-0.412	-0.179	0.606**	-0.428	-0.423	-0.484*
Protein yield					0.638*	0.641	-0.757**	-0.225	0.494*	0.162	-0.639*	-0.304	0.563*	-0.774**	-0.749**	-0.546*
Fat corrected yield						0.999**	-0.731**	-0.301	0.391	0.195	-0.565*	-0.401	0.532*	-0.766**	-0.733**	-0.534*
Energy corrected yield							-0.741**	-0.287	0.394	0.199	-0.556*	-0.383	0.545*	-0.772**	-0.740**	-0.542*
Carotenes								0.380	-0.458	-0.011	0.592**	0.348	-0.337	0.964**	0.992**	0.767**
Xanthophylls									-0.153	0.172	0.899**	0.957**	0.343	0.430	0.383	0.387
Retinol										0.580*	-0.412	-0.215	0.569*	-0.501*	-0.444	-0.541*
α-tocopherol											-0.063	0.043	0.646**	-0.045	0.009	0.077
Lutein												0.923**	-0.084	0.640**	0.609**	0.554*
Zeaxanthin													0.176	0.432	0.356	0.353
β-cryptoxanthin														-0.332	-0.341	-0.226
α-carotene															0.963**	0.837**
β-carotene																0.786**

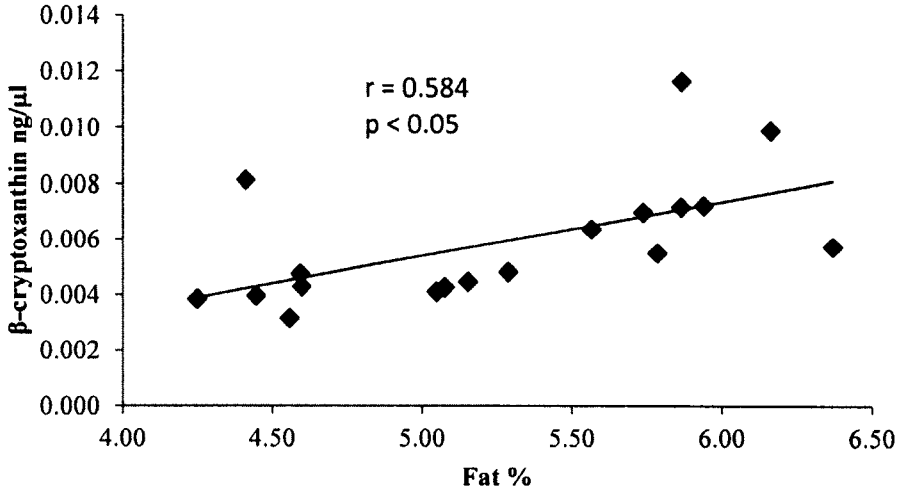
[1]Overall mean values from 18 cows
**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)

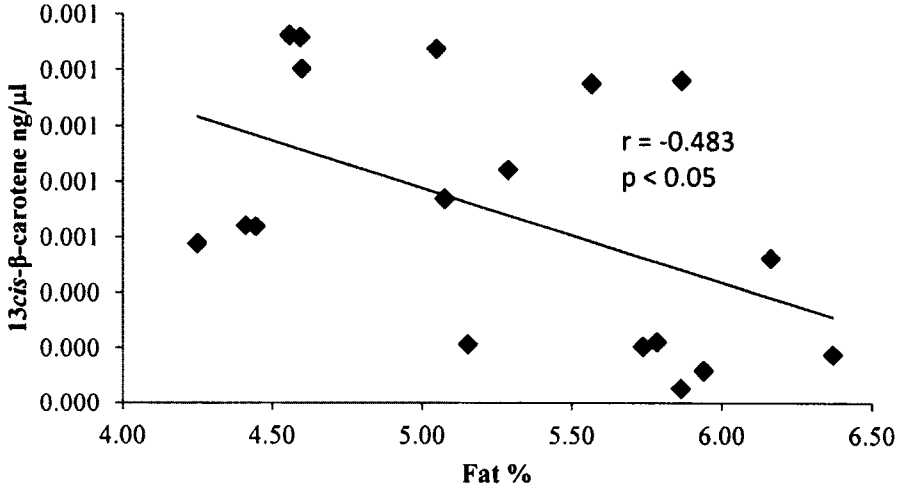

Yield kg/morning milking
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
r = -0.702
p < 0.01
0.050
0.100
0.150
0.200
0.250
0.300
0.350
0.400
0.450
Carotenes ng/µl


Retinol ng/µl
0.350
0.300
0.250
0.200
0.150
0.100
r = 0.494
p < 0.05
4.00
4.50
5.00
5.50
6.00
6.50
Fat %


Lutein ng/µl
0.025
0.020
0.015
0.010
0.005
0.000
r = -0.693
p < 0.01
4.00
4.50
5.00
5.50
6.00
6.50
Fat %


0.005
0.004
0.003
0.002
0.001
0.000
-0.001
Zeaxanthin ng/µl
r = -0.487
p < 0.05
4.00
4.50
5.00
5.50
6.00
6.50
Fat %


0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
β-cryptoxanthin ng/µl
r = 0.584
p < 0.05
4.00
4.50
5.00
5.50
6.00
6.50
Fat %


0.001
0.001
0.001
0.001
0.000
0.000
0.000
13cis-β-carotene ng/µl
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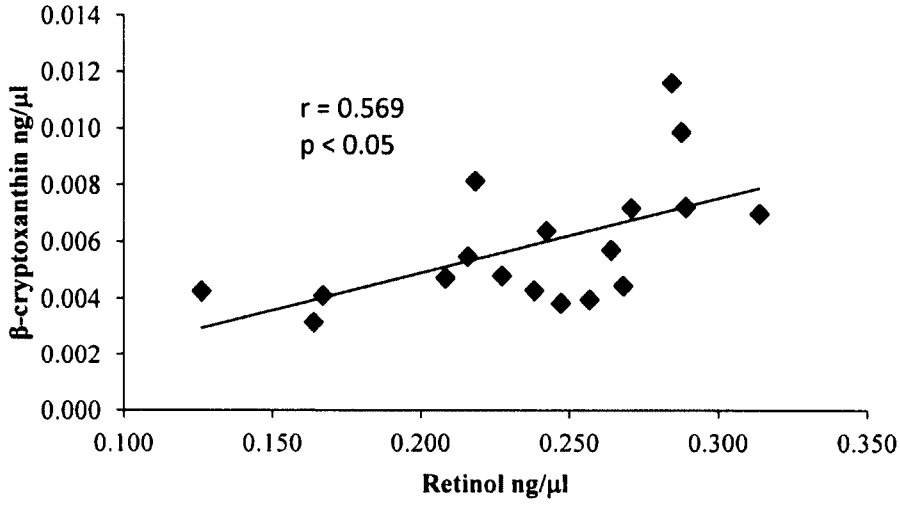
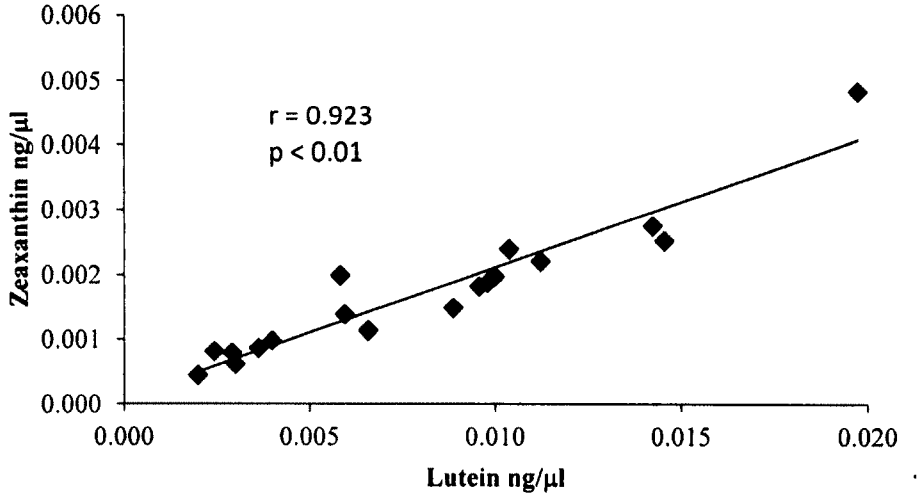
p < 0.05
4.00
4.50
5.00
5.50
6.00
6.50
Fat %


0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
β-cryptoxanthin ng/µl
r = 0.569
p < 0.05
0.100
0.150
0.200
0.250
0.300
0.350
Retinol ng/µl


0.006
0.005
0.004
0.003
0.002
0.001
0.000
Zeaxanthin ng/µl
r = 0.923
p < 0.01
0.000
0.005
0.010
0.015
0.020
Lutein ng/µl

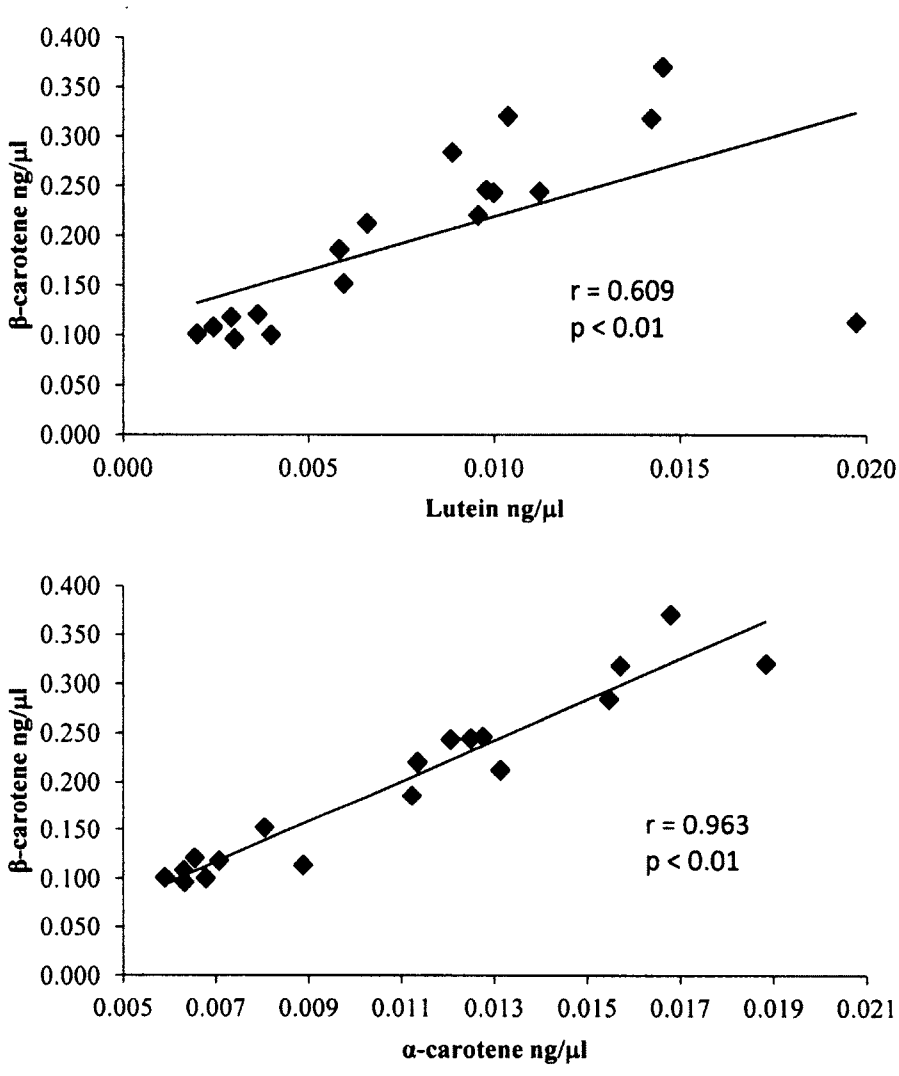


Figure 8. Relation between milk yield, concentrations of vitamins and concentrations of carotenoids; values are means for 18 cows over all 7 time-points

Cheese Correlations and Color

Mean concentrations and yields of nutrients and carotenoids in raw milk, pasteurized milk, whey and cheese, as well as mean total cheese yield at the OD and FD can be found in Appendix F. The correlations between the carotenoid and nutrient concentrations in cheese and the concentrations in raw milk, pasteurized milk, and whey are reported in Table 9, while the key significant correlations are illustrated in Figure 9. As expected, total carotenes in cheese were positively correlated with the milk, both raw and pasteurized, and with the whey. Total xanthophylls in cheese were only correlated with total xanthophylls in pasteurized milk. None of the other individual components in cheese were correlated with raw milk, pasteurized milk, or whey.

Table 9. Correlations between cheese components, and components in raw milk, pasteurized milk and whey

	Raw milk	Pasteurized milk	Whey
Carotenes	0.988**	0.974**	0.992**
Xanthophylls	0.337	0.789*	-0.373
Retinol	0.264	0.506	0.556
α-tocopherol	-0.265	-0.381	-0.312
Lutein	0.340	0.386	-0.459
Zeaxanthin	0.275	0.658	-0.426
β-cryptoxanthin	-0.206	0.518	-0.262
α-carotene	0.210	-0.065	0.495
β-carotene	0.383	0.084	0.472
13*cis* β-carotene	0.389	0.619	0.470

**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)

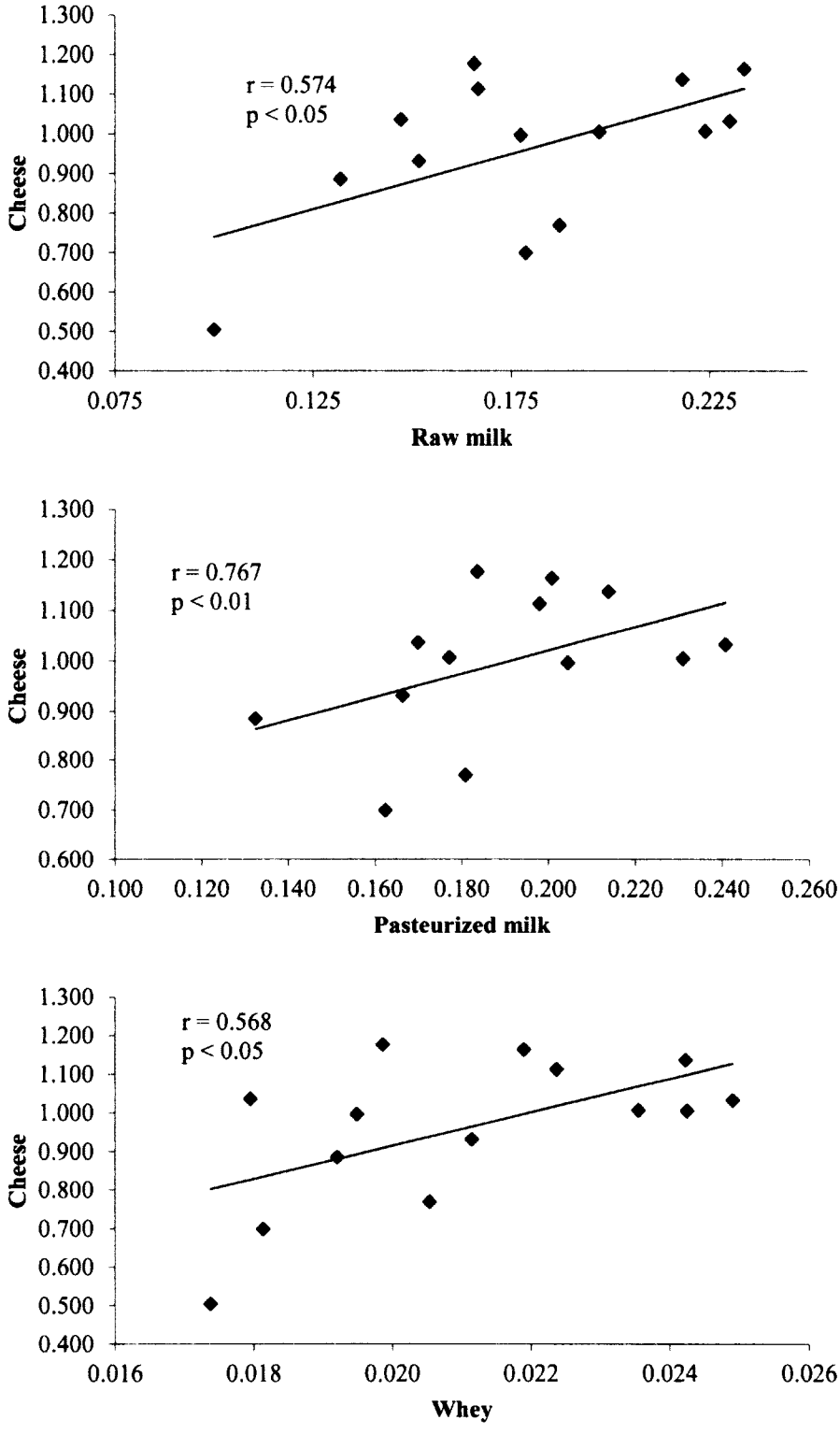


Figure 9. Relation between carotene concentrations in cheese and concentrations in raw milk, pasteurized milk, and whey; values are means of both dairies at each collection point (reported in ng/μl)

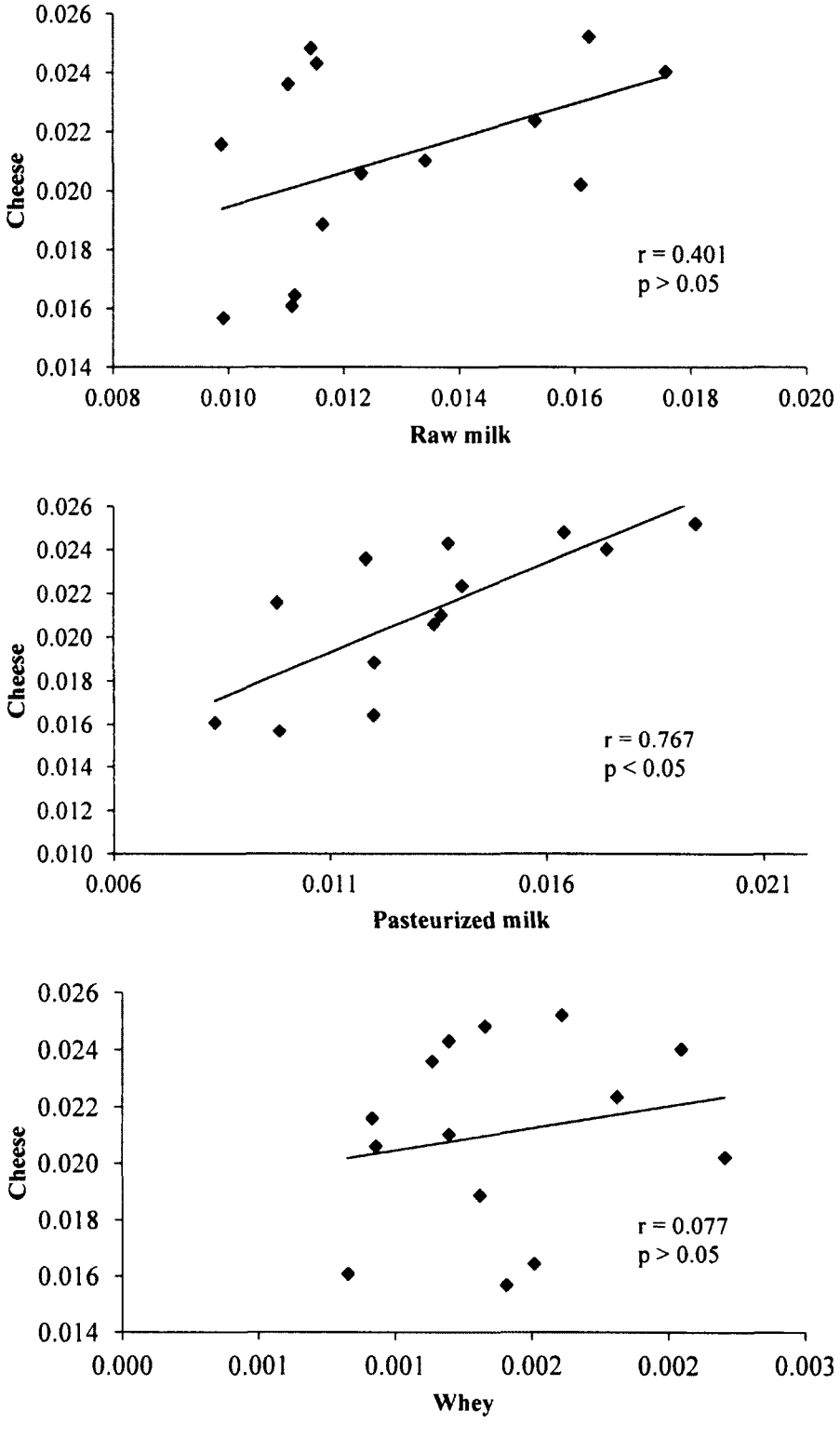


Figure 10. Relation between xanthophyll concentrations in cheese and concentrations in raw milk, pasteurized milk, and whey; values are means of both dairies at each collection point (reported in ng/μl)

Table 10 reports the variations in color index of the mozzarella cheese produced at each collection point throughout the entire time period (May-November). Time 1 reflects the color index of cheese before the cows at the OD went on pasture, and time 14 the color index of cheese after the cows at the OD came off pasture.

Table 10. Variation in color index of mozzarella cheese over time and between dairies[1]

Time[2]	OD	FD
1	6.32	6.31
2	14.64	6.49
3	15.82	5.73
4	12.53	5.41
5	14.06	5.46
6	13.49	6.77
7	11.66	5.81
8	11.83	6.06
9	15.90	5.97
10	15.47	4.87
11	17.57	7.37
12	15.93	5.74
13	16.16	4.54
14	12.98	5.56

[1]Color index reported as ΔE*ab
[2]Cheese produced at each collection point over the entire time period

Between time 1 (before pasture) and 13 (right before coming off pasture) the difference in color index increased in cheese made with milk from the OD from 6.32 to 16.16, respectively (Table 10). The difference in color index did not increase over the season in cheese made with milk from the FD. Figure 10 illustrates the variation between dairies over time.

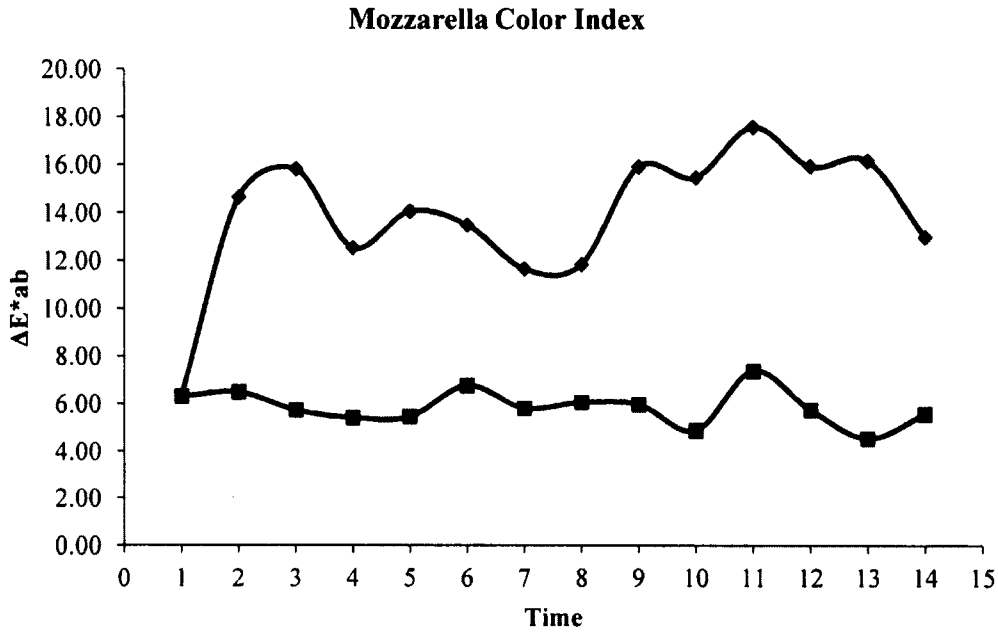


Figure 10. Variation in color index of mozzarella cheese made biweekly with milk at each collection May-November from the Organic Dairy (◆) and Fairchild Dairy (■)

Figure 11 displays the overall mean values for protein yield (g) and mozzarella cheese yield (g) from milk at the Organic and Fairchild Diary (Appendix F). As hypothesized, mean yields of protein and cheese were higher at the FD than the OD.

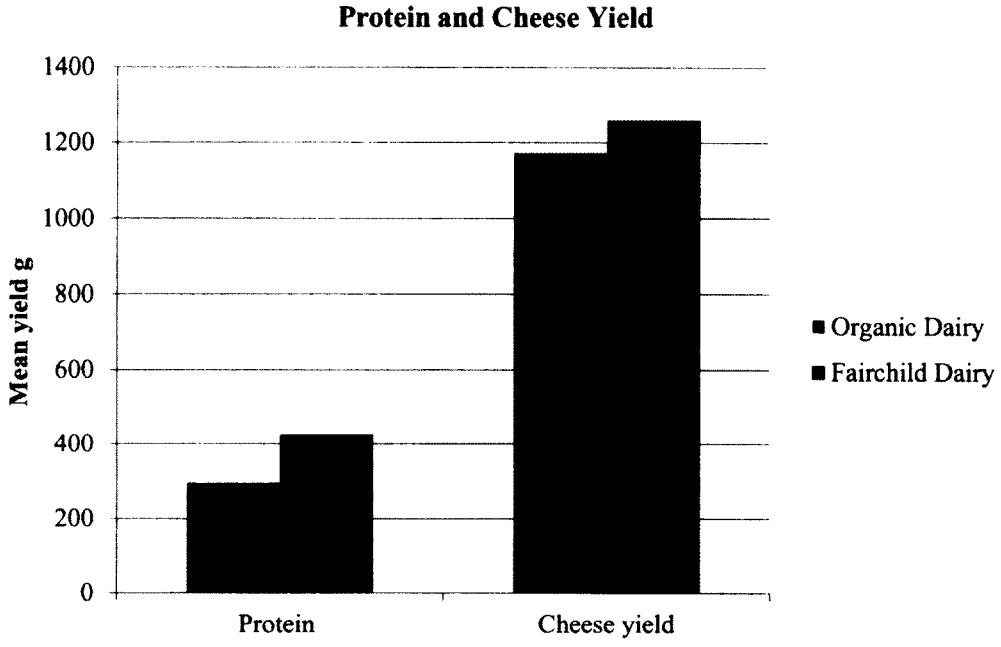


Figure 11. Comparison of mean protein and mozzarella cheese yields between the Organic Dairy and the Fairchild Dairy

Regression Analysis

Multiple regression analyses were conducted with mean data from all 18 cows across all time-points to examine the relationship between each carotenoid concentration in milk and three predictors: group (OD and FD), milk yield, and fat percent. Table 11 summarizes the analysis results. The regression models that were significant with all three predictors were total carotenes ($R^2 = 0.872$, $F = 31.807$, and $p < 0.001$), retinol ($R^2 = 0.426$, $F = 3.458$, and $p < 0.05$), lutein ($R^2 = 0.446$, $F = 3.761$, and $p < 0.05$), β-cryptoxanthin ($R^2 = 0.367$, $F = 4.284$, and $p < 0.05$), α-carotene ($R^2 = 0.809$, $F = 19.762$, and $p < 0.001$), β-carotene ($R^2 = 0.856$, $F = 27.667$, and $p < 0.001$), and 13*cis* β-carotene ($R^2 = 0.516$, $F = 4.984$, and $p < 0.05$).

Table 11. Summary of multiple regression analysis for variables predicting carotenoid concentrations

	β-Coefficients			Model		
	Group	Yield lbs	Fat %	R^2	F	*P*-value
Carotenes	-0.958**	-0.214	0.307*	0.872	31.807	0.000
Xanthophylls	0.178	-0.155	-0.424	0.147	0.805	0.512
Retinol	0.67-	-0.208	0.122	0.426	3.458	0.046
α-tocopherol	0.183	0.019	0.034	0.048	0.235	0.870
Lutein	-0.089	-0.159	-0.524-	0.446	3.761	0.036
Zeaxanthin	0.309	-0.243	-0.607-	0.131	1.858	0.183
β-cryptoxanthin	0.495	0.002	0.253	0.367	4.284	0.024
α-carotene	-0.793**	-0.288-	0.000	0.809	19.762	0.000
β-carotene	-0.931**	-0.217	0.276-	0.856	27.667	0.000
13*cis* β-carotene	-0.734*	0.031	-0.005	0.516	4.984	0.015

**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)

CHAPTER VII

DISCUSSION

Dietary carotenoids play an important biological role in human health. The focus of this lab's research has been on the variations and bioavailability of carotenoids in various plants and egg yolk, with an interest in factors that impact concentration in food and carotenoid uptake in the human body. The current research investigated milk and cheese as another nutritionally and economically valuable source of dietary carotenoids.

The purpose of this study was to investigate the changes in nutritional value of milk and cheese as a function of feeding practice and season, with the goal of comparing what is gained and/or lost in product quality. The variables specifically examined were carotenoids, retinol, fat, protein, and overall milk yield.

Interest in and desire for foods with health promoting properties is increasing among consumer. As scientific discoveries continue to inform our understanding of how food and its components affect human health, our ability to meet the demand for nutrient rich foods also increases. Functional foods, with added components of interest, have become common placed in today's market. Discovering ways to increase beneficial components of food in a cost effective and natural way could increase marketability for the farmer and cheesemaker and could increase consumption of those beneficial components in the consumer, either by promoting an increase in consumption of the milk/cheese, or simply

by the greater concentration of the components of interest in the milk/cheese already being consumed.

Milk Composition

Many factors have the potential to impact the composition of milk. There has been extensive research investigating the effects of feeding practice specifically on milk composition. Milk from cows fed on pasture has been shown to contain different components than milk from cows fed a total mixed ration, and the composition of milk from cows fed on pasture can also change considerably throughout the seasons. Less research has focused specifically on the changes in carotenoid concentration in milk as a result of changes in the factors.

Since the composition of dairy products is influenced by the nature of the diet ingested by cows, this factor is an important consideration in consumer perception of product quality. Dairy products derived from animals fed grass-based diets are often perceived in a positive way as having a "clean and green image" in relation to both nutritional and sensorial interest (101). In the last decade or so, a new market niche has emerged for grass-fed meat and dairy products, a market segment that before then didn't exist (130). Where grass-fed products used to be sold at a discount, they now sell at a premium. Consumers are increasingly demanding clear information regarding production systems, and are interested in sustainable agriculture, in how animals are treated, and in the nutritional quality of their food (130).

Another contributing factor to the rising popularity of pasture feeding for dairy farmers in the US is the growing organic industry. According to the USDA Certified Organic

standards, producers are required to feed their cows on pasture during the pasture season. More specifically, they must provide pasture as a minimum of 30 percent of a ruminant's dry matter intake, on average, over the course of the pasture season (131).

Organic agriculture is developing rapidly and is now practiced in more than 120 countries of the world (132). In North America specifically, there has been an increase of almost 30 percent in the amount of land used for organic agriculture since 2004, the highest rate of growth of any continent. In the US, the organic industry grew at a rate of nearly eight percent in 2010 (to over $28.6 billion), compared to the growth of total food sales that year, which was only 0.6 percent (133). Organic dairy is the second largest growing organic food category, and it experienced nine percent growth to achieve a value of $3.9 billion – and captured nearly six percent of the total US market for dairy products.

One of the primary reasons for the growth of this market is, again, the growing consumer demand. Demand remains concentrated in North America, which is currently experiencing undersupply because production is not meeting demand (132), suggesting the need for this area of the market to continue to grow.

Multiple studies have examined the economic advantages and disadvantages to pasture based management systems (134). Dairy farmers are always being challenged to find ways to increase farm profitability to stay competitive. One of the largest costs for dairy farmers is animal feed. A pasture-based system has the potential to lower those costs by replacing much of the stored forages in the ration, and by lowering capital costs for machinery, manure systems and facilities (134). Cows fed pasture have been shown to produce less milk than cows fed a total mixed ration. However, despite lower milk yields,

economic models and farm surveys have shown that pasture-based systems can have lower operating expenses than confinement systems (134). Long term comparisons of these systems are needed.

The main finding of the current study is that the concentrations of carotenoids (all but zeaxanthin $p = 0.086$) in milk, as well as retinol and α-tocopherol, were significantly different between dairies. Milk production and fat percent were both significantly higher at the FD, which is consistent with previous literature on the effect of pasture on these variables. Concentrations of retinol and β-cryptoxanthin were also significantly higher in milk from the FD. The higher retinol may be due to the higher concentration of vitamin A supplementation in the total mixed ration at the FD (Appendix B) compared to the concentration in the diet at the OD (Appendix A), and the higher β-cryptoxanthin may be due to the higher content of corn in the total mixed ration at the FD. Concentrations of each of the remaining carotenoids, however, were significantly higher in milk from the OD, mostly likely due to an increase in consumption of these components from the pasture. Even with lower overall milk yields at the OD, the mean yields were still numerically higher (Appendix C) in milk from the OD than in milk from the FD, showing that the increase in carotenoids was not simply due to the dilution effect. These results are valuable because they demonstrate the potential nutritional benefits of milk produced under a pasture-based feeding system.

When analyzed separately, results showed that the concentration of each component of interest in milk from the OD, except zeaxanthin ($p = 0.29$) significantly changed in over time ($p < 0.01$) independent of any group effects. The overall milk yield and fat percent also significantly changed over time ($p < 0.01$). In the milk from the FD, lutein was the

only carotenoid that changed significantly over the season ($p = 0.004$); lutein + zeaxanthin changed significantly over time, as did the total xanthophylls, but this was primarily due to the lutein, as neither the zeaxanthin nor the β-cryptoxanthin change over time were significant. Retinol and α-tocopherol also significantly changed over time ($p < 0.05$) in milk from the FD. These results confirm the hypothesis that milk from cows fed on pasture is subject to great variation in its carotenoid and nutrient composition throughout the pasture season, to a greater degree than milk from cows fed a total mixed ration. It is important to note that time-point 1 is a mean of the first two collections, one before the cows at the OD began their pasture diet and one after. Thus, an even greater increase in carotenoid concentrations would be reflected in the results had the first collection alone been compared to the last collection taken before the cows came off pasture.

These findings are significant because they confirm that not only are the concentrations of these nutrients of interest highly variable throughout the season, variations for which the current nutrient databases do not currently account, but that change is also dependant on the management system/farming practices of the dairy from which it comes. Because there are many factors that cannot always be taken into account with studies when looking at two different farms, it is not possible to attribute these variations completely to the pasture feed compared to the total mixed ration. However, feeding practice was the primary difference between the dairy and is likely the cause. This information is valuable because dairy farmers trying to increase the quality of their milk need to understand the farming practices that may increase concentrations of beneficial components. Future

research should continue to shed light on the changes that occur as a result of breed, varying feeding systems and feed types, and over extended periods of time.

A regression analysis was run for each of the nutrients of interest with group (OD and FD), total milk yield and fat percent as the predictors. Several of the components of interest resulted in significant regression models, including total carotenes ($p < 0.001$), retinol ($p < 0.05$), lutein ($p < 0.05$), β-cryptoxanthin ($p < 0.05$), α- and β-carotene ($p < 0.001$), and 13*cis* β-carotene ($p < 0.05$). This is valuable to farmers as these predictors are easily known to them and equations made with these coefficients could provide them with the ability to predict levels of these beneficial components without the cost/expertise needed for analysis.

Advantages of the Study

Previous studies that have examined changes in carotenoid concentrations in milk have compared milk from multiple breeds or breeds other than Jerseys, and have been conducted primarily in Europe. They have also looked at differences from either one time-point, over a very short period of time, or with only a few collection points over a longer period of time.

Recently, Noziere et al., (2006) found that the concentrations of β-carotene in milk, as well as the amount secreted in the milk, were higher in cows fed grass silage compared with hay diets (98). The study included thirty-two Montbeliarde and Holstein dairy cows that were either fed diets high in carotenoids (grass silage-based diet) or low in carotenoids (hay-based diet) over an 8 wk experimental period beginning in March. The results also showed an effect of time on the carotenoids. In the cows fed the grass silage

diets, there was an increase in both concentration and yield of carotenoids in the milk between d 1 and 50. Around the same time Calderon et al., (2007) found similar results when examining the variations in carotenoid, vitamins A and E concentrations in milk of dairy Montbeliarde cows following a shift from a hay diet to diets containing increasing levels of grass silage over a 6wk period (101). There was a positive relationship between the amount of grass silage in the diet and the concentrations and amounts secreted of β-carotene and vitamin E in the fluid milk and milk fat.

Agabriel et al., (2007) conducted a large scale study looking at 204 farms throughout France distinguished by their forage system (grassland vs. corn silage-based diets) as well as their altitudes (103). The cows on these farms were Montbeliarde and Holstein breeds, and milk was sampled from the bulk tanks 5 times: twice during the winter (February-March) and three times during the grazing period (May-September). Bulk tank milk was examined to quantify the variability of carotenoids and vitamins A and E, and result showed that β-carotene and lutein concentrations were higher during the grazing period than during the winter feeding period.

These studies support the idea that diet and season impact the concentration and secretion of carotenoids in milk. They were, however, done over short periods of time and with grass silage as opposed to pasture. The advantage of the current study is that milk was examined over a six month period of time before, during, and after the pasture season. As different types of forage contain varying amounts of carotenoids it is valuable to see how pasture impacts the milk composition over the whole season, as would be realistic for farmers who choose this method of feed. Breed and location have also been shown to affect milk composition and it is important to understand how carotenoids in milk from

Jersey cows vary, as they are known to secrete higher levels of β-carotene than some other breeds, and to see how these effects might impact farmers in the Northeastern parts of the US.

Slots et al., (2009) also looked at milk from different farms to find out how composition of milk produced by dairy farmers could be differentiated by the management at the dairy farm (102). Milk was collected from 30 commercial dairy herds, with a mix of Holstein-Friesian and Jersey cows, covering three milk production systems in Denmark and the United Kingdom: conventional (total mixed ration-based diets) and organic (pasture- and grass silage-based diets supplemented with total mixed ration) milk production and an extensive (exclusively pasture-based diets) milk production system. Milk samples were taken from the bulk tanks at the conventional and organic diaries at five time-points and from the bulk tanks at the extensive milk production system at 4 time-points. Beta-carotene was the only carotenoid examined in the milk, and the results showed no significant difference in concentration between the conventional and organic systems. It did, however, show a significant increase in concentration in milk from the extensive system – possibly due to the lower daily milk yield in the extensive system, resulting in less dilution.

Although these results showed a lack of significant variation between the β-carotene concentrations in milk from the two feeding systems investigated in the present study, there are several reasons why this may have occurred. There are many confounding variables that may impact results when looking at farms all over the country, and especially in different countries. It is difficult to attribute effects, or lack of effects, directly to feed when so many other factors are involved – such as location, other

differences in management practices, and multiple breeds of cow etc. In the current study, although two different farms were used and management variability may have impacted results, the effect of location is most likely not relevant as they are both in New Hampshire, and the breed of cow was the same at both farms.

The significantly higher concentrations of β-carotene found in the milk from extensive milk production dairies, as in the research conducted by Slots et al., supports the idea that pasture increases this component in milk, however, this type of feeding system is not used in Northeastern United States and so it is necessary to conduct research on practices that are currently used and possible to begin using by farmers here.

This is the first study to look at carotenoids variations in milk from Jersey cows only as a function of season and feeding practice. By comparing milk from a single breed, this study removed the potential confounding effect of breed, which has been shown to affect milk composition. Thus far, no research has been conducted examining carotenoid concentration in milk from cows fed pasture versus a total mixed ration in New Hampshire. The value of this study is that farmers in this region will be able to apply these results more directly to their own practices. Samples of milk from individual cows were taken biweekly over a seven month period, which allowed for acute and long term changes to be observed. Samples were also taken from individual cows, as opposed to just from the bulk tank. This allowed for greater specificity in the results with the possibility that one or more cows were driving the overall concentrations in the milk.

Cheese Composition

Few studies have shown empirical evidence of associations between cheese quality and color and variations in farming practice, using panels to measure sensory criteria (123). Evidence from objective measurements is even more limited, because of the difficulty in accurately discriminating between specific effects of factors within the farm setting (i.e., feed, management, etc.) and those linked to the cheesemaking process (124).

The goal of this study to determine the relationship between the carotenoid concentrations in a fresh, Mozzarella cheese and the carotenoid concentrations in the milk from which it was made. While no connections are being made directly between the cheese and the farming practices or feed, this would be a beneficial area of future research for cheesemakers. The advantage to using a fresh cheese in the current study, as opposed to an aged cheese, was that we were able to examine the nutrient stability throughout the immediate cheese making process, as a first step to understanding how carotenoids transfer from milk to cheese. The majority of the research thus far has examined aged cheeses, which must take into account any changes that also occur during the ripening period. In the current study, an additional benefit of examining carotenoids in a fresh cheese is that this type of cheese is economically valuable to a local farmer. Mozzarella cheese can be made and sold immediately. If beneficial components in the cheese are increased, it can be sold as a 'value added' product, and information on variations of these components is an important aspect of the farmer's ability to market.

With regards to the cheesemaking process, it is possible that degradation of carotenoids and retinol, having been shown to be sensitive to different physico-chemical factors

including air, oxidizing agents and ultraviolet light, may be accelerated by the environment created during the cheesemaking process. Retinol can also be quite unstable at pH 4.5 or lower (87). Consequentially, treatments such as heating and acidification used when processing milk to produce cheese, as well as the immediate processing and storage environment, are likely to influence the concentration of these components in the resulting dairy products. Because the processing of milk to produce cheese involves selective transfer of constituents from the milk to the cheese, it is possible that some of the carotenoids and/or retinol are lost to the whey during the cheesemaking process.

The current study showed a significant positive correlation between concentration of total carotenes in milk and cheese. The xanthophylls in cheese were less related to the xanthophylls in milk, with concentration in pasteurized milk being the only significant correlation with concentration in cheese. Other significant correlations were found between raw milk, pasteurized milk and whey, but no other correlations with cheese were shown to be significant.

As previously noted, current nutrient databases (84) do not account for variations in food components. Table 12 shows the differences in vitamin and carotenoid content in Mozzarella cheese between those reported in the *USDA National Nutrient Database for Standard References* and those found in the current study. For an artisan cheesemaker, it is valuable to understand how and why these variations might occur.

Table 12. Nutrient composition of Mozzarella cheese, whole milk[1]

		Results of current study	
	Database	OD	FD
Component of interest	Value per 100.0 g	Value per 100.0 g	Value per 100.0 g
Retinol μg	174.00	32.06	42.27
α-tocopherol μg	190.00	57.96	77.79
β-carotene μg	57.00	87.52	36.30
α-carotene μg	0.00	19.02	8.39
Lutein + zeaxanthin μg	0.00	0.79	0.48
β-cryptoxanthin μg	0.00	1.19	1.54

[1]Adapted from USDA National Nutrient Database for Standard References, Release 24 (2011)

Sensory and Color

Numerous studies have investigated the factors that influence the sensory quality of cheese (135). Differences in cheese sensory characteristics have been found to be associated with differences in feed and forage types and between differing herd management systems. Cheesemakers have frequently reported these differences and these reports have been backed up by larger studies analyzing the sensory properties of various types of cheeses and relating the diversity to the conditions under which the milk and cheeses were produced (123). Multiple factors can influence the sensory characteristics, including individual animal genetics, breed, health status of the animal, stage of lactation, and dietary factors. Even different species of forages can result in varying effects (136). The evidence of these changes is drawn from studies on aged cheeses, however, not fresh cheeses. To our knowledge, the sensory quality of Mozzarella cheese has not been extensively researched. For the objectives of the current study, a focus on this type of cheese is important because this is a product that could be adding to the economic base of local farms. As consumer acceptance is important when producing food, future research

should be conducted to investigate the effects of carotenoid variations, as well as other compositional changes in milk that result from pasture feeding and seasonal changes, on the sensory characteristics of Mozzarella cheese produced.

In many cases, color is the first criterion to be perceived by the consumer. A recent review by Martin et al., (2005) indicates that multiple observational studies have shown that feed and forage type lead to variations in the color of cheese (123). As diets vary from hay, to silage, to pasture, so the sensory panels in the studies reported increased yellow color of cheeses. Conversely, studies looking at both butter and cheeses showed that maize in the diet led to products that were rated as whiter by tasters when compared to products from animals consuming diets higher in grasses (123).

Once again, however, the reported research has been done on aged cheeses. None have examined the changes in color over the pasture season in fresh cheeses. There is also limited research using objective color measurements on dairy products; most objective color measurements have been done on fat and muscle in animals.

One study conducted by Noziere et al., (2006) used objective color measurement on cow's plasma and milk (98). Results showed that concentrations of β-carotene explained 58 and 40 percent of the variability in color index in plasma and milk, respectively. Although such a simple method of color determination was unable to accurately assess the concentration of β-carotene in plasma and milk, the utilization of such color index in plasma and milk to trace feeding management of dairy cows remains an interesting perspective. Although concentration of β-carotene was not determined, the measurements in plasma and milk, together, were able to accurately distinguish between the types of

forage fed to animals in the study (grass silage vs. hay). This very simple method of color measurement has the potential be useful in distinguishing products from animals under various feeding systems.

In the present study, we were able to measure the color objectively using a spectrophotometer (Konica Minolta CM-600d) to determine the changes in color over the season and between farms (Figure 10). Although statistical analyses were not run on the color data, these measurements add a unique perspective on color variations in this type of cheese. Additional research should be conducted to evaluate the use of color measurement to determine carotenoid content in this type of cheese and/or the diet of the animals from which the milk was produced.

Consumer demand for food products with health promoting properties is increasing, and the organic market continues to grow throughout the US. This research is necessary to determine whether the changes in fluid milk and cheese composition from cows fed on pasture are substantial enough to result in health benefits to the consumer and support this method of feeding in agriculture. The findings of this study suggests that a pasture-based feeding systems results in some 'losses', primarily in yield of milk and milk fat. However, there are also 'gains', with increased concentration and yield of carotenoids, components of nutritional interest. There is also the potential economic incentive to the dairy farmer, as the amount of feed necessary for purchase would decrease as pasture intake increases.

Although protein and Mozzarella cheese yield were not analyzed statistically, there were decreases in milk protein yield in milk from the OD compared to the FD. However,

cheese that is made with higher levels of beneficial components, like carotenoids, may be marketed as a 'value added' product, which is economically beneficial to the farmer and/or cheesemaker and nutritionally beneficial to the consumer, as functional foods are becoming increasingly popular.

The results here show that the milk from the OD produced cheese with an increasing yellow color throughout the pasture season. If farmers choose a pasture-based diet for their cows, they will need to understand these changes to be able to use them to their advantage. A market strategy that focuses on the nutritional quality may be useful.

It is also important to note that while there is strong evidence that dietary forms of β-carotene are associated with certain health benefits, there is also evidence that this may not be the case with β-carotene in supplemental form, as discussed previously. Thus, increasing the carotenoid concentrations in whole food, as done in the current study, is a more desirable way to promote increased consumption.

Limitations of the Study

Some research has also shown geographic location to play a role in milk composition, although, those effects may also be attributed to feeding, breed or calving period depending on the production region and the regional effects may also interact with seasonal effects. Factors not controlled for in this study that may have influenced the milk composition include but are not necessarily limited to, the changes in weather, differing management practices unrelated to feed type, stage of lactation and/or parity, and health of the animals. The differing locations of the two farms in this study were a limitation because of the increased potential for these confounding variables.

Although not a part of the study design, we did not have a panel to evaluate the sensory characteristics and/or color of the cheese produced, which would be beneficial in future studies. We saw changes in the color index of cheese, but it would be valuable to understand consumer perception of those changes.

Future Direction

As Mozzarella cheese has the potential to become a 'value added' product when made with milk produced by animals grazing on pasture, it would be valuable for future research to focus on the bioavailability and consumer acceptance of such a cheese.

Research shows that bioavailability of fat soluble components increases in the presence of fat, suggesting that the carotenoids in the milk and Mozzarella cheese would be highly bioavailable. Feeding studies comparing bioavailability of carotenoids from milk and milk products and other sources would be useful, as well as studies that focus on the kinetics of movement of these components upon consumption. Retinol and carotenoids are stored in fat globules and are thus present in a presumably highly bioavailable form, but future research could confirm this assumption with regards to this Mozzarella cheese.

It is also important to recognize that the yellow color resulting from increased β-carotene will vary throughout the season and may influence consumer perception and acceptance. Mozzarella cheese is traditionally very white, and although a more yellow color could indicate higher levels of carotenoids, and thus health promoting properties, it may take strategic marketing and/or education of the consumer because of its unfamiliarity. The results of this study suggest an opportunity for this marketing and education, and future

research should investigate consumer perception of a yellow Mozzarella cheese, and ways that might increase acceptability.

CHAPTER VIII

CONCLUSION

Market data indicates that consumers are increasingly interested in foods with health promoting properties. With the potential health benefits of dietary carotenoids, increasing these components in milk and cheese would be economically valuable to the farmer and cheesemaker and nutritionally valuable to the consumer. The market for organic milk and milk products is growing, and with it, the requirement for pasture-based feeding systems. Understanding how pasture influences the carotenoids in milk is important for farmers who are interested in following this type of feeding system. The results of this study show significant changes in carotenoid concentration in milk throughout the pasture season, and between different feeding systems. The composition of the Mozzarella cheese was also different than the composition reported in the USDA nutrient database. The variations in milk and cheese composition reported in this study provide valuable information to local farmers and artisan cheesemakers in particular, as their products are likely to vary as well.

CHAPTER IX

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APPENDIX A

ORGANIC DAIRY FEED

Diet summary on a dry matter basis for cows at the Organic Dairy

Ingredient	% DM
1st crop baleage	37.12
Pasture	34.88
Molasses	2.21
Bunk pellet	
Organic corn meal	13.15
Organic barley	6.42
Organic midds	2.50
Organic roasted soy	0.80
Dikal-21 16Ca 21P	0.27
Limestone 35.5Ca	0.73
Sodium bicarbonate	0.42
Sel-Plex/Yea-Sacc 1026	0.04
Salt-Plain	0.50
Potassium/mg Sulfate	0.14
MAGOX 51.5mg	0.36
Sodium bentonite	0.24
CFD Vit A-D-E PX	0.11
Certifeed TM #303	0.10

[1]Total diet provides 206.7 mg/kg Fe, 15.49 mg/kg Cu, 68.43 mg/kg Mn, 71.0 mg/kg Zn, 0.738 mg/kg Co, 0.822 mg/kg I, 1.49 mg/kg Mo, 0.3 mg/kg Se, 40,540 IU/kg Vit A, 1,880 IU/kg Vit D, 60 IU/kg Vit E

Nutrient analysis of total mixed ration at the Organic Dairy

Nutrient	% DM
Dry matter	35.94
Crude protein	15.55
R soluble protein	5.34
RDP CNCPS	12.65
RUP CNCPS	3.60
ADF	23.83
NDF	41.30
Starch	15.67
Crude fat	3.57
Calcium	0.85
Phosphorus	0.40
Magnesium	0.40
Potassium	2.00
NE$_l$[1] CNCPS	

[1]Provides 0.726 Mcal/kg

Diet summary on a dry matter basis for hi producing cows at the Organic Dairy

Ingredient	% DM
1st crop baleage	31.75
Pasture	32.25
Molasses	1.80
Bunk pellet	21.59
Hi pellet	
Organic cornmeal	4.34
Organic barley	3.13
Organic soy meal	0.77
Organic midds	1.49
Organic roasted soy	1.95
Dikal-21 16Ca 21P	0.13
Limestone 35.5Ca	0.33
Sodium bicarbonate	0.14
Salt-plain	0.09
Potassium/mg Sulfate	0.08
MAGOX 51.5mg	0.05
Sodium bentonite	0.11
Sel-Plex/Yea-Sacc 1026	0.01
Certifeed TM #303	0.01

[1]Total diet provides 193.24 mg/kg Fe, 14.95 mg/kg Cu, 66.75 mg/kg Mn, 71.0 mg/kg Zn, 0.722 mg/kg Co, 0.809 mg/kg I, 1.48 mg/kg Mo, 0.3 mg/kg Se, 36,700 IU/kg Vit A, 1,580 IU/kg Vit D, 55 IU/kg Vit E

Nutrient analysis of total mixed ration for hi producing cows at the Organic Dairy

Nutrient	% DM
Dry matter	37.78
Crude protein	15.90
R soluble protein	5.23
RDP CNCPS	12.72
RUP CNCPS	3.92
ADF	21.80
NDF	38.53
Starch	18.56
Crude fat	3.95
Calcium	0.90
Phosphorus	0.44
Magnesium	0.40
Potassium	1.90
NE$_l$[1] CNCPS	

[1]Provides 0.726 Mcal/kg

APPENDIX B

FAIRCHILD DAIRY FEED

Diet summary on a dry matter basis for cows at the Fairchild Dairy

Ingredient	% DM
Corn silage bunk pellet	42.20
Haylage	7.85
Alfalfa Hay	3.51
Straw	0.92
Bloodmeal + methionine	2.00
Fat	0.92
Energy mix	
Corn meal	10.07
Citrus pulp	7.36
Steam flaked corn	3.79
Soy hulls	3.16
Molasses	0.88
Protein mix	
Soybean meal	9.56
Canola meal	3.02
Distillers	1.02
Urea	0.27
LactatMin624[1]	3.45

[1]Vitamin + mineral Premix includes 13.27% Ca, 1.78% P, 6.03% Mg, 0.04% K, 0.30% S, 14.05% Na, 8.38%, Cl, 9.14 mg/kg Se, 148.02 mg/kg Fe, 168.45 mg/kg Cu, 766.04 mg/kg Zn, 629.78 mg/kg Mn, 28.81 mg/kg Co, 5.82 mg/kg I, 200,000 IU/kg Vit A, 46,000 IU/kg Vit D, 1,500 IU/kg Vit E

Nutrient analysis of total mixed ration at the Fairchild Dairy

Nutrient	% DM
Dry matter	47.96
Protein	16.26
RUP CNCPS	6.65
RDP CNCPS	9.62
ADF	20.15
NDF	31.50
Starch	23.70
Fat	4.17
Calcium	0.89
Phosphorus	0.37
Magnesium	0.39
Potassium	1.32
NEl[1] CNCPS	

[1]Provides 1.72 Mcal/kg

APPENDIX C

MEAN CAROTENOID AND NUTRIENT YEILDS OVER TIME

Mean nutrient and carotenoids yields over time[1]

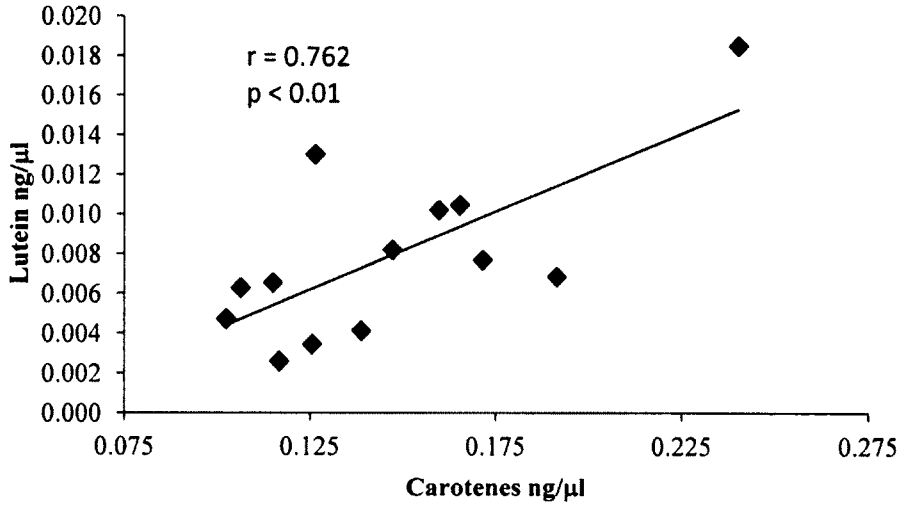
Time-point	1		2		3		4		5		6		7		Mean	
Group	OD	FD	OD	FD	OD	FD	OD	FD	OD	FD	OD	FD	OD	FD	OD	FD
Milk yield kg	10.89	12.90	8.81	10.72	9.08	12.11	7.47	10.77	7.59	10.59	7.99	8.65	6.76	8.30	8.37	10.58
Fat corrected yield kg	11.61	15.97	9.55	13.43	9.59	14.88	8.21	13.76	8.50	12.69	9.60	11.18	8.55	9.56	9.37	13.07
Energy corrected yield kg	12.54	17.20	10.27	14.56	10.37	16.26	8.89	15.02	9.23	14.07	10.50	12.29	9.53	10.47	10.19	14.27
Fat g	483.6	720.5	401.6	609.2	397.0	669.0	348.5	630.4	363.9	563.8	427.3	514.9	389.7	452.7	401.7	594.4
Milk fat g/kg	44.45	55.867	45.9	56.57	44.46	54.48	47.59	58.43	47.92	52.51	54.15	59.31	57.95	65.89	48.92	57.58
Protein g	355.2	477.22	285.9	413.1	295.8	475.1	252.4	436.0	265.8	432.2	307.8	365.0	297.4	306.9	294.3	415.1
Milk protein g/kg	32.72	37.081	32.55	38.34	32.87	38.96	34.34	40.58	35.13	40.78	38.79	42.33	44.72	44.73	35.87	40.4
Carotenes	2.090	1.743	2.506	1.488	2.616	1.550	1.728	1.378	2.396	1.259	3.139	1.163	2.259	1.045	2.391	1.375
Xanthophylls	0.168	0.246	0.135	0.163	0.170	0.169	0.116	0.141	0.139	0.136	0.170	0.114	0.097	0.080	0.142	0.150
Retinol	2.854	5.465	1.875	3.369	2.097	3.202	1.388	2.245	1.569	2.290	1.489	2.022	1.209	2.080	1.783	2.953
α-tocopherol	3.565	5.543	2.380	3.595	3.255	4.638	2.828	3.766	2.640	3.410	2.625	2.889	1.942	2.930	2.748	3.825
Lutein	0.116	0.120	0.080	0.051	0.109	0.057	0.078	0.051	0.083	0.051	0.103	0.046	0.050	0.023	0.089	0.057
Zeaxanthin	0.020	0.024	0.018	0.016	0.021	0.017	0.015	0.013	0.016	0.013	0.019	0.012	0.009	0.005	0.017	0.014
β-cryptoxanthin	0.033	0.101	0.038	0.095	0.040	0.095	0.023	0.077	0.039	0.073	0.048	0.056	0.037	0.052	0.037	0.078
α-carotene	0.103	0.103	0.114	0.082	0.122	0.086	0.094	0.082	0.121	0.071	0.147	0.067	0.124	0.059	0.118	0.078
β-carotene	1.986	1.636	2.388	1.402	2.486	1.460	1.625	1.292	2.264	1.185	2.979	1.092	2.121	0.985	2.264	1.293
13*cis* β-carotene	0.002	0.003	0.004	0.004	0.007	0.004	0.008	0.004	0.011	0.003	0.014	0.005	0.013	0.001	0.008	0.003

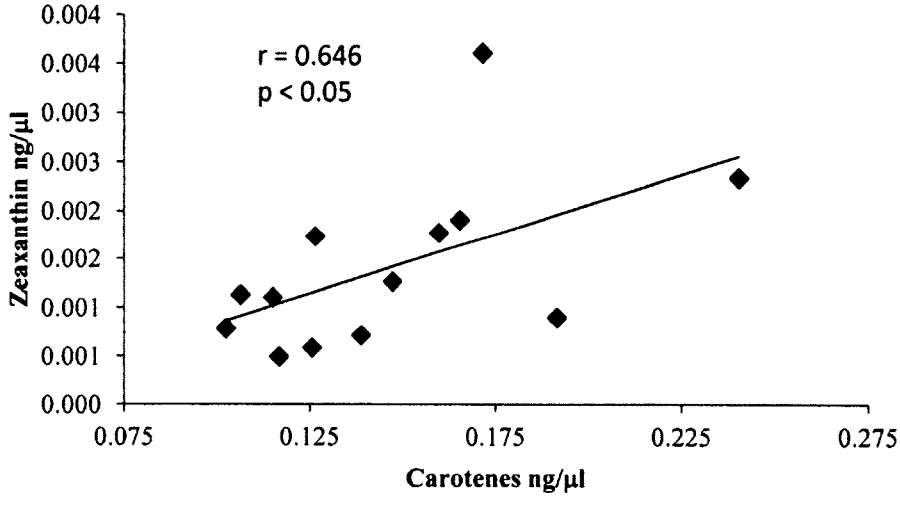
[1]Values are means for 9 cows per group at 7 time-points representing AM milkings only

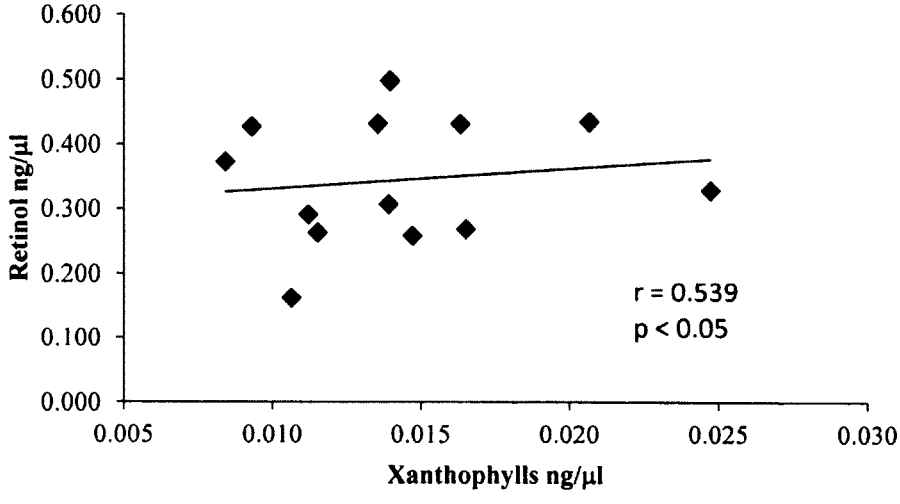
All carotenoid values are reported in mg

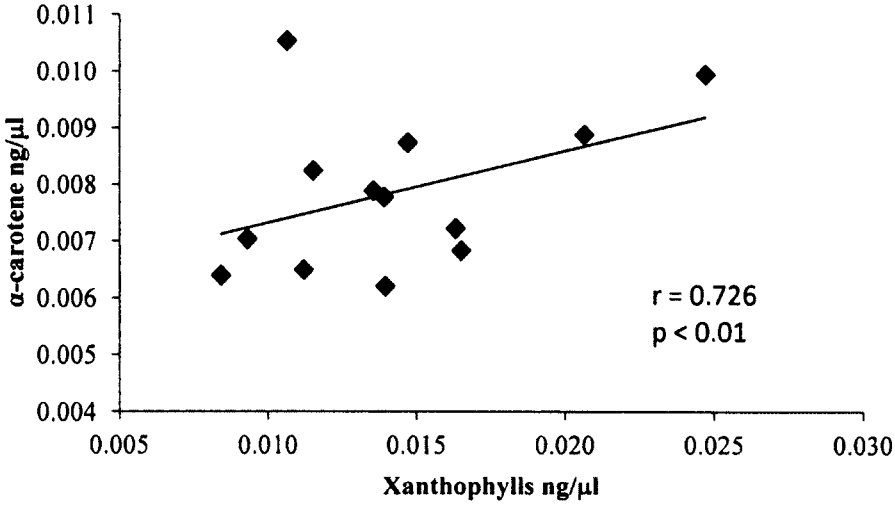
APPENDIX D

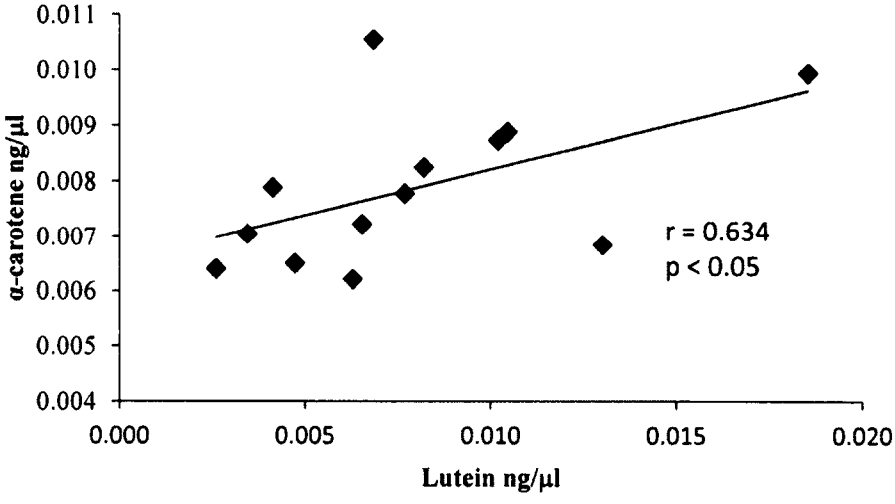
CAROTENOID AND NUTRIENT CORRELATIONS IN MILK AT TIME-POINT 1


r = 0.762
p < 0.01
Lutein ng/µl
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.075
0.125
0.175
0.225
0.275
Carotenes ng/µl


r = 0.646
p < 0.05
Zeaxanthin ng/µl
0.004
0.004
0.003
0.003
0.002
0.002
0.001
0.001
0.000
0.075
0.125
0.175
0.225
0.275
Carotenes ng/µl

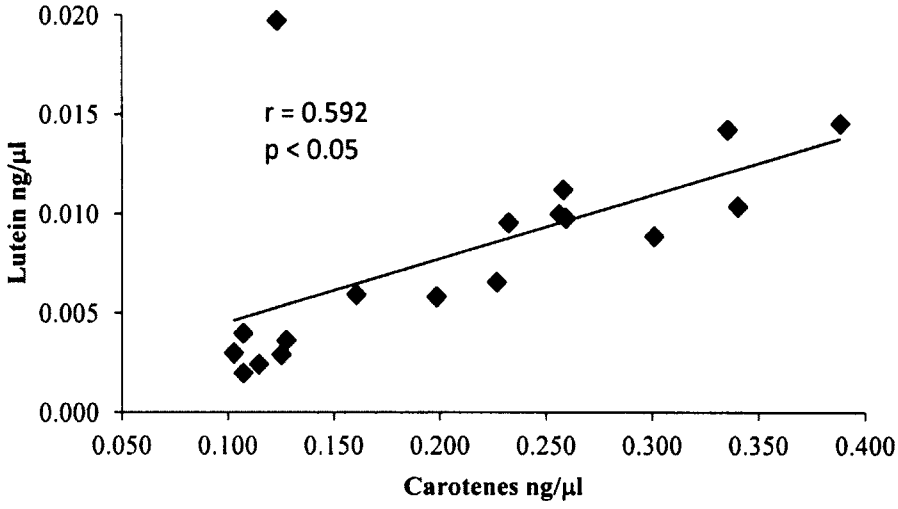

r = 0.539
p < 0.05
Retinol ng/µl
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.005
0.010
0.015
0.020
0.025
0.030
Xanthophylls ng/µl

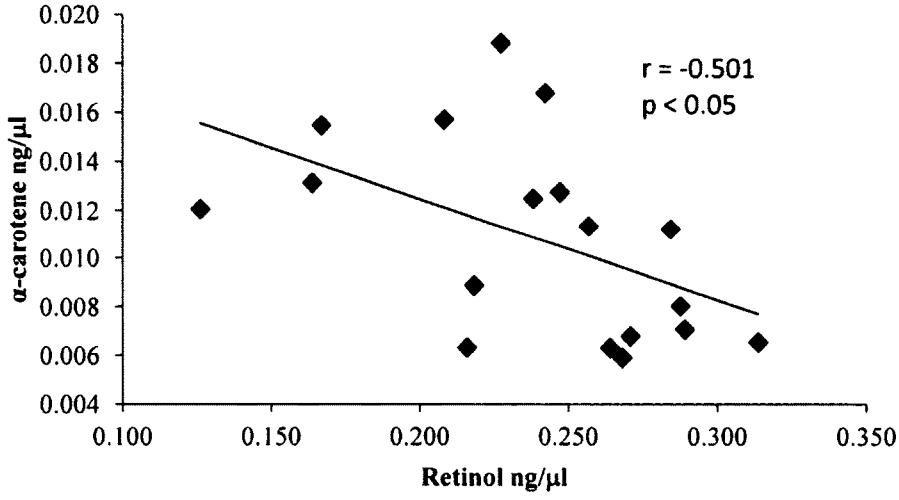

0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
α-carotene ng/µl
r = 0.726
p < 0.01
0.005
0.010
0.015
0.020
0.025
0.030
Xanthophylls ng/µl

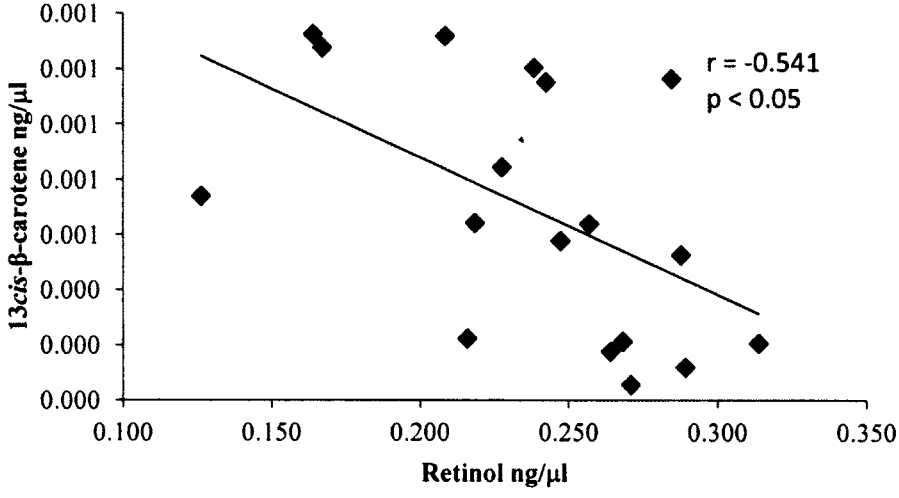

0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
α-carotene ng/µl
r = 0.634
p < 0.05
0.000
0.005
0.010
0.015
0.020
Lutein ng/µl

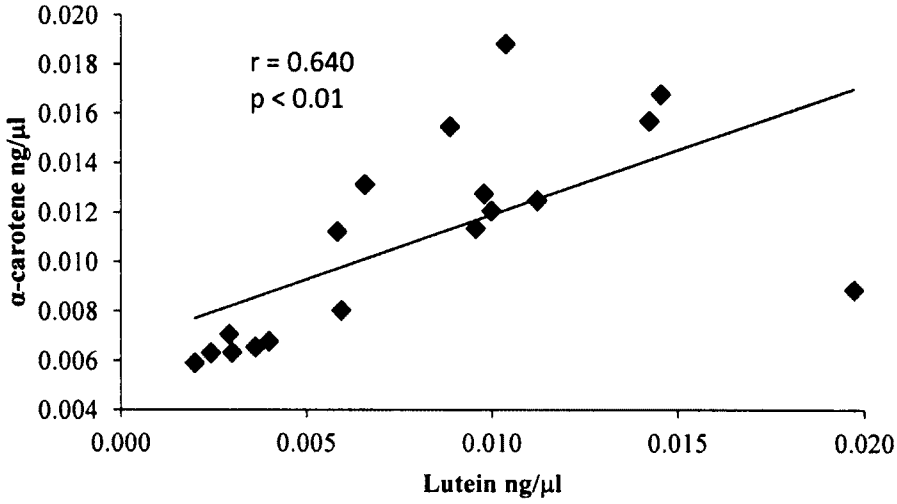
APPENDIX E

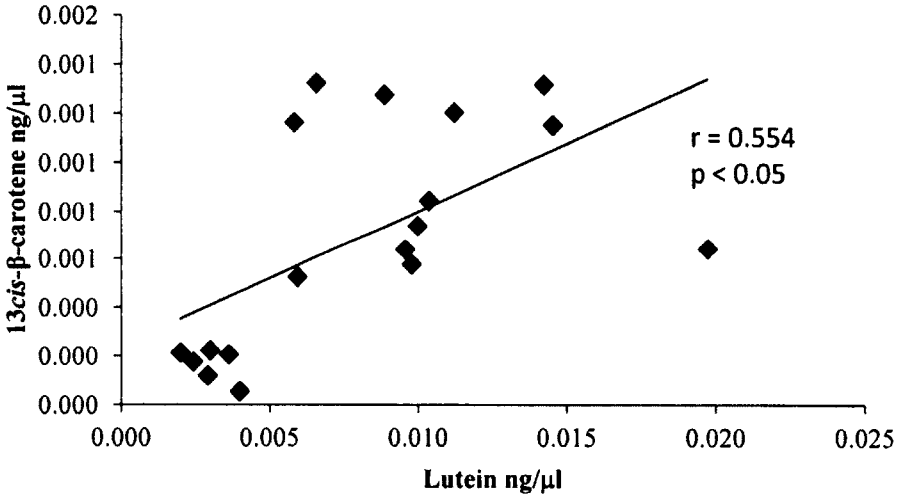
MEAN CAROTENOID AND NUTRIENT CORRELATIONS IN MILK

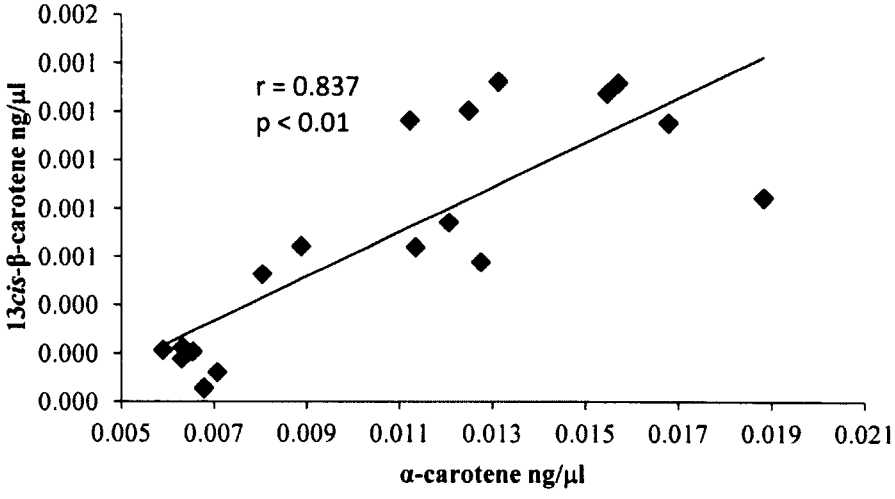

0.020
0.015
0.010
0.005
0.000
Lutein ng/µl
r = 0.592
p < 0.05
0.050
0.100
0.150
0.200
0.250
0.300
0.350
0.400
Carotenes ng/µl


0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
α-carotene ng/µl
r = -0.501
p < 0.05
0.100
0.150
0.200
0.250
0.300
0.350
Retinol ng/µl


0.001
0.001
0.001
0.001
0.000
0.000
0.000
13cis-β-carotene ng/µl
r = -0.541
p < 0.05
0.100
0.150
0.200
0.250
0.300
0.350
Retinol ng/µl


r = 0.640
p < 0.01
α-carotene ng/µl
Lutein ng/µl
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.000
0.005
0.010
0.015
0.020


r = 0.554
p < 0.05
13cis-β-carotene ng/µl
Lutein ng/µl
0.002
0.001
0.001
0.001
0.001
0.000
0.000
0.000
0.005
0.010
0.015
0.020
0.025


r = 0.837
p < 0.01
13cis-β-carotene ng/µl
α-carotene ng/µl
0.002
0.001
0.001
0.001
0.001
0.000
0.000
0.005
0.007
0.009
0.011
0.013
0.015
0.017
0.019
0.021

APPENDIX F

CAROTENOIDS AND NUTRIENTS IN MILK, WHEY AND CHEESE

Mean concentrations and yields of carotenoids and nutrients in milk, whey and cheese[1]

	OD				FD			
	Raw	Past[2]	Whey	Cheese	Raw	Past	Whey	Cheese
Yield g				1174.43				1261.43
Carotenes								
ng/µl	0.261	0.272	0.024	1.258	0.098	0.109	0.018	0.488
mg	2.084	2.174	0.156	1.563	0.784	0.873	0.112	0.648
Xanthophylls								
ng/µl	0.014	0.016	0.001	0.019	0.011	0.011	0.001	0.019
mg	0.113	0.115	0.008	0.023	0.091	0.081	0.008	0.026
Retinol								
ng/µl	0.174	0.169	0.013	0.306	0.219	0.219	0.020	0.403
mg	1.390	1.352	0.085	0.374	1.748	1.754	0.127	0.534
α-tocopherol								
ng/µl	0.296	0.279	0.040	0.553	0.309	0.301	0.052	0.742
mg	2.369	2.231	0.257	0.695	2.468	2.407	0.330	0.982
Lutein								
ng/µl	0.009	0.009	0.001	0.006	0.005	0.003	0.001	0.003
mg	0.068	0.073	0.007	0.008	0.037	0.028	0.005	0.005
Zeaxanthin								
ng/µl	0.002	0.002	0.000	0.001	0.001	0.001	0.000	0.001
mg	0.013	0.013	0.000	0.001	0.008	0.007	0.000	0.001
β-cryptoxanthin								
ng/µl	0.004	0.004	0.000	0.011	0.006	0.006	0.000	0.015
mg	0.034	0.030	0.001	0.015	0.045	0.050	0.002	0.019
α-carotene								
ng/µl	0.012	0.016	0.003	0.181	0.006	0.007	0.002	0.080
mg	0.098	0.128	0.017	0.225	0.048	0.058	0.012	0.106
β-carotene								
ng/µl	0.247	0.234	0.022	0.835	0.092	0.093	0.016	0.346
mg	1.978	1.872	0.140	1.037	0.735	0.743	0.100	0.459
13cis β-carotene								
ng/µl	0.001	0.001	0.000	0.242	0.000	0.000	0.000	0.062
mg	0.009	0.010	0.000	0.301	0.001	0.002	0.000	0.082

[1]Values are means of each, over the entire time period

[2]Pasteurized milk