ENHANCING THE PROPORTIONS OF HEALTHY FATTY ACIDS IN MILK FROM DAIRY COWS

by

Korie A.S. Nelson

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Approved:

Dr. Randy Wiedmeier Major Professor	Dr. Allen Young Committee Member
Dr. Silvana Martini Committee Member	Dr. Donald McMahon Committee Member
Dr. Thomas D. Bunch Acting Department Head	Dr. Byron Burnham Dean of Graduate Studies

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Logan, Utah

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ABSTRACT

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Korie A.S. Nelson, Master of Science

Utah State University, 2008

Major Professor: Randy Wiedmeier
Department: Animal, Dairy, and Veterinary Sciences

Twenty cows were used in a repeated measures, block design experiment for 9 wk to determine the effects of feeding partially ruminally inert calcium salts (Ca-salts) of fish oil (FO) and a general fatty acid (FA) supplement (EnerGII) at varying levels. The effects on cow health, milk components, composition of milk FA, and sensory evaluation of milk were evaluated. Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**). Cow health was not negatively affected by treatment diets. Treatment only significantly affected dry matter intake (DMI) and net energy of lactation (NE_L), with FH83 having the lowest DMI. Week of trial significantly affected all milk components except protein percent, which did not change. Dry matter intake, milk yield, fat yield, fat percent, and protein yield demonstrated a net decrease over time. Lactose, solids, and somatic cell count all shared a net increase over time.

Milk urea displayed no definitive trend over time. Content of conjugated linoleic acid (CLA) isomers $C_{18:2}$ *cis*-9, *trans*-11 and *trans*-10, *cis*-12 combined over time was 0.54, 0.68, 1.18, and 0.82 g/100 g FA for CTL, FH41, FH83, and FL, respectively. Vaccenic acid (VA) $C_{18:1}$ *trans*-11 content over time was 1.04, 1.51, 2.28, and 1.68 g/100 g FA; and total omega-3 FA content over time was 0.52, 0.76, 0.82, and 0.80 g/100 g FA for CTL, FH41, FH83, and FL, respectively. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels increased by as much as 6- and 2.7-fold, respectively, over CTL for the duration of the experiment. Although levels of EPA, DHA, VA, and CLA increased for treatments FH41, FH83, and FL over CTL, a trained sensory panel detected no difference in milk flavor between treatments with little or no intensity of off-flavors. Results suggest that feeding FO and EnerGII at varying levels enhanced CLA, VA, EPA, DHA, and total omega-3 FA in milk over the length of the experiment without negatively affecting cow health, milk composition, or flavor of milk.

(84 pages)

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Korie A.S. Nelson

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LIST OF ABBREVIATIONS

ADF = acid detergent fiber

AI = atherogenic index

ALA = alpha-linolenic acid

BW = body weight

Ca-FO43 = calcium salts of 43% fish oil

Ca-FO71 = calcium salts of 71% fish oil

CLA = conjugated linoleic acid

CP = crude protein

CTL = control

DHA = docosahexaenoic acid

DIM = days in milk

DM = dry matter

DIM = days in milk

DMI = dry matter intake

ECM = energy-corrected milk

EnerGII = calcium salts of palm oil

EPA = eicosapentaenoic acid

FA = fatty acid

FCM = fat-corrected milk

FDA = U.S. Food and Drug Administration

FH41 = treatment that received Ca-FO71 at 0.41% DM

FH83 = treatment that received Ca-FO71 at 0.83% DM

FL = treatment that received Ca-FO43 at 0.83% DM

FO = fish oil

LDL = low-density lipoprotein

NDF = neutral detergent fiber

NE_L = net energy for lactation

$PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$

SCC = somatic cell count

TI = thrombogenic index

TMR = total mixed ration

VA = vaccenic acid

INTRODUCTION

It has been demonstrated that omega-3 fatty acids (**FA**), specifically eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**), can significantly decrease the risks of cardiovascular disease, hypertension, certain autoimmune and inflammatory diseases, behavior problems, and cancer (Simopoulos, 1991; Ruxton, 2004). They are also essential to the development of the brain and retinas, and to normal cell function and growth (Simopoulos, 1991). The possible health benefits of conjugated linoleic acid (**CLA**) are anticarcinogenicity, antiatherogenicity, growth promotion, antiobesity, and immunomodulation, with the anticarcinogenic properties among the most desirable and pertinent (Chouinard et al., 1999; Baer et al., 2001; Hughes and Dhiman, 2002).

While the aforementioned FA carry the potential to be beneficial to humans, the difficulty lies in determining the vehicle that will provide *all* of these FA. Dairy products, along with ruminant meat, are the greatest source of CLA in human diets (Hughes and Dhiman, 2002; Dhiman et al., 2005). Simple diet manipulation in cows can increase CLA and subsequently the benefits of dairy products (Chouinard et al., 2001). While fish oil (**FO**) has been recognized as the best supplemental source for omega-3 FA, it is inconducive to fortification in respect to sensory evaluations, especially when added to dairy products with low fat content, such as milk (Simopoulos, 1991; Kolanowski and Wieβbrodt, 2007). Fortification of dairy products with FO can result in a host of problems including the following: it is hard to get levels high enough to be effective

without causing fishy or other off-flavors, high susceptibility to oxidation of FO can cause further formation of off-flavors, and masking of off-flavors with desirable flavoring (e.g. strawberry or garlic) creates potential health risks (Kolanowski and Wieβbrodt, 2007). Since fortification of dairy products with FO appears to be an undesirable mode of consuming combined beneficial doses of omega-3 FA and CLA, eating dairy products from animals fed a diet including FO and linoleic acid-rich feed seems to be the best alternative. This provides the consumer with a more healthful and acceptable product while aiding in meeting the suggested beneficial levels of intake for omega-3 FA and CLA without significantly altering the consumer's diet.

The objective of this experiment was to enhance the CLA and omega-3 FA content of milk through diet manipulation, while maintaining milk fat content, feed intake, overall health of the cow, the flavor quality of the milk, and observing the effect of time on the aforementioned characteristics.

LITERATURE REVIEW

Introduction

The demand for, and interest in functional, or nutraceutical, foods is increasing in the U.S., with beverages on the forefront (Campbell et al., 2003). Although diet manipulation for product improvement is commonplace in the food-animal world, it is becoming more so in the human realm. With new research exposing the potential health benefits of an array of foodstuffs each day, people are seeking functional foods in lieu of, or before turning to, conventional methods such as medicine to treat or prevent certain diseases. The vast benefits of omega-3 FA have taken a stronghold in the consumer market. Food sources consisting of adequate omega-3 FA levels, however, are extremely limited to only a few food items, the majority of which are marine-based, such as algae and fish. Although green leafy plants (Simopoulos, 1991) and flaxseed do provide omega-3 FA, they provide only α-linolenic acid (**ALA**) the precursor to EPA and DHA. The problem is that, although marine-based foods contain the highly desirable omega-3 FA, the foods themselves are minimally consumed due to undesirable flavor. Thus, researchers are continuing to explore various ways for consumers to increase their intake of omega-3 FA without significantly changing their diets, which ultimately requires manipulating existing foodstuffs. Milk is already widely consumed and a natural source of CLA thus, it is an obvious choice for further nutritional improvement by increasing levels of CLA and adding the desired omega-3 FA via FO. In general, omega-3 FA and FO do not lend themselves to fortification. Kolanowski and Wieβbrodt (2007) found that fortification at beneficial levels creates the same problem as the natural sources of

omega-3 FA themselves – a fishy and undesirable flavor. Thus, manipulation of food animal diets is the most feasible way of achieving the aforementioned improvements.

Dhiman et al. (2005) showed that while other sources of FA, such as extruded soybeans, sunflower oil, and linseed, provided more CLA than FO, only FO could provide both CLA and omega-3 FA at levels that could be potentially beneficial. This makes FO highly desirable as a fat supplement. It can, however, cause milk fat depression and off-flavors in byproducts. Ramaswamy et al. (2001), however, found that a combination of high CLA feed and FO yielded significant amounts of CLA and omega-3 FA, as well as lessened the milk fat depression often seen with FO alone.

Conjugated linoleic acid also has marked health benefits and is highly desired by the consumer. Although CLA, specifically, is less well known to the consumer than omega-3 FA, consumers seek out increased CLA by looking for products under the guise of pasture-fed animals and their byproducts. Achieving increased consumer consumption of CLA is much easier than for omega-3 because it already occurs in red meat and dairy products (Dhiman et al., 2005). The goal then, is to increase levels of CLA without changing consumer acceptability of the product or jeopardizing the health of the animal.

Greatly increasing the levels of omega-3 FA and CLA in dairy products, specifically milk, will create a more healthful product, increase consumption of these FA, and potentially stimulate the dairy industry. Diet manipulation of food animals is a means to achieve increased levels of both CLA and omega-3 FA. The poultry industry has already marketed eggs from chickens fed sources of omega-3 FA. Milk, however, can offer CLA in addition to omega-3 FA and may increase consumption of dairy products.

Current research is now looking at how to achieve the desirable and beneficial levels of omega-3 and CLA FA without negatively affecting the flavor or content of the product.

Fish Oil

Compound Background. Omega-3 FA (as well as omega-6 FA) are essential FA since they cannot be synthesized by mammals in adequate amounts (Simopoulos, 1991). There are 3 isomers that make up the omega-3 FA: ALA (α-$C_{18:3}$), EPA ($C_{20:5}$), and DHA ($C_{22:6}$) (Ramaswamy et al., 2001; see Figure 1). Although EPA and DHA are synthesized

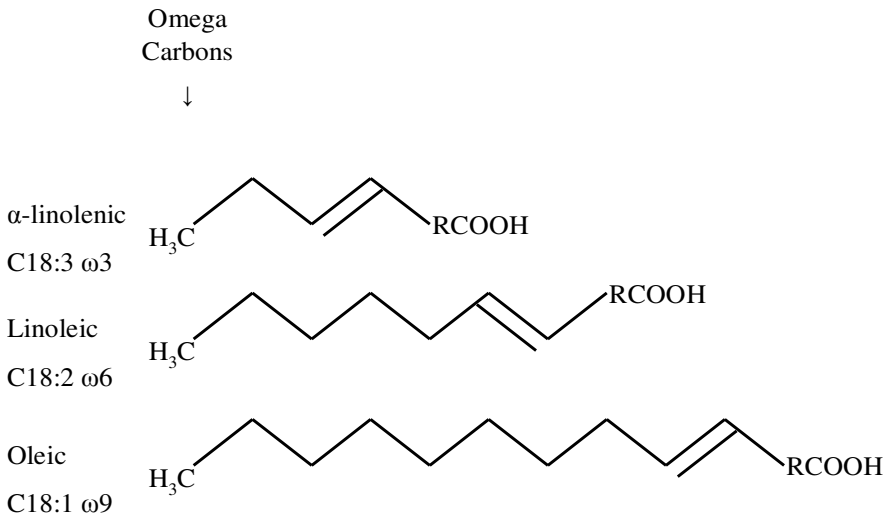

Figure 1. Structures of parent omega fatty acids (FA). Adapted from Simopoulos (1991).

from ALA (Petit et al., 2002), FO allows this to be bypassed by providing significant amounts of EPA and DHA. This is advantageous since the synthesis of DHA from EPA in humans is minimal due to poorly understood and inefficient peroxisomal oxidation (Attar-Bashi et al., 2007). α-linolenic acid is naturally found in the chloroplasts of green leafy vegetables (Simopoulos, 1991). Although humans can synthesize EPA from ALA and DHA from EPA, albeit poorly, the omega-6 FA compete with the omega-3 FA for the desaturase enzymes (Simopoulos, 1991; see Figure 2). People with medical conditions such as diabetes and hypertension, as well as premature infants lack complete synthesis of EPA and DHA from ALA (Simopoulos, 1991). Attar-Bashi et al. (2007) have shown that although humans can synthesize DHA from ALA via peroxisomal oxidation, this process is inadequate and fails to yield significant levels of DHA. Certain medical conditions, competition from the omega-6 FA for common enzymes, and failure of peroxisomal oxidation result in the need for dietary intake of EPA and DHA.

Benefits for Humans. The health benefits of omega-3 FA for humans are widespread and significant; most notable are its potential to prevent cardiovascular disease, preterm labor, and cancer, and its essentiality for proper growth and function in infants (Simopoulos, 1991; Ruxton, 2004; Oh, 2005). Ruxton (2004) noted that a decreased level of omega-3 FA consumption can cause mental illnesses such as depression, severity of symptoms linked with depression, dementia, and increased risk of Alzheimer's disease. Oh (2005) noted that the most compelling reason for the use of FO in primary care is its cardiovascular benefits. Oh (2005) reviewed several cardiovascular

Omega-6 FA	Omega-3 FA
↓	↓
Linoleate Series	Linolenate Series
C18:2 ω6 linoleic acid	C18:3 ω3 α-linolenic acid (ALA)
↓ Δ^6-desaturase	↓ Δ^6-desaturase
C18:3 ω6 γ-linoleic acid	C18:4 ω3
↓	↓
C20:3 ω6 dihomo-γ-linoleic acid	C20:4 ω3
↓ Δ^5-desaturase	↓ Δ^5-desaturase
C20:4 ω6 arachidonic acid	C20:5 ω3 eicosapentaenoic acid (EPA)
↓	↓
C22:4 ω6	C22:5 ω3 docosapentaenoic acid
↓ Δ^4-desaturase*	↓ Δ^4-desaturase
C22:5 ω6 docosapentaenoic acid	C22:6 ω3 docosahexaenoic acid (DHA)

* The presence of Δ^4-desaturase is unconfirmed in humans.

Figure 2. Elongation of omega-3 and omega-6 fatty acids (FA) from parent FA. Adapted from Simopoulos (1991).

studies and found the overall consensus to be that FO greatest benefit was the reduction of cardiovascular deaths, by as much as 30% in only 3 months.

Dosing. While the benefits of consuming omega-3 FA are clear, the dosing to achieve these benefits is not. Wistuba et al. (2005) stated that the estimated intake of DHA and EPA by humans in general is only 0.1 to 0.2 g/d. The FDA has not officially stated a minimum daily value or whether it should be based off of EPA and DHA specifically or of a product, such as FO; it has stated, however, that no more than 3 g/d of FO should be consumed via diet or supplement (Kolanowski and Wieβbrodt, 2007). Despite the remarkable safety and benefits of omega-3 FA, an upper limit has been set due to the potential of increased bleeding time and low-density lipoprotein (**LDL**), as well as worsening of glycemic profile in diabetics (Oh, 2005). Research has suggested various levels as being beneficial. Ruxton (2004) stated that 1 to 4 fish meals a week (450 mg to 900 mg EPA and DHA, or 0.45 g to 0.9 g) is adequate, whereas Oh (2005) found that a minimum of 1 fish meal a week was sufficient enough to reduce sudden cardiac death by 52%. The American Heart Association suggests 1 g of FO a day for patients with coronary artery disease, while one study demonstrated that 2 to 4 g/day can significantly reduce hypertriglyceridemia (Oh, 2005). Lastly, Wistuba et al. (2005) reported that 0.15 to 0.65 g/d is required to achieve desired benefits.

Whatever the recommended dose, Western-style diets in general contain an average of 0.15 g/d of omega-3 FA, a level that is below the majority of the suggested doses that would be beneficial (Kolanowski and Weiβbrodt, 2007). In addition, intake of omega-3 FA has been decreasing while omega-6 intake has been increasing, creating an

unhealthy ratio of omega-6 to omega-3 FA of 10:1, in general, and 17:1 in the U.S. (Ruxton, 2004; Kolanowski and Weiβbrodt, 2007). Because omega-6 FA do not possess the same health benefits as omega-3 FA, as well as compete for the same enzymes, it has been recommended that this ratio should not exceed 4:1 (Ruxton, 2004; Oh, 2005; Kolanowski and Weiβbrodt, 2007). Simply increasing daily fish intake proves to be more difficult than thought for several reasons. First, the amount of fish needed to reach the recommended levels is well above that normally consumed and would require large dietary changes, which are difficult (Kolanowski et al., 1999). Additionally, levels of EPA and DHA vary greatly among fish and methods of preparation. Fresh salmon contains 1.2 g DHA and 0.5 g EPA per serving, while canned salmon has 0.06 g DHA and 0.09 g EPA per serving (average serving size of 80-120 g; Ruxton, 2004). Lastly, certain fish, such as mackerel, can contain significant levels of mercury, thereby creating a potential health risk outweighing the benefits of the omega-3 FA (Oh, 2005).

***Effects on Cows*.** Although the health benefits of omega-3 FA for humans are clear, the possible negative or positive effects on food animals whose byproducts serve as vehicles for these FA are not. Ideally, animals would not be negatively affected, the efficiency of transfer of the omega-3 FA would be high, and the byproduct itself would also not be negatively affected. Wistuba et al. (2005) noted that when supplementing FO to cattle that are on a corn-based diet, their average daily feed intake and average daily gain decreased, but had no effect on a wheat-midd based diet. Additionally, Giesy et al. (2002) noted that the milk fat depression often caused by FO supplementation can

actually be beneficial in that it can be used as a management tool to reduce energy requirements for lactating cows due to the high costs associated with milk fat secretion.

One potential benefit of omega-3 FA is their effect on the immune system. Although other studies in humans and rats have shown a decrease in immune activity via interleukin-1 and tumor necrosis factor-α production (both are types of cytokines activated in immune response and are made by leukocytes, and macrophages and T-cells, respectively), it was found that immune activity was actually stimulated in cattle when presented with concanavalin A, phytohemmagglutinin, and pokeweed mitogen (Wistuba et al., 2005). Conversely, Petit et al. (2002) found that feeding FO did not decrease DMI. They also found that supplementing FO decreased prostaglandins (specifically $PGF_{2\alpha}$) and increased corpora lutea thereby improving gestation rates in cows.

***Calcium Salts*.** Calcium salts are a form of protection for supplemental FA. Lacasse et al. (2002) and Jenkins and Palmquist (1984) stated that unprotected FO causes decreased DMI, fiber digestibility, milk fat content, and animal performance. Fatty acids tend to inhibit rumen microbes and, therefore, decrease fiber digestion causing milk fat depression by lowering the ratio of acetic to propionic acids (Jenkins and Palmquist, 1984). The addition of minerals to supplemented fats counteract the decreased fiber digestibility by creating a salt formed from the FA and divalent cations of the minerals (Jenkins and Palmquist, 1984). This process occurs naturally when fats and minerals are fed separately, but is unreliable due to type and amount of minerals, type of fat, rumen pH, and turnover rate of solids (Jenkins and Palmquist, 1984). Thus, preformed calcium salts were created and designed to completely bypass the rumen and completely

dissociate post-ruminally (Jenkins and Palmquist, 1984). Castañeda-Gutiérrez et al. (2007) emphasize, however, that calcium salts render unsaturated FA inert only to their effects on the rumen microbial population, not rumen biohydrogenation. Jenkins and Palmquist (1984) noted that the processes required for proper utilization of calcium salts are as follows: dissociation in abomasum acid, absorption of calcium in the duodenum, and absorption of FA in the jejunum and ileum. In addition, if calcium is improperly absorbed or in excess, insoluble salts will reform in the large intestine and then be excreted via feces (Jenkins and Palmquist, 1984).

Protected FO, while still causing milk fat depression, did not cause decreased DMI or milk production as noted by Lacasse et al. (2002) with unprotected FO. Interestingly, regardless of protection, protein content was variable. Although Lacasse et al. (2002) found protein to decrease when compared to a control while supplementing with either protected or unprotected FO, both Baer et al. (2001) and Ramaswamy et al. (2001) found that it was unaffected when using a protected source. Recent research by Castañeda-Gutiérrez et al. (2007) found that the protection calcium salts provided was correlated to rumen pH and FA $pK_{d.}$ In addition, as unsaturation increased, ruminal biohydrogenation also increased, while the difference between protected and unprotected FO decreased. While calcium salts do not protect against ruminal biohydrogenation, specifically of EPA and DHA, they still prevent decreased DMI and milk yield that is seen when using unprotected unsaturated FA supplements (Castañeda-Gutiérrez et al., 2007). Thus, protected supplemental FA are currently used, despite incomplete

protection, due to their ability to lessen or negate some of the effects that unprotected sources create.

Conjugated Linoleic Acid

Compound Background. Conjugated linoleic acid has 17 identified isomers (Dhiman et al., 2005), but the *cis*-9, *trans*-11 isomer is not only the most prevalent, making up 80% of the isomers, but also the most biologically active, with its potential to be anticarcinogenic the most notable of these activities (Donovan et al., 2000). While not as substantial in quantity, the isomer *trans*-10, *cis*-12 of CLA has also shown anticancer effects in animals, and is thought to be a cause for milk fat depression (Dhiman et al., 2005; see Figure 3). Conjugated linoleic acid is naturally synthesized via 2 processes. Biohydrogenation in the rumen creates CLA as an intermediate from linoleic acid. When this process is incomplete, CLA is left as a byproduct. The majority of CLA, however, is made endogenously in adipose tissues and the mammary gland from *trans*-11 $C_{18:1}$, or vaccenic acid (**VA**) (Donovan et al., 2000; Abu-Ghazaleh et al., 2002). These 2 syntheses are illustrated in Figure 4. This latter synthesis involves the Δ^9-desaturase enzyme (Lynch et al., 2005). Interestingly, VA is also an intermediate in biohydrogenation. It is thought that FO can stimulate CLA production from other components in the diet, despite FO itself having very little linoleic or linolenic acids itself (Abu-Ghazaleh et al., 2002).

Although CLA is stable under normal cooking and storage conditions, several factors influence the amount of CLA in the consumer product, including animal age,

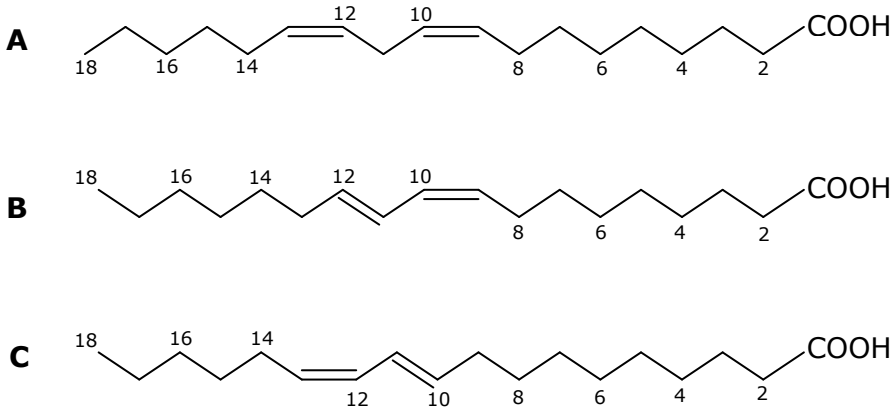

Figure 3. Abridged structures of conjugated linoleic acid (CLA) and its isomers. (A) linoleic acid, $C_{18:2}$ (B) the most prevalent CLA isomer, $C_{18:2}$ *cis*-9, *trans*-11 (C) the second most potentially healthful isomer, $C_{18:2}$ *trans*-10, *cis*-12. Adapted from Dhiman et al. (2005).

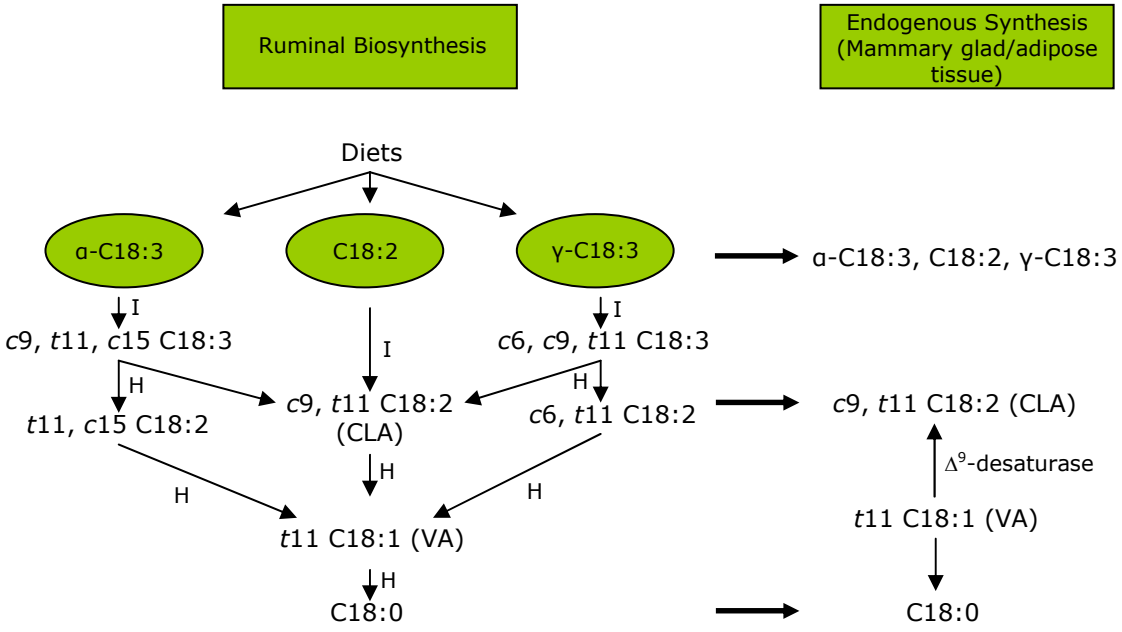

Figure 4. Two forms of synthesis of conjugated linoleic acid (CLA). VA = vaccenic acid; I = isomerization; H = hydrogenation. Adapted from Dhiman et al. (2005).

animal breed, animal diet, and management of feed supplements that affect the diet (Dhiman et al., 2005). Dhiman et al. (2005) note that the following yielded the most CLA in their group: pasture versus other diets, sunflower oil (5.3%) versus other plant seed oils, full fat extruded soybean (1.65 kg) versus other intact oil seeds, FO (1 to 3%) versus other marine sources, combination of 5.3% soybean and 1% FO versus other combinations, summer versus other seasons, higher elevations (1275-2120 m) versus lower, restricted feeding versus free, Montebeliarde cows (on pasture) versus other dairy breeds on TMR or pasture, older cows (4 lactations or more) versus younger, and a mixture of synthetic CLA supplements given post-ruminally versus other types and combinations given post-ruminally or fed. In summary, pasture feeding at high elevations in the summer is the best type of diet, although immature forage harvested as silage also yields high CLA. Holstein cows, particularly those that are older, yield the second most CLA among breeds (Dhiman et al., 2005). Lastly, despite the availability of synthetic CLA supplements, those naturally occurring are better, specifically those naturally occurring from FO (Dhiman et al., 2005; Jones et al., 2005).

Benefits for Humans. Conjugated linoleic acid has demonstrated clear health benefits including prevention of atherosclerosis and thrombosis (Dhiman et al., 2005). While these benefits are significant, other positive effects, such as decrease in body fat while increasing lean body mass, enhancement of immune function, and antidiabetic properties, have also been noted (Hughes and Dhiman, 2002; Dhiman et al., 2005; Jones et al., 2005). Among the most significant and promising is the potential for CLA to prevent or slow cancer. Hughes and Dhiman (2002) reported that these effects occur by

feeding CLA at certain levels and durations which inhibit cell growth pre-cancer, suppress tumors pre-cancer, suppress cancerous tumors, and block both local growth and systemic spread of human breast cancer in mice. It should be noted, however, that many of these benefits have been extrapolated from animal studies and have yet to be researched in humans. Human studies would account for the different (higher) fat content of our diets and would potentially change the significance of the noted benefits (Jones et al., 2005).

Dosing. The main source of CLA in the consumer's diet is from ruminant meat and dairy products (Dhiman et al., 2005; Jones et al., 2005). Hughes and Dhiman (2002) stated that dairy products naturally have CLA levels ranging from 2.9 to 8.9 mg/g of fat, with 73 to 93% of that the *cis*-9, *trans*-11 isomer. Similarly, Khanal et al. (2005) reported levels of CLA as 0.30 to 0.55 g/100 g FA in whole milk. In general, Chilliard et al. (2001) noted that milk from ruminants is only about 1 to 5% linoleic acid when looking at all FA. Despite these natural levels of CLA in dairy products, more is needed to attain health benefits from CLA. To achieve significant effects, Jones et al. (2005) state that one would need >1.2 g/d of CLA to see decreased lymphocyte activation and lowered LDL-to-HDL and total cholesterol-to-HDL ratios. Additionally, only 0.8 g/d of CLA would be needed to inhibit tumor growth (Jones et al., 2005). Dhiman et al. (2005) reported that 1 serving of whole milk (227 mL) and 1 serving of cheese (30 g) a day can provide up to 90 mg of CLA. This would only represent, however, 25% of the lowest effective dose of CLA, based on a 600 g diet (Dhiman et al., 2005). Campbell et al. (2003) suggest that 3 g/d of CLA would yield beneficial results. Animal studies have shown that increasing

levels in dairy products would achieve desired health benefits with little or no risk to humans (Hughes and Dhiman, 2005).

***Effects on Cows*.** Conjugated linoleic acid is clearly favorable for human health, but the effects of providing feeds high in CLA to cows must be considered. Abu-Ghazaleh et al. (2002) reported that somatic cell counts (**SCC**) were similar among all treatments where fish meal, extruded soybeans, or a combination thereof were fed. It was also found that treatment did not affect DMI; in fact, those cows fed a source of CLA (extruded soybean) alone or in combination with FO actually had higher milk yields (Abu-Ghazaleh et al., 2002). Even though Dhiman et al. (2005) found that cows grazed on pasture yielded more CLA in their milk, Khanal et al. (2005) found that those cows on pasture actually had lowered DMI and, therefore, decreased milk yields. In contrast, both Abu-Ghazaleh et al. (2002) and Whitlock et al. (2006) found that feeding a source of CLA did not change the DMI among treatments. Hughes and Dhiman (2005) noted that feeding CLA, even at increased levels, has little or no negative effects on the cow herself.

***Δ^9-desaturase Enzyme*.** Conjugated linoleic acid can be synthesized in the mammary gland or adipose tissue from VA via the Δ^9-desaturase enzyme (see Figure 4). This enzyme is part of a multienzyme complex that is made of reduced nicotinamide adenine dinucleotide (NADPH)-cytochrome b_5 reductase, cytochrome b_5, acyl-coenzyme A (CoA) synthase, and Δ^9-desaturase enzyme on the terminus (Hughes and Dhiman, 2005). Although many saturated and unsaturated acyl-CoA units can serve as a substrate to Δ^9-desaturase, stearoyl-CoA and palmitoyl-CoA are the main substrates (Hughes and

Dhiman, 2005). Figure 5 shows the general reaction between a FA and a desaturase enzyme.

There are also differences among species and tissues in regards to Δ^9-desaturase activity and level. Hughes and Dhiman (2005) stated that mRNA levels and activity are

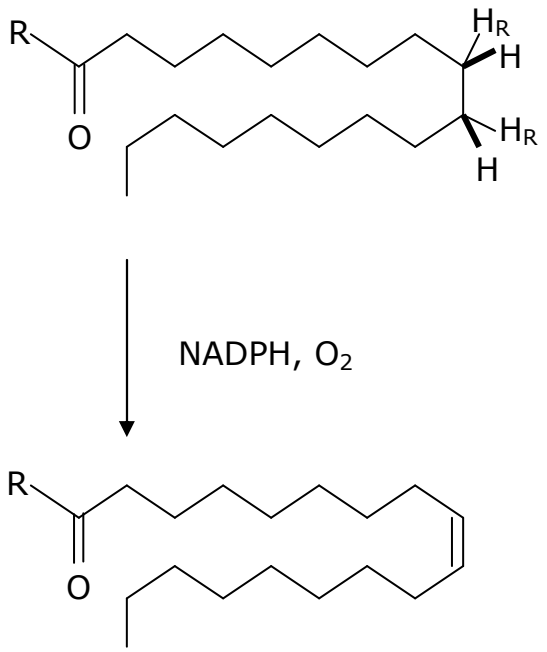

Figure 5. A representative reaction of a fatty acid (FA) and a desaturase enzyme. Adapted from Fox et al. (2004). H_R = hydrogen and main substrate.

highest in the liver for rodents, while the same is true for adipose tissue in growing cattle and sheep. Diet, hormone balance, physiological state, and other inhibiting and activating factors also affect the activity of Δ^9-desaturase and mRNA amounts in either a negative or positive manner (Hughes and Dhiman, 2005).

Fortification

Achievement of nutraceutical, or functional foods, is typically sought through fortification initially due to ease and speed. Flavor, stability, composition, and cost of the product must be considered. Campbell et al. (2003) confirmed the demand for these foods in the consumer market with a survey that revealed that taste (78%) and nutrition (70%) are the main reasons for consumption and purchase of milk. Adding 2% CLA to milk would increase the cost by $0.20 to $0.25 per 8 oz. serving, but certain consumers, such as women, those with a family history of cancer, and those over the age of 25, would be willing to pay more (Campbell et al., 2003). Acceptability, however, of products fortified with CLA and omega-3 FA is low (Campbell et al., 2003; Kolanowski and Weiβbrodt, 2007). Fish oil tends to yield a fishy flavor and is susceptible to oxidation, causing more off-flavor if oxidation occurs. A study by Campbell et al. (2003) found that, although most consumers bought 2% milk, the milk which contained 1 or 2 % CLA in place of normal milk fats contained low intensities of "grassy/vegetable oil" off-flavors and lowered consumer acceptability and perceived freshness. Campbell et al. (2003) also found that CLA changed the color of milk slightly when fortified, negatively affecting the perceived taste. Although flavoring can often mask slight off-flavors and colors, Campbell et al. (2003) found that chocolate flavoring helped acceptability scores for

lower levels of fortified CLA (1%), but had little impact on the 2% CLA milk. The most frequently purchased chocolate milk was 2%, thus negating the benefits of flavoring (Campbell et al., 2003). An additional problem with flavored milk is that although it is liked, it is bought and consumed less frequently than regular milk (Campbell et al., 2003). Lynch et al. (2005) stated that, despite all the benefits of fortification with CLA, it is expensive and may not contain the same isomers or isomer ratios that are found in milk when CLA is naturally obtained through diet.

Fortification of products with FO has even more problems than fortification with CLA. Kolanowski and Weiβbrodt (2007) stated that the natural off-flavor of FO and its susceptibility to oxidation, which enhances the off-flavor if it occurs, greatly limits its use in fortification. In a study by Kolanowski and Weiβbrodt (2007), several dairy products were fortified with FO and then subjected to sensory analysis. Solid products and those high in fat, like butter and cheese, were able to be fortified without adverse sensory evaluations, but the level of FO in these products was limited to achieve acceptability (Kolanowski et al., 1999; Kolanowski and Weiβbrodt, 2007). Although flavoring can mask off-flavors (Kolanowski et al., 1999; Campbell et al., 2003; Kolanowski and Weiβbrodt, 2007), Kolanowski and Weiβbrodt (2007) found that masking off-flavors caused by oxidation could jeopardize the safety of foods fortified with FO. Antioxidants can be added to lessen the effects of oxidation in foods fortified with FO and CLA, but this is not always effective (Campbell et al., 2003) and is restricted by food law in many countries, including the U.S., as to the types of antioxidants used in what products (Kolanowski et al., 1999; Jacobsen et al., 2008). Limited levels of beneficial FA,

increased risk of oxidation, lowered acceptability, and higher costs clearly eliminate fortification of CLA and omega-3 FA in milk as a viable option.

Interestingly, only minimal research exists looking at the effects of supplemented CLA and FO on dairy cows and their byproducts over an extended period of time. The present study attempted to enhance the CLA and omega-3 FA content of milk through diet manipulation while maintaining milk fat content, feed intake, overall health of the cow, and flavor quality of the milk. The hypothesis for this study was that desired FA contents would be enhanced, milk fat content would be maintained or only slightly decreased, and feed intake, cow health, and the flavor of the milk would be maintained, if not improved, over time.

MATERIALS AND METHODS

Experimental Design and Treatments

Twenty multiparous Holstein cows averaging 156 ± 45 DIM were selected from the general herd at Utah State University's Caine Dairy. Cows were blocked into 5 different groups according to average milk yield 10 d prior to the start of the experiment. Cows had an average BW of 750.5 ± 58.6 kg at the start of the experiment. Cows within each group were randomly assigned to 1 of 4 treatments. Experimental duration was 9 wk, including a 3-wk diet adaptation at the beginning of the experiment. The experiment ran from late May until the end of July, 2007. Cows were fed non-experimental TMR in a tie-stall barn 1 wk prior to start of the experiment for adjustment to the barn. Measurements were made during the last 6 wk of the experiment. Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**). Virtus Nutrition Inc. (Corcoran, CA) supplied all FA supplements. Diets were formulated to meet the nutrient requirements of cows producing 48 kg of 3.5% FCM/d according to NRC (2001) recommendations and fed as TMR. Composition of diets for each treatment are given in Table 1. Fresh water was available at all times. Cows were fed twice daily

Table 1. Ingredient and chemical composition of diets

Composition	Treatment[1] CTL	FH41	FH83	FL
	---------- g/100 g of DM ----------			
Ingredient				
Alfalfa hay	25.72	25.72	25.72	25.72
Corn silage	31.08	31.08	31.08	31.08
Steam rolled corn	13.38	13.38	13.38	13.38
Almond hulls	1.60	1.60	1.60	1.60
Corn dried distillers grain	1.83	1.83	1.83	1.83
Corn, hominy	3.65	3.65	3.65	3.65
Canola meal Mech. Extr.	1.37	1.37	1.37	1.37
Whole-linted cottonseed	5.28	5.28	5.28	5.28
Soybean meal expeller	1.10	1.10	1.10	1.10
Blood meal, ring dried	0.73	0.73	0.73	0.73
Ca-salts of palm oil (EnerGII)	1.65	1.24	0.82	0.82
Ca-salts of fish oil - 71%	0.00	0.41	0.83	0.00
Ca-salts of fish oil - 43%	0.00	0.00	0.00	0.83
Molasses, sugar beet	2.14	2.14	2.14	2.14
Minerals & vitamin mix	1.39	1.39	1.39	1.39
Chemical, g/100 g of DM				
NE_L, Mcal/kg of DM	1.30	1.30	1.30	1.30
CP	17.70	17.70	17.70	17.70
Total fatty acids	5.31	5.31	5.31	5.31
NDF	29.60	29.60	29.60	29.60
ADF	18.70	18.70	18.70	18.70

[1]Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**).

(0430 h and 1530 h) ad libitum with amounts fed and orts recorded daily. Orts were targeted to 5 to 10% of daily intake as-fed. Supplements were weighed according to each treatment for each feeding

and added to TMR, then promptly fed. Animal care and procedures were approved and conducted under established standards of the Utah State University Institutional Animal Care and Use Committee.

Daily TMR samples were frozen at -20°C for subsequent analysis. Orts from each cow (1 to 1 ½ cups each) were composited by treatment to create a representative sample for that treatment each day. These samples were also frozen at -20°C for subsequent analysis. Daily samples of both TMR and orts were then composited by treatment to create weekly samples that were then analyzed for DM content. The DM content of the feed ingredients was determined by oven-drying at 60°C for 48 h.

Dried feed samples were ground through a Wiley Mill (1 mm screen, Arthur H. Thomas, Philadelphia, PA). Samples from every 3 wk were dried at 60°C for 48 h individually, composited (creating Periods A, B, and C), ground, then analyzed for CP, NDF, ADF, FA content, and FA profile. The CP contents of the dietary ingredients and orts samples were determined using an elemental analyzer (LECO TruSpec N, St. Joseph, MI, USA) and AOAC (2000) procedure number 990.03. The NDF and ADF contents were determined with the ANKOM^{200} Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY, USA), using the basic procedure of Van Soest et al. (1991). Sodium sulfite was not used in the procedure for NDF determination, but pre-treatment with heat stable amylase (Type XI-A from *Bacillus subtilis*; Sigma-Aldrich Corporation,

St. Louis, MO, USA) was included. Total FA content and FA profile analysis was determined by using the procedure described by Sukhija and Palmquist (1988). During analysis the samples were further dried at 105°C for 8 h to determine the absolute DM, and chemical analyses were expressed on the basis of absolute DM.

The chemical composition of the TMR was calculated from the chemical composition of individual ingredients of the diet. Daily DMI for individual cows was calculated by subtracting the weekly mean for orts from the weekly mean for feed offered. The NE_L content of the diet was calculated by using the NE_L table values (NRC, 2001) for the individual dietary ingredients (Table 1). Weekly mean NE_L intakes were calculated by multiplying the NE_L values of the diet by the mean DMI of the individual cows for that week. The CP, NDF, and FA intakes were calculated by subtracting CP, NDF, and FA amounts in orts from feed offered. The amount of CP, NDF, and FA in orts were calculated by multiplying weekly mean orts for individual cows by treatment average CP, NDF, and FA content in orts during that week. Chemical composition of the treatment diets are in Table 1. The metabolizable protein contents of the diets were calculated using the NRC (2001) model. Diets were formulated to be iso-energetic and iso-nitrogenous.

Milk Collection and Processing

Milk yields were recorded daily on an individual cow basis. Weekly milk samples were collected from each cow from 4 consecutive a.m. and p.m. milkings (0445 h and 1645 h) at the end of each week during wk 4 through 9. A Broad Spectrum

Microtabs II (D & F Control Systems Inc., San Ramon, CA) preservative was added to each sample in tablet or liquid form and then stored at 4°C until analyzed for FA content. Analysis was conducted within 1 wk of collection. Leftover milk was stored at -20°C for future reference.

During wk 5 and 8, milk from 2 consecutive milkings for each cow was collected in vacuum-sealed milking cans. Milk was collected during these weeks to evaluate the effect and possible differences created by time. The milk was transferred into plastic 20-L buckets, labeled by treatment and cow, and transported within 45 min of cessation of milk collection from the dairy to the Gary H. Richardson Dairy Products Laboratory at Utah State University. Upon arrival at the Dairy Laboratory, the milk was cooled to 4°C within 2 h after collection. Approximately 8 kg of milk/cow per milking was combined for each treatment. Each treatment was homogenized using a Gaulin model CGC 2-stage homogenizer (Gaulin, Everette, MA) at 787 and 196 per square centimeter, and then pasteurized at 73°C for 16 s using an APV Model SR15-S (APV Equipment Inc., Tonawanda, NY). Approximately 19 kg of homogenized and pasteurized milk for each treatment was stored in 10-gallon stainless steel milking cans. Sensory evaluation of milk was conducted within 72 h of procurement and 36 h of processing the milk without standardizing for fat content. An additional sensory evaluation of the same milk for each indicated week was conducted 7 d after the initial 72 h (10 d of storage) to simulate aging of milk in consumer's fridge and the effects time has on flavor.

Compositional Analysis of Milk

Individual milk samples from each cow for each week were analyzed for fat percent, true protein percent, lactose percent, solids, SCC, and urea by the Rocky Mountain Dairy Herd Improvement Association Laboratory (Logan, UT) with mid-infrared wave-bands (2 to 15 µm) procedures using a Bentley 2000 (Bentley Instruments, Chaska, MN). The infrared instrument was calibrated weekly using raw milk standards based on chemistry analysis (Eastern Laboratory Services Limited, Fairlawn, OH, USA). The fat measurement channel used was a combination of Fat A and Fat B. An enzymatic procedure was used to determine milk urea nitrogen using a Chemspec 150 instrument (Bentley Instruments, Chaska, MN). Final milk composition for each week was expressed on weighted milk yield of a.m. and p.m. samples. Average fat and protein yields was calculated by multiplying milk yield from the respective week by fat and protein content of the milk on an individual cow basis. Energy corrected milk (**ECM**) was calculated on an individual cow basis using milk yield, fat and protein content (Tyrrell and Reid, 1965). Gross feed efficiency was calculated by dividing daily ECM by feed DMI on an individual cow basis.

Weekly weighted composite milk samples from individual cows were analyzed for FA composition, including CLA, VA, and omega-3 FA. Milk fat was extracted by boiling the milk in a detergent solution (Hurley et al., 1987). Extracted fat was derived to methyl esters using an alkaline methylation procedure by mixing 40 mg of fat with sodium methoxide methylation reagent ($NaOCH_3$/MeOH) as described by Chouinard et al. (1999) with minor modifications. After FA methyl esters were formed, anhydrous

calcium chloride pellets were added and the samples were allowed to stand for 1 h to remove water. Samples were then centrifuged at 2600 *g* at 5°C for 5 min.

Separation of individual FA was achieved by using a gas chromatograph (Model QP2010, Shimadzu Co., Columbia, MD) fitted with a flame ionization detector. Samples containing methyl esters in hexane (1 µl) were injected onto an HP-88 fused silica 100 m x 0.25 mm column, 0.20 µm film (Agilent Technologies, Palo Alto, CA). The injection port was maintained at 250ºC in the split mode, and the sample was split at a 100:1 ratio with a 3.0 ml/min purge flow. Hydrogen was used as the carrier gas at a flow rate with a linear velocity of 41.1 cm/s. The temperature program was as follows: initial temperature 50°C and hold for 1 min, ramp at 40°C/min to 175ºC and hold for 4 min, ramp at 3.5 ºC/min to 250ºC and hold for 3 min. The detector was operated at 250ºC and makeup gas was nitrogen 30 ml/min. Air and hydrogen flow to the detector was 450 ml/min and 40 ml/min, respectively. Total run time was 32.55 min/sample.

Each peak was identified using FA and FA methyl esters (Nu-Chek Prep, Elysian, MN; Matreya, Pleasant Gap, PA and Supelco^{TM} 37 Component fatty acid methyl ester mix, Supelco, Bellefonte, PA). Heptadecanoic acid was added as an internal standard. Percentage of each FA was calculated by dividing the area under the FA peak (minus the area under the peak for heptadecanoic acid) by the sum of the areas under the total reported FA peaks. Fatty acids are reported as g/100 g of FA methyl esters. The CLA yield was calculated by multiplying CLA content with total fat yield corrected for glycerol content (Chouinard et al., 2001) on an individual cow basis. The VA is converted to CLA in the mammary gland (Corl et al., 2001) via the Δ^9-desaturase

enzyme. The Δ^9-desaturase index was calculated for selected milk FA using product-to-substrate ratios of FA. The FA ratios to determine the Δ^9-desaturase index were $C_{14:1}$:$C_{14:0}$, $C_{16:1}$:$C_{16:0}$, $C_{18:1}$ *cis*-9:$C_{18:0}$, and CLA *cis*-9, *trans*-11:$C_{18:1}$ *trans*-11(VA).

Fatty acids can promote or prevent atherosclerosis and coronary thrombosis based on their effects on serum cholesterol and LDL cholesterol concentrations (Ulbright and Southgate, 1991). The equations proposed by Ulbright and Southgate (1991) for the atherogenic and thrombogenic indices indicate that the $C_{12:0}$, $C_{14:0}$, and $C_{16:0}$ FA are atherogenic, and $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ are thrombogenic, while the omega-3, omega-6, and monounsaturated FA are antiatherogenic and antithrombogenic. The ratio between the 2 is used to calculate the atherogenic index (**AI**) and thrombogenic index (**TI**). The AI and TI indices were calculated in the present study using the equations described by Ulbright and Southgate (1991). In these equations, the $C_{14:0}$ FA is considered to be 4 times more atherogenic than the other FA, thus the coefficient "4" was assigned to it. The $C_{18:1}$ omega-6 and monounsaturated FA was assigned coefficients of 0.5 because they are less antiatherogenic than the omega-3 FA, which were assigned a coefficient of 3.

Sensory Evaluation of Milk

A trained panel of judges evaluated pasteurized and homogenized fluid milk samples from wk 5 and 8 for each treatment on 3 and 10 d of storage for grassy (feedy), fishy, oily, oxidized, and rancid flavors. Data was collected using an electronic data collection system (SIMS2000) and analyzed using SAS 9.1.3 (1999-2000). Judges were trained for the proposed study, but many were already familiar with the scoring

process for fluid milk flavor. Panelists were trained for a total of 5 h and training procedures followed the guidelines described by Meilgaard et al. (2007). The training began 1 wk prior to sensory evaluation and continued once weekly until the last evaluation (10 d of refrigeration) for wk 8 samples. When a training session occurred on the same day as an evaluation, a 20 min break was given before testing. Training was performed at a round table enabling the interaction of the judges. The sensory evaluation was conducted using a continuous 5-point scale for flavor characteristics with the following categories: highly pronounced flavor, moderate, slight, barely perceptible, and no flavor. For statistical analysis, numerical scores were given to the categories where 5 = highly pronounced flavor and 1 = no flavor. Whole milk fortified with vitamin D in a clear plastic container was purchased from the store with at least 14 d shelf life remaining and was used as a positive control. Reference samples for specific attributes were provided for all flavor characteristics during sampling in training (see Table 2).

Refrigerated (22.0 ± 1.0°C) fluid milk from each treatment and control were served in plastic cups to the trained panel. Random code numbers were assigned for identification to each sample. Samples were also presented in a randomized and balanced way among panelists. Sample tasting was performed in individual booths under fluorescent white light. Water and spittoons were provided to panelists to cleanse the palate between samples.

Table 2. Preparation of off-flavor reference samples

Off-flavor	Preparation
Rancid	Mix 1 part raw milk with 3 parts homogenized milk and store at 40°F (~4.4°C) for 2 days
Oxidation	Dissolve 1 g copper sulfate (blue vitriol, $CuSO_4$ $5H_2O$) in 99 ml of water. Add 0.2 ml of this 1% solution to 1 quart pasteurized non-homogenized milk. Store at 40°F (~4.4°C) for 2 days. Since cream line milk varies in susceptibility toward oxidation with the season, more or less of the 1% solution may be used
Grassy	Add 20 mg of hexanal to 1L of milk
Oily	Add 1 drop (0.02 g) soybean oil to 50 ml of milk
Fishy	Add 2, 5 or 7 drops of DHA oil to 900 ml of milk

Statistical Analyses

The design of the experiment was a repeated measures, randomized block design with 4 treatments in 5 blocks. Blocks were defined by average daily milk yield of each cow 10 d prior to the experiment. Block 1 held the 4 cows with the highest average milk yield of the 20 cows selected, block 2 the second highest, and so on. Assignment of treatments was randomized among blocks such that each treatment had a representative cow from each block. Statistical analyses were performed using the PROC MIXED procedure of SAS (1999-2000). Significance level was declared at $P < 0.05$, unless otherwise noted. Trends for significance were declared at $0.05 \leq P < 1.0$. Analysis of intake data, milk yield, milk composition, FA composition, Δ^9-desaturase enzyme index,

atherogenic index, and thrombogenic index were determined using PROC MIXED in a repeated measures design. Treatment, block, week, and treatment × week were included in the model as fixed effects with week as the repeated measure on the cows. Covariance structure was autoregressive (1) [AR(1)].

For the sensory panel, PROC MIXED was also used under the same significance levels. Covariance structure was diagonal. Design for analysis was a split-plot where week was the whole plot, storage day the sub-plot, and treatment (to include store-bought control) the block. Treatment, week, storage day, treatment × week, storage day × week were included in the model.

RESULTS AND DISCUSSION

Intake, Milk Yield, and Milk Composition

There was no week × treatment interaction for any production variable. The effect of treatment and week on milk yield, fat yield, protein yield, fat percent, protein percent, lactose, urea, solids, and SCC were then analyzed separately. Treatment was significant only for DMI ($P = 0.0076$; see Figure 6) and consequently NE_L ($P < 0.001$; see Table 3). The DMI for FL was significantly different from FH41 over the length of the experiment (40.6 kg versus 38.3 kg, respectively) and, most notably, the DMI for FH83 was significantly lower than the other treatments (32.0 kg versus 38.9 kg, 38.3 kg, and 40.6 kg for CTL, FH41, and FL, respectively). FH83 having a significantly lower DMI than the rest is interesting but not surprising because, although FH41 and FH83 contained the same percent of FO (71%), FH41 contained less FO than FH83 (0.41% versus 0.83% DM, respectively); therefore, one would expect FH83 to have a lower DMI, versus FH41, if higher levels of FO are the cause for lower DMI. The lower DMI in FH83 disagrees with the findings of Whitlock et al. (2006), who saw no difference in DMI when feeding FO at 0.33%, 0.67%, and 1.0% DM and Allred et al. (2006) who saw no difference when feeding calcium salts of palm and FO alone or in combination with soybeans. Giesy et al. (2002) also found no difference in DMI among treatments when feeding calcium salt CLA supplement at 0, 12.5, 25, 50, and 100 g. One possible contribution to the differing DMI may be the cows themselves. This experiment ran through the summer months. It

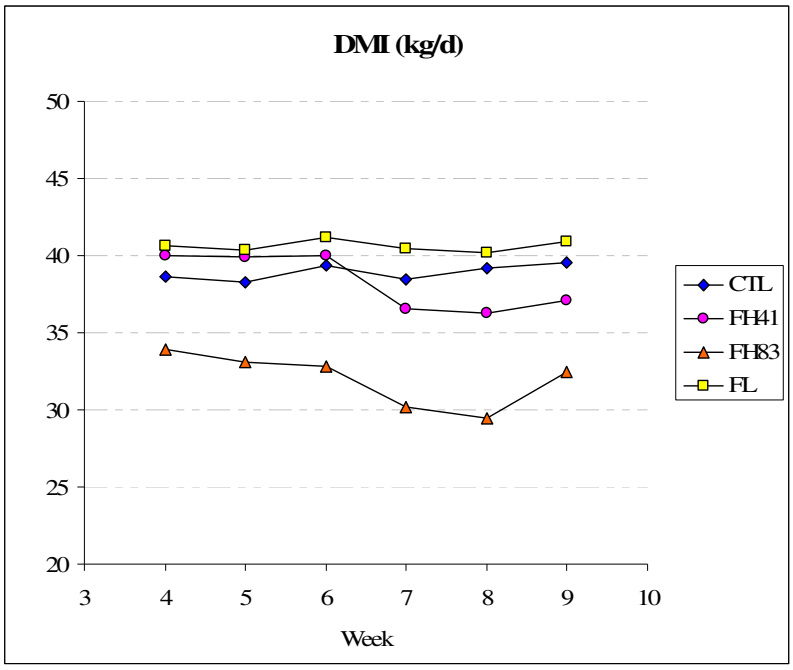

Figure 6. Dry matter intake (DMI) for treatments over time. Values equal mean for each treatment. $P < 0.0001$ for week and $P = 0.008$ for treatment.

was observed that on a daily basis 3 out of the 5 cows in treatment FH83 splashed water from their drinking vessels in such large amounts that their feed troughs contained up to about 1 1/2 inches of water. This occurred for at least 2 wk, most notably wk 6 and 7. During this time period, ambient temperatures in the area were higher than normal; therefore, it is assumed temperatures in the barn were also higher. Excessive panting for the same 3 cows was also observed. Although the cause of these behaviors is undetermined, the possible increased water consumption and excessive panting are

Table 3. Nutrient intake and milk production of cows fed varying amounts of calcium salts of fish oil (FO) and EnerGII

	Treatment[1]					
Item	CTL	FH41	FH83[3]	FL	*P*[4]	SEM[6]
Intake, kg/d						
DM	38.91[a]	38.29[a]	31.98[b]	40.62[a]	***	1.49
NE_L (Mcal/d)	50.58[a]	49.78[b]	41.57[b]	52.81[b]	***	1.94
CP	5.44	5.57	4.07	6.14	-----[5]	
NDF	8.57	8.39	10.14	6.94	-----	
Fat	2.07	2.03	1.70	2.16	-----	
Production, kg/d						
Milk yield	35.15	34.07	31.14	35.47	0.389	0.05
ECM[2]	34.78	33.01	27.68	33.17	0.139	2.08
ECM/DMI	0.88	0.86	0.87	0.81	0.605	0.04
Fat yield	1.21	1.12	0.82	1.08	0.086	0.10
Protein yield	1.06	1.03	0.96	1.05	0.472	0.05
Milk Composition, %						
Fat	3.41	3.28	2.75	3.15	0.068	0.20
Protein	3.02	3.04	3.02	3.01	0.898	0.08
Lactose	4.67	4.65	4.61	4.72	0.531	0.05
Milk urea N (mg/dL)	14.38	13.38	12.34	12.92	0.448	0.54

[a,b,c]Means in the same row with different superscripts differ significantly for treatment effect with *P* value as mentioned in column for significance.
[1]Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**).
[2]ECM = 0.327 x milk(kg) + 12.95 x fat(kg) +7.20 x protein(kg); equation derived from Table 4 of Tyrrell and Reid (1965).
[3]Data for cow 9968 still included despite erroneous blocking. Did not change statistical significance.
[4]Significance of effects of treatments.
[5]Unable to statistically analyze because laboratory analysis was done in periods of 3 weeks instead of by week.
[6]SEM = standard error of least square means.
*** $P < 0.001$

indicative of heat stress (West et al., 2003). Additionally, the excess water in the troughs alone is another possibility as to what may have caused the lowered DMI. Consequently, regardless of the cause, FH83 exhibited a significantly lower NE_L, CP intake, and increased NDF intake in conjunction to the lowered DMI.

The lack of effect of treatment on milk yield, protein yield, protein percent, solids, and SCC is in agreement with Abu-Ghazaleh et al. (2003). The unaltered fat yield and fat percent by treatment, however, contrasts with the findings of Abu-Ghazaleh et al. (2003) when they fed various fat supplements at 2% DM and FO at 1% DM (a total of 3%), but agrees with Allred et al. (2006), who fed various levels of soybean products with 2.7% FO or FO alone (2.7%). Although milk fat depression is typical of cows fed FO, as demonstrated in Ramaswamy et al. (2001), findings in this experiment did not indicate a significant difference between treatments (see Figure 7 and 8). Despite the lack of significance for the overall treatment effect for fat yield and percent, a trend was present for both ($P = 0.086 \pm 0.10$ for fat yield and $P = 0.068 \pm 0.20$ for fat percent). Means comparison for treatment shows that CTL had a higher fat yield than FH83. In fact, CTL had the highest fat yield of all treatments (1.21 kg versus 1.12 kg, 0.82 kg, and 1.08 kg for FH41, FH83, and FL, respectively). Additionally, CTL also had the highest fat percent when compared to the other treatments (3.41% versus 3.28%, 2.63%, and 3.15% for FH41, FH83, and FL, respectively). This supports common findings that milk fat depression can be caused by FO.

Week significantly affected ($P < 0.0001$) all of the components tested except for protein percent, which was unaffected by treatment or week ($P = 0.898$ for treatment and

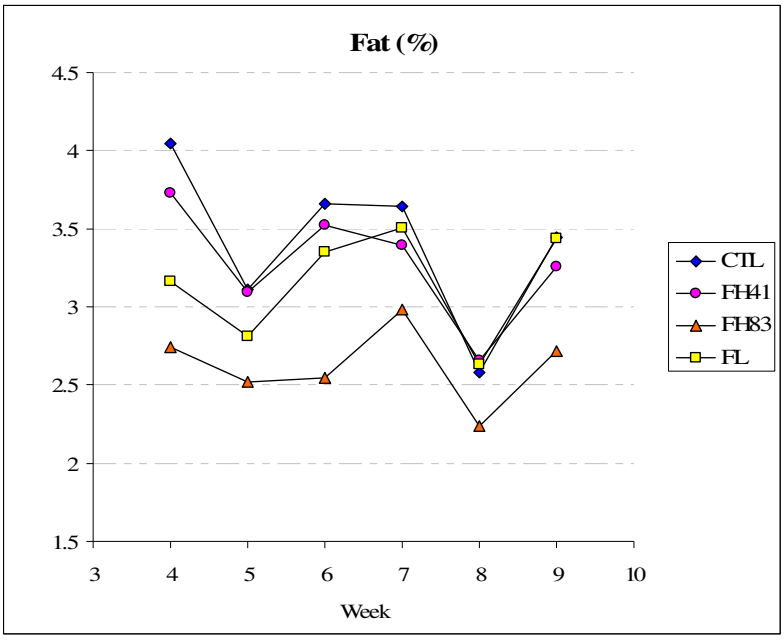

Figure 7. Fat percent for treatments over time. Values equal mean for each treatment. P <0.0001 for week and P = 0.068 for treatment.

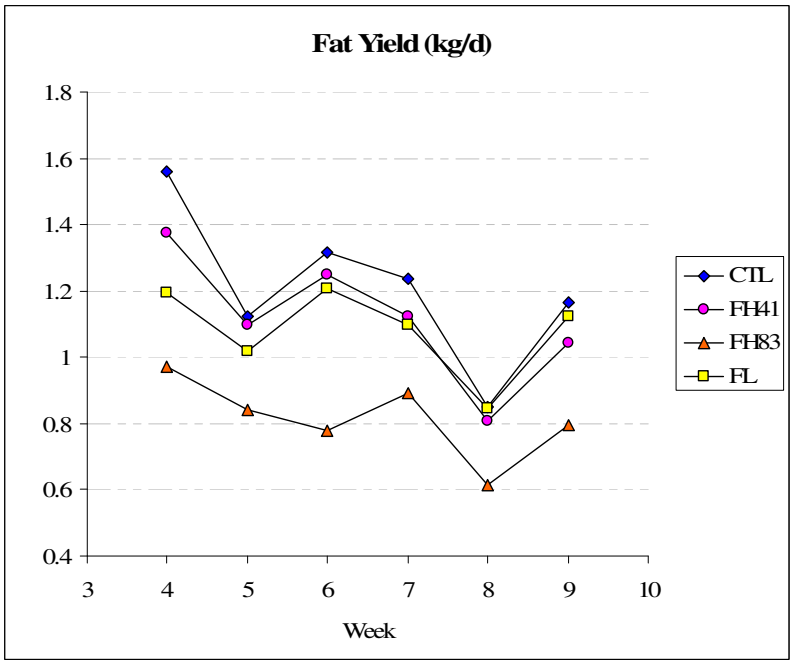

Figure 8. Fat yield for treatments over time. Values equal mean for each treatment. P <0.0001 for week and P = 0.086 for treatment.

P = 0.140 for week; see Figure 9). This disagrees with Shingfield et al. (2006), who found protein yield, not percent, unaffected by time. Milk yield values required a log transformation when statistically analyzed because the data did not meet the assumption of normality. As time continued, milk yield decreased until it hit nadir on wk 7 for CTL and FL and wk 8 for FH41 and FH83 (see Figure 10). After hitting nadir, all treatments seemed to increase slightly. The general decrease in milk yield may be attributed to the stage of lactation that the cows were in (mid-lactation at the start of the experiment), and not FO, because CTL also decreased, but DMI for CTL did not significantly change. In

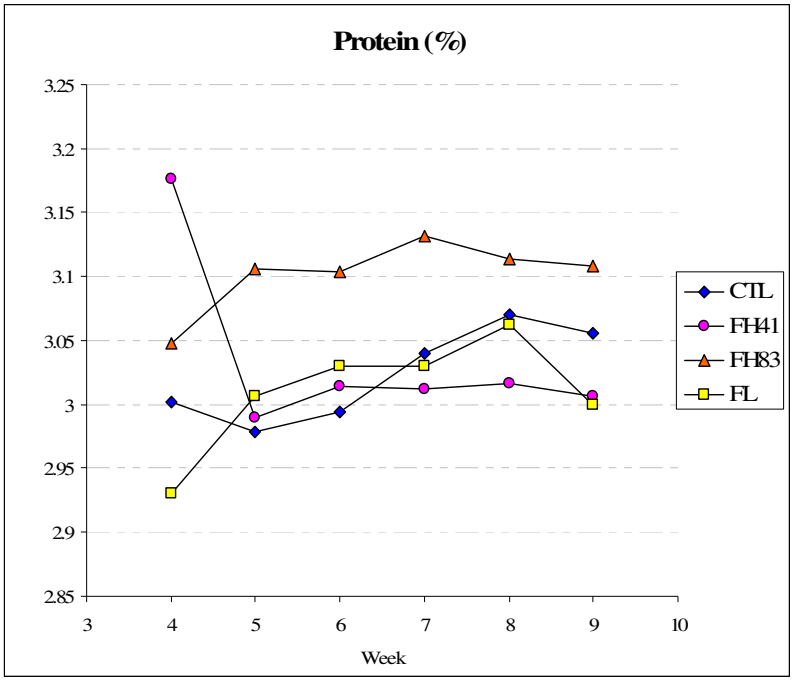

Figure 9. Protein percent for treatments over time. Values equal mean for each treatment. *P* = 0.140 for week and *P* = 0.898 for treatment.

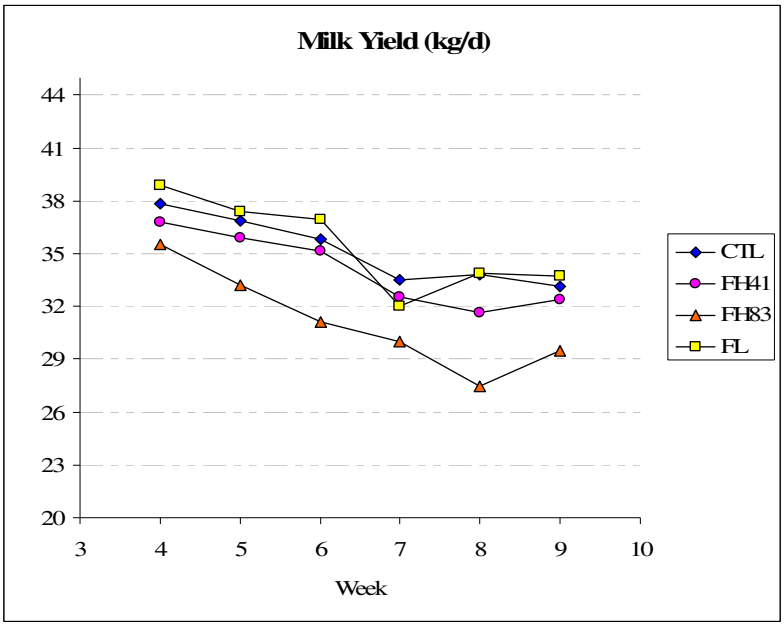

Figure 10. Milk yield for treatments over time. Values equal mean for each treatment. P <0.0001 for week and P = 0.389 for treatment.

accordance with the lowered DMI, means comparisons of treatment showed FH83 had a lower milk yield than the other treatments (31.1 kg versus 35.2 kg, 34.1 kg, and 35.5 kg for CTL, FH41, and FL, respectively).

There was also a time-dependent decrease in protein yield (P <0.0001), displaying a similar pattern as milk yield. Again, nadir was on wk 7 for CTL and FL and wk 8 for FH41 and FH83 (see Figure 11).

Over time DMI did significantly differ (P <0.0001; see Figure 6) for all treatments. FH41 and FH83 had a significant decrease in DMI on wk 7. Both treatments had 71% FO. The higher level of FO, which is known to cause decreased DMI, may

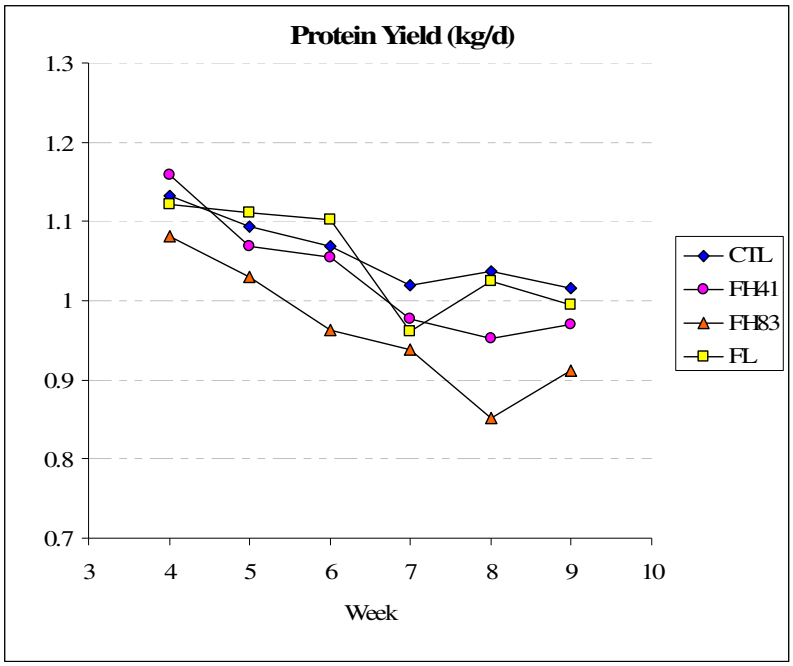

Figure 11. Protein yield for treatments over time. Values equal mean for each treatment. P <0.0001 for week and P = 0.452 for treatment.

explain this decrease because CTL and FL did not display a similar drop. In fact, both CTL and FL increased slightly on wk 6 and then drop back to previous intake values on wk 7. All treatments increased slightly after wk 7.

While wk 4 had the greatest amount of fat yield (see Figure 8) and fat percent (see Figure 7), and wk 8 the least, wk 5, 6, 7, and 9 were variable for all the treatments. There was, however, a general trend of decreased fat yield and percent as time went on. Means comparison of treatment showed that fat percent was lower for FH83 when compared to the other treatments (2.63% versus 3.41%, 3.28%, and 3.15% for CTL, FH41, and FL, respectively) and that FL and CTL differed. Fat and protein percents usually increase as

the DIM increase, but this was not observed in this experiment. As expected, the general decrease of fat yield follows that of milk yield, but the high variability between weeks is curious and could be attributed to the changing of lactation stages and possible heat stress.

The findings for lactose were the inverse of those for fat: wk 4 had the lowest amount of lactose while wk 8 had the greatest (see Figure 12). Weeks 5 and 6, and wk 7 and 9 shared similar values (4.71% and 4.7%, and 4.63% and 4.62%, respectively). The spike between wk 4 and 5, 7 and 8, and generally the high variability seen in this

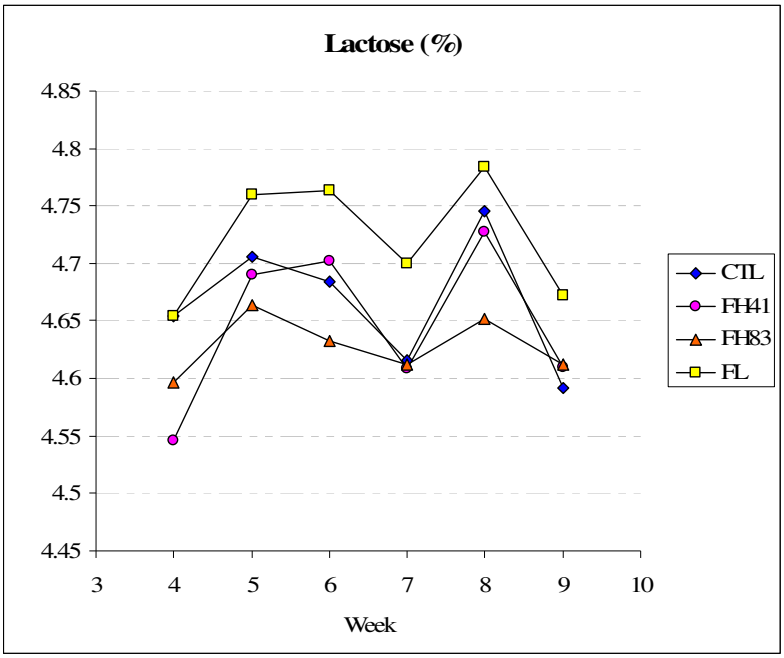

Figure 12. Lactose for treatments over time. Values equal mean for each treatment. P <0.0001 for week and P = 0.531 for treatment.

experiment for lactose do not correlate with the time-dependent decrease of milk yield and also disagree with Cant et al. (1997), who note that the majority of milk volume is determined by lactose. This is also inconsistent with the findings of Baer et al. (2001) and Ramaswamy et al. (2001), who note that lactose is generally unaffected when compared to the control, regardless of FO or other fat supplement fed. It does, however, agree with Giesy et al. (2002), who note that as lactose changes, solids follow a similar pattern. This is demonstrated in the current experiment with both having a slight net increase.

Milk urea was generally consistent over time with the exception of wk 5 holding nadir and wk 7 the highest point (see Figure 13). In general, there was no discernible pattern. Week 7 had the most significant difference in milk urea, with its levels being the highest at this point for all treatments. Means comparison of treatment showed that CTL displayed urea levels above treatments FH83 and FL (14.4% versus 12.3% and 12.9% for FH83 and FL, respectively). It should be noted that there were 3 samples from this week (7) that could not be analyzed by the Rocky Mountain DHIA because they were rotten. Interestingly, they were all from FH41 at the same milking. After consultation with the DHIA, contamination was determined to be the most likely cause. The experiment cows, however, were milked randomly amongst each other. The collection bottles were assigned randomly, as well as the sample containers that were sent to the DHIA. Samples from all 20 experimental cows were exposed to the same temperatures and conditions. The possibility of it being treatment-based is unlikely as

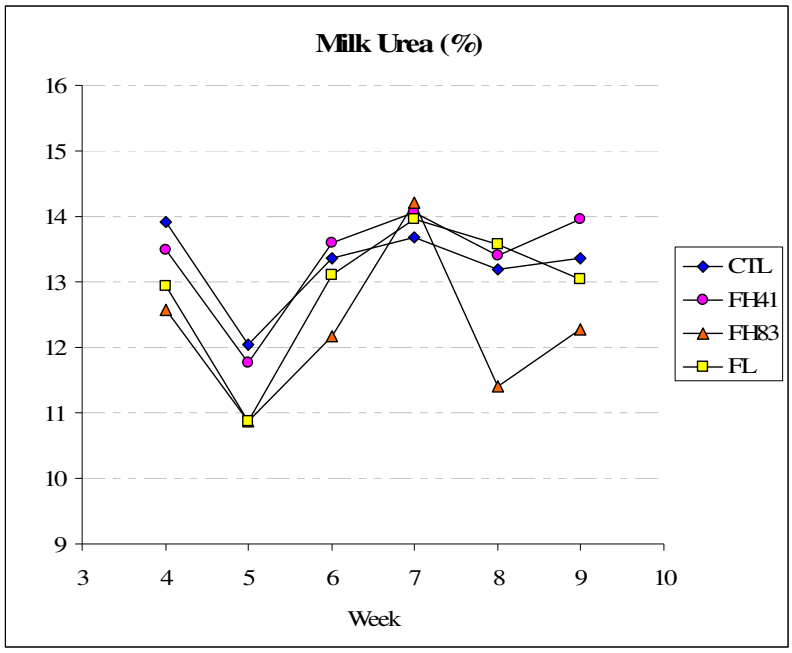

Figure 13. Milk urea for treatments over time. Values equal mean for each treatment. P <0.0001 for week and P = 0.448 for treatment.

only 3 of the 5 cows in that treatment were affected and for only 1 milking. The fact that all the samples came from FH41 may be simple coincidence.

Solids and SCC were also significantly affected by week (P <0.0001 and 0.020, respectively). Week 4 held nadir and wk 8 the highest values for solids. Although there was substantial variation between each week, there was a general net increase (see Figure 14). Expectedly, this trend corresponds to that of lactose and follows that of fat percent. Not surprisingly, values for SCC were highly variable and required a log transformation when statistically analyzed to meet the assumption of normality (see Figure 15). There was a slight net increase over time while all treatments experienced a significant spike in

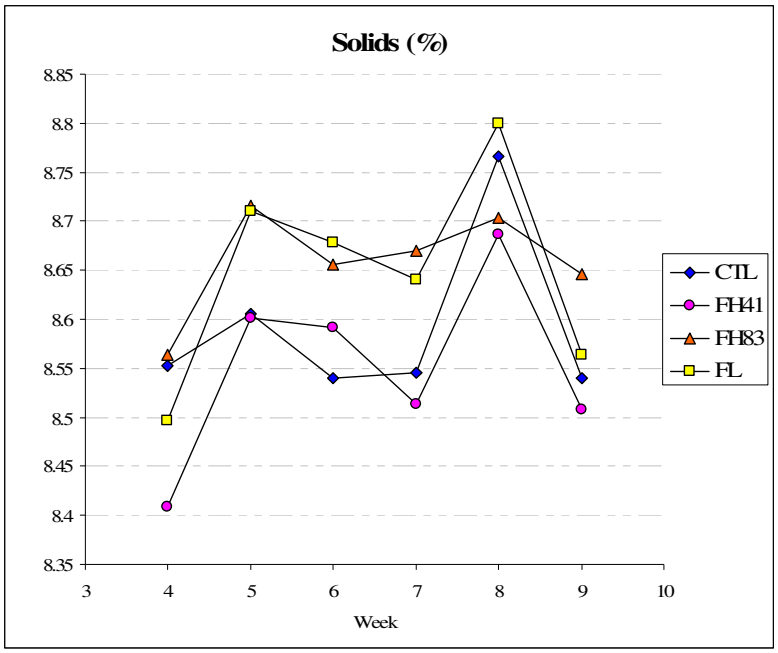

Figure 14. Solids for treatments over time. Values equal mean for each treatment. P <0.0001 for week and P = 0.931 for treatment.

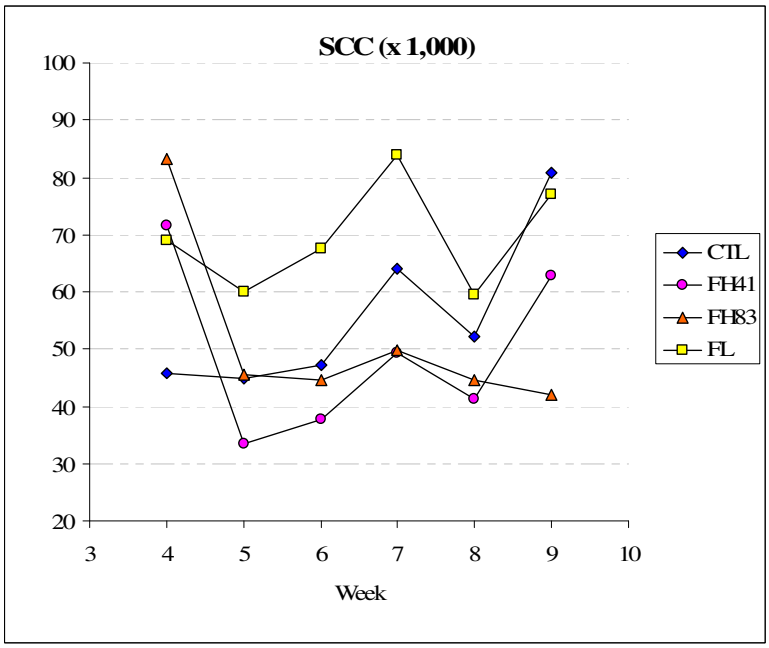

Figure 15. Somatic cell count (SCC) for treatments over time. Values equal mean for each treatment. P = 0.020 for week and P = 0.765 for treatment.

SCC values on wk 7. This spike is most likely from increased oxidative stress caused by heat stress.

It should be noted that cow 9968 belonging to block 1 and treatment FH83 was improperly blocked. This error occurred due to incorrect preliminary data. Although her average daily milk yield 10 d prior to the project met the criterion to be blocked into block 1 (the block with the highest milk yield), for unknown reasons her actual average daily milk yield during the project was significantly lower, and was better fit for block 4. Despite this discrepancy, her data remains in the pool since removing it negatively affected the statistical analysis by unbalancing the experimental design and did not change significance for any measurements. It would have been more advantageous to look at a *prior* lactation in its completeness, rather than 10 d prior, for a more accurate determinant of milk yield.

Fatty Acid Composition of Milk

Short-chain FA concentrations did not significantly decrease with FO supplementation. Some medium-chain FA decreased and the majority of long-chain FA concentrations were increased when compared to CTL. In general, levels of CLA, EPA, and DHA increased over CTL when FO was fed. Total saturated FA did not decrease and total unsaturated FA did not increase when fed FO, unlike similar research. There was no statistically significant interaction between week and treatment for any of the FA. Fatty acid concentrations averaged for each treatment over time are presented in Table 4.

Table 4. Fatty acid composition of milk from cows fed varying amounts of calcium salts of fish oil (FO) and EnerGII

Fatty Acid[2]	Treatment[1] CTL	FH41	FH83	FL	P^3	SEM[4]
	--------- g/100 g of fatty acids reported -------					
$C_{4:0}$[5]	1.94	2.02	1.49	1.88	0.20	0.18
$C_{6:0}$	1.20	1.19	0.89	1.18	0.32	0.13
$C_{8:0}$	0.71	0.68	0.53	0.70	0.42	0.08
$C_{10:0}$	1.65	1.54	1.24	1.67	0.37	0.18
$C_{12:0}$	2.02	1.92	1.72	2.07	0.44	0.16
$C_{14:0}$	8.75	8.50	8.47	8.95	0.86	0.45
$C_{14:1}$	0.76^{ab}	0.58^{a}	1.00^{b}	0.77^{ab}	**	0.07
$C_{15:0}$	0.84	0.78	0.85	0.85	0.23	0.02
$C_{16:0}$	32.9^{ab}	31.5^{a}	33.5^{b}	32.2^{ab}	*	0.45
$C_{16:1}$	1.29	1.30	2.00	1.51	0.10	0.21
$C_{17:0}$	0.42	0.45	0.45	0.44	0.26	0.01
$C_{18:0}$	12.3^{ab}	12.9^{a}	9.46^{b}	11.4^{ab}	*	0.72
$C_{18:1}$ *t*-11 (VA)	1.04^{a}	1.51^{a}	2.28^{b}	1.68^{ab}	**	0.18
$C_{18:1}$ *c*-9	25.6^{a}	25.4^{ab}	23.5^{b}	24.1^{ab}	*	0.50
$C_{18:2}$	2.68^{a}	3.17^{b}	2.82^{ab}	3.08^{b}	**	0.08
$C_{18:3}$ *c*-6,9,12	0.04^{a}	0.02^{ac}	0.01^{b}	0.02^{bc}	***	0.003
$C_{18:3}$ *c*-9,12,15	0.42	0.50	0.46	0.50	0.05	0.02
CLA *c*-9,*t*-11	0.52^{a}	0.67^{ab}	1.15^{c}	0.80^{b}	***	0.08
CLA *t*-10,*c*-12	0.02^{ab}	0.00^{a}	0.02^{b}	0.01^{ab}	*	0.30
Total CLA	0.54^{a}	0.68^{a}	1.18^{b}	0.82^{a}	***	0.08
$C_{20:2}$	0.03	0.03	0.04	0.03	0.06	0.002
$C_{20:3}$ *c*-8,11,14	0.13^{ab}	0.11^{b}	0.06^{c}	0.09^{abc}	**	0.11
$C_{20:3}$ *c*-11,14,17	0.01^{a}	0.01^{ab}	0.04^{b}	0.02^{ab}	***	0.12
$C_{20:4}$	0.12^{a}	0.11^{ab}	0.08^{b}	0.09^{ab}	**	0.006
$C_{20:5}$ (EPA)	0.03^{a}	0.07^{b}	0.08^{b}	0.08^{b}	*	0.01
$C_{22:4}$	0.02	0.02	0.02	0.02	0.09	0.001
$C_{22:5}$	0.04^{a}	0.09^{b}	0.11^{b}	0.10^{b}	**	0.01
$C_{22:6}$ (DHA)	0.02^{a}	0.09^{b}	0.12^{b}	0.11^{b}	**	0.02
Total n-3[6]	0.52^{a}	0.76^{b}	0.82^{b}	0.80^{b}	**	0.04
Total n-6[7]	2.99^{a}	3.43^{b}	2.98^{a}	3.30^{ab}	*	0.10
n-3:n-6	0.17^{a}	0.20^{b}	0.27^{c}	0.24^{bc}	***	0.01
Saturated fatty acids	62.8	61.5	58.6	61.3	0.17	1.23
Unsaturated fatty acids	32.8	33.7	33.7	33.0	0.70	0.68

[a,b,c]Means in the same row with different superscripts differ significantly for treatment effect with *P* value as mentioned in column for significance.

[1]Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**).

[2]Expressed as number of carbons: number of double bonds; *c* = *cis*, *t* = *trans.*

[3]Significance of effects of treatments.

[4]SEM = standard error of least square means.

[5]$C_{4:0}$ to $C_{15:0}$ may have significant response factors to convert relative area to relative percent. $C_{16:0}$ to $C_{22:6}$ are generally close to 1 and do not statistically differ from recorded values. See appendix for transformed values.

[6]Sum of $C_{18:3}$ *c*-9,12,15; $C_{20:3}$ *c*-11,14,17; $C_{20:5}$ (EPA); and $C_{22:6}$ (DHA).

[7]Sum of $C_{18:2}$; $C_{18:3}$ *c*-6,9,12; $C_{20:3}$ *c*-8,11,14; $C_{20:4}$; and $C_{22:4}$.

*P <.05; **P <0.01; ***P <0.001.

There was no treatment effect for the short-chain FA ($C_{4:0}$ to $C_{12:0}$). Week, however, was significant for $C_{4:0}$ and $C_{6:0}$, when both decreased after wk 5. Treatment effect for medium-chain FA ($C_{14:0}$ to $C_{17:0}$) was present in only $C_{14:1}$ and $C_{16:0}$ with treatment FH41 having much smaller concentrations than FH83 for both FA. A decrease in short- and medium-chain FA is common in diets supplemented with FO, with a greater decrease seen with larger amounts of FO, as shown by FH41 and FH83 for $C_{14:1}$ and $C_{16:0}$. The lack of decreased short- and overall medium-chain FA with FO supplementation are unlike those found by Baer et al. (2001), Donovan et al. (2000), and Allred et al. (2006), who found short-and medium-chain FA concentrations to decrease when FO was added to the diet. The effect of week on medium-chain FA was seen in $C_{14:1}$, $C_{16:1}$, and $C_{17:0}$ with high variation between weeks creating no discernible pattern.

The effect of treatment was seen in all the long-chain FA ($C_{18:0}$ and longer) except for $C_{18:3}$ *cis*-9,12,15 (omega-3); $C_{20:2}$; and $C_{22:4,}$ although these had trends of significance. Fatty acids CLA *trans*-10,*cis*-12; $C_{20:3}$ *cis*-11,14,17 (omega-3); and EPA required a log transformation when statistically analyzed to meet the assumption of normality. All treatments showed an increase in VA, $C_{18:2}$, $C_{22:5}$, EPA, and DHA when compared to CTL. Specifically, FH41, FH83, and FL showed a 1.5-, 2.2-, and 1.6-fold increase over CTL when evaluating VA. When compared separately, all omega-6 FA decreased, as desired. Treatment FH41 had increased levels of stearic and oleic acids, while FH83 and FL had decreased levels when compared to CTL. CTL and FH41 shared similar concentrations of $C_{20:3}$ *cis*-11,14,17 (omega-3) while FH83 and FL displayed increased levels. Week was significant for most of these FA, excluding $C_{18:1}$ *trans*-11(VA); $C_{18:1}$

cis-9; $C_{18:3}$ *cis*-6,9,12 (omega-6); CLA *cis*-9,*trans*-11; $C_{20:3}$ *cis*-8,11,14 (omega-6); $C_{20:4}$; and $C_{22:5}$. Those long-chain FA that were affected by time displayed a significant change after wk 4, either decreasing or increasing to wk 5 from their lowest or highest point. Week 6 was also notable in that, except for $C_{22:4}$, it was the inverse of wk 4. In general, however, long-chain FA displayed relatively unremarkable variation.

Although the levels of VA were unaffected by time, differences among treatments were apparent. Treatment FH83 had significantly higher (P <0.01) concentrations of VA when compared to CTL and FH41 (2.28 versus 1.04 and 1.51 g/100 g, respectively). This agrees with Allred et al. (2006) who saw increased levels of VA when FO was fed. Fish oil increases levels of VA by inhibiting the enzyme that converts VA to stearic acid in ruminal biohydrogenation, in turn creating an accumulation of VA (Allred et al., 2006). Therefore, it would follow that more FO would create more accumulation. This was observed in this experiment. Treatment FH83 had a greater concentration of VA than any of the other treatments.

Both CLA isomers were affected by treatment, but week affected only CLA *trans*-10,*cis*-12 with wk 4 (0.010 g/100 g) being significantly lower than wk 6 (0.011 g/100 g; P <0.01). In general, CLA levels did not vary significantly over time (P = 0.066 $\pm$ 0.08), but did demonstrate a trend of significance. Means comparison revealed that total CLA levels increased over time until wk 7 when they hit nadir. Week 8 showed an increase with levels reaching their highest at this point and then dropped again wk 9. Treatment FH83 had more of the *trans*-10,*cis*-12 isomer when compared to FH41 (0.022 and 0.003 g/100 g, respectively). FH83 also had significantly greater levels (P <0.001) of

the *cis*-9,*trans*-11 isomer when compared to the other treatments (1.16 versus 0.517, 0.674, and 0.805 g/100 g for CTL, FH41, and FL, respectively), as well as total CLA (see Table 4). Treatments FH41, FH83, and FL displayed a 1.3-, 2.2-, and 1.5-fold increase of total CLA when compared to CTL. These higher levels of CLA can be partially attributed to the higher level of VA found in FH83 and the activity of the Δ^9-desaturase enzyme (Allred et al., 2006; Whitlock et al., 2006). Whitlock et al. (2006) noted that FO is needed to maximize CLA *cis*-9,*trans*-11 in milk, even if it is provided in small amounts. This is supported by the finding that all treatments fed FO in this experiment had greater concentrations of CLA than CTL.

Because the majority of CLA is made endogenously in the mammary gland from VA via the Δ^9-desaturase enzyme, it is important to estimate the index of this enzyme (Corl et al., 2001; Allred et al., 2006). This is done by estimating the ratios of FA products and substrates requiring Δ^9-desaturase, as presented in Table 5. There was no week × treatment interaction and week was not a significant effect. Treatment effect was significant for only the $C_{14:1}$:$C_{14:0}$ ratio. This differs from Allred et al. (2006) who saw effect of treatment only in $C_{18:2}$ *cis*-9, *trans*-11:$C_{18:1}$ *trans*-11. This difference, however, may be due to composition of diet and FA supplements because Allred et al. (2006) fed FO at 2.7% DM with varying levels of soybean meal. While CTL and FL were similar for $C_{14:1}$:$C_{14:0}$, they had a significantly higher index of the Δ^9-desaturase enzyme than FH41 and less index than FH83. Although not statistically significant, means comparison showed that CTL and FH41 had similar enzyme index for $C_{16:1}$:$C_{16:0,}$ while both FH83

Table 5. Estimated Δ^9-desaturase index of selected fatty acid ratios in the mammary gland of cows fed varying levels of calcium salts of fish oil (FO) and EnerGII

	Treatment[1]					
Fatty Acid Ratios[2]	CTL	FH41	FH83	FL	*P*[3]	SEM[4]
$C_{14:1}$:$C_{14:0}$	0.09[a]	0.07[b]	0.12[c]	0.09[a]	**	0.01
$C_{16:1}$:$C_{16:0}$	0.04	0.04	0.06	0.05	0.14	0.01
$C_{18:1}$ *c*-9:$C_{18:0}$	2.11	2.04	2.55	2.18	0.16	0.16
$C_{18:2}$ *c*-9,*t*-11:$C_{18:1}$ *t*-11	0.50	0.45	0.51	0.48	0.44	0.03

[a,b,c]Means in the same row with different superscripts differ significantly for treatment effect with *P* value as mentioned in column for significance.
[1]Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**).
[2]Expressed as number of carbons: number of double bonds; *c* = *cis*, *t* = *trans.*
[3]Significance of effects of treatments.
[4]SEM = standard error of least square means.
***P* <0.01.

and FL had a slightly higher index. FH83 had the highest index than the other treatments when comparing means for $C_{18:1}$ *cis*-9:$C_{18:0}$. Means comparison showed that FH41 had a lower index when compared to CTL and FH83 for $C_{18:2}$ *cis*-9, *trans*-11;$C_{18:1}$ *trans*-11, while FH83 had a higher index when compared to FL, although not statistically significant. The activity of the Δ^9-desaturase enzyme seemed to be greatest in FH83 overall and may account for the discrepancies seen between the long-chain FA for FH83 and, FH41 and FL. This is especially evident when comparing stearic and oleic acid concentrations. FH83 had, numerically, the greatest amount of both these FA in the supplements when compared to the other treatments. Most importantly, however, FH83 had the most of stearic acid, which is converted to oleic acid via the Δ^9-desaturase enzyme; therefore, the index of Δ^9-desaturase was greatest for FH83 when evaluating the

$C_{18:1}$ *cis*-9:$C_{18:0}$ ratio. These higher levels of FA are most likely due to the type of FA supplement (71% FO) and the level at which it was fed (0.83% DM). It is possible that the majority of the Δ^9-desaturase enzyme was bound converting the larger amounts of oleic acid from stearic acid, thus decreasing the availability of Δ^9-desaturase to convert VA to CLA.

Both EPA and DHA were affected by treatment ($P = 0.014$ for EPA and $P = 0.003$ for DHA), but only DHA was affected by week ($P < 0.0001$). There was an increase in concentration of DHA after wk 4 followed by a plateau at wk 5. As expected, EPA was significantly lower for CTL when compared to the other treatments due to the absence of FO. Because FO provides both EPA and DHA, it isn't surprising that DHA follows the same pattern with CTL having a significantly smaller amount than the other treatments. DHA was increased as much as 6-fold when fed FO and EPA by 2.7-fold when compared to CTL. Interestingly, means comparison reveals that EPA and DHA levels did not significantly differ among the different concentrations (43% and 71%; see Table 4) and amounts of FO fed (FH41, FH83 and FL). These observations for EPA counter those Donovan et al. (2000) found when feeding FO at 1, 2 or 3% DM. They observed that EPA levels increased as the amount of FO increased. In agreement with our findings, however, Ramaswamy et al. (2001) found that varying levels of FO (1 and 2% DM) and soybean meal produced similar levels of EPA.

Time affected only total omega-3 FA concentrations ($P < 0.0001$), not total omega-6 FA ($P = 0.078$), with wk 4 having significantly lower values than wk 5 and 6. Not surprisingly, CTL contained significantly lower levels of total omega-3 FA when

compared to the other treatments and supports other research that demonstrates similar findings when feeding FO. Curiously, treatment FH41 exhibited significantly higher levels of total omega-6 FA when compared to CTL and FH83 (3.43 versus 2.99 and 2.98 g/100 g for CTL and FH83, respectively). The ratio of omega-3-to-omega-6 FA was affected by both time and treatment ($P = 0.0002$ for treatment and $P < 0.0001$ for week). Weeks 8 and 9 contained lower values when compared to wk 5 and 6 (0.217 and 0.216 versus 0.241 and 0.233, respectively). Treatment CTL had significantly lower values for the ratio when compared to the other treatments, and treatments FH41 and FH83 significantly differed (see Table 4). In general, the ratio improved with the supplementation of FO when compared to the control.

As stated previously, AI and TI indices indicate the healthfulness of foodstuffs in regards to FA and their potential to prevent or cause atherosclerosis and thrombosis. As demonstrated by FH41, FH83, and FL, although not statistically different, supplemental FO seemed to slightly improve the TI when compared to CTL (see Table 6). The values obtained from treatment diets are similar to those indicated by Ulbright and Southgate (1991) (lower values indicate a more healthful product).

A recommended intake of EPA and DHA to obtain healthful benefits is proposed to be 650 mg/d (Allred et al., 2006). While the concentrations of EPA and DHA in the milk from the cows in this experiment are only 3.1% of this requirement, based on a 480 mL serving (or about 2 cups) of 2% milk, levels were significantly improved over the CTL. Additionally, CLA levels were increased and the omega-3-to-omega-6 FA ratio improved. Treatment FL, when considering health of the cow, DMI, milk yield, and

Table 6. Influence of treatment diet fed to dairy cows on the atherogenic and thrombogenic indices of milk

	Treatment[1]					
Index	CTL	FH41	FH83	FL	P^2	SEM[3]
Atherogenic	2.19	2.06	2.14	2.19	0.65	0.08
Thrombogenic	1.54	1.44	1.40	1.44	0.16	0.04

[1]Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**).
[2]Significance of effects of treatments.
[3]SEM = standard error of least square means.

production variables, seems to be the most viable diet for actual use in production. Enhanced milk like that created in this experiment could prove to be an important factor and benefit to a balanced and healthy diet.

Sensory Evaluation

There was no statistical significance between treatments or days of storage alone for any of the off-flavors. Only week made a difference for fishy off-flavor ($P = 0.001$). Week 5 contained no intensity of this off-flavor (1.03 $\pm$ 0.07) while wk 8 displayed a higher average intensity of 1.33 ($\pm$ 0.07). Note, however, that although these are statistically different, both weeks had ratings close to 1, which indicates no off-flavor. There was a week × storage day interaction only for oxidative off-flavor ($P = 0.044$). This occurred during wk 8, with d 10 having the more intense off-flavor of oxidation (1.6 $\pm$ 0.11) when compared to 3 d of storage (1.36 $\pm$ 0.10) of the same week. Again, both are rated as having no off-flavor despite statistical differences. There was also a significant interaction between week and treatment, occurring only for rancid off-flavor ($P = 0.023$).

Both FH83 and FL displayed significantly higher intensities in wk 8 versus wk 5 (1.35 $\pm$ 0.10 and 1.81 $\pm$ 0.01 for wk 5 versus 2.08 $\pm$ 0.10 and 2.36 $\pm$ 0.10 for wk 8, respectively), thus wk 5 ratings indicated no off-flavor detected, while the rancid off-flavor increased to slight detection in wk 8. This interaction probably occurred in only these 2 treatments because they both yielded the highest amounts of CLA, EPA, and DHA. Polyunsaturated FA, such as these, are susceptible to rancid off-flavor. It should be noted that, although CTL served as our control for the diets, the store-bought whole milk (**STORE**) served as our control for the sensory evaluation and should not be confused with the control of the diets.

Sensory evaluation for wk 5 milk after 3 d of storage yielded generally favorable results. Of the 5 flavor characteristics tested (oxidative, fishy, oily, grassy, and rancid), FL was the only treatment that seemed to yield a fishy flavor when compared to the other treatments, but this difference was not statistically significant ($P = 0.674$) for treatment effect. This difference, however, was found to be at an intensity between 1 (no off-flavor detected) and 1.5 (see Figure 16). The mean intensity for fishy off-flavor for all treatments combined during this period was 1.04 ($\pm$ 0.10). FL also displayed a grassy flavor when compared to CTL and STORE in the same testing period ($P = 0.093$), but, again, this was not statistically significant for the overall treatment effect. The intensity was rated between 1 and 2 with a mean intensity for grassy off-flavor of 1.29 ($\pm$ 0.07), no off-flavor detected. Treatments FH41, FL, and STORE all had intensities for both

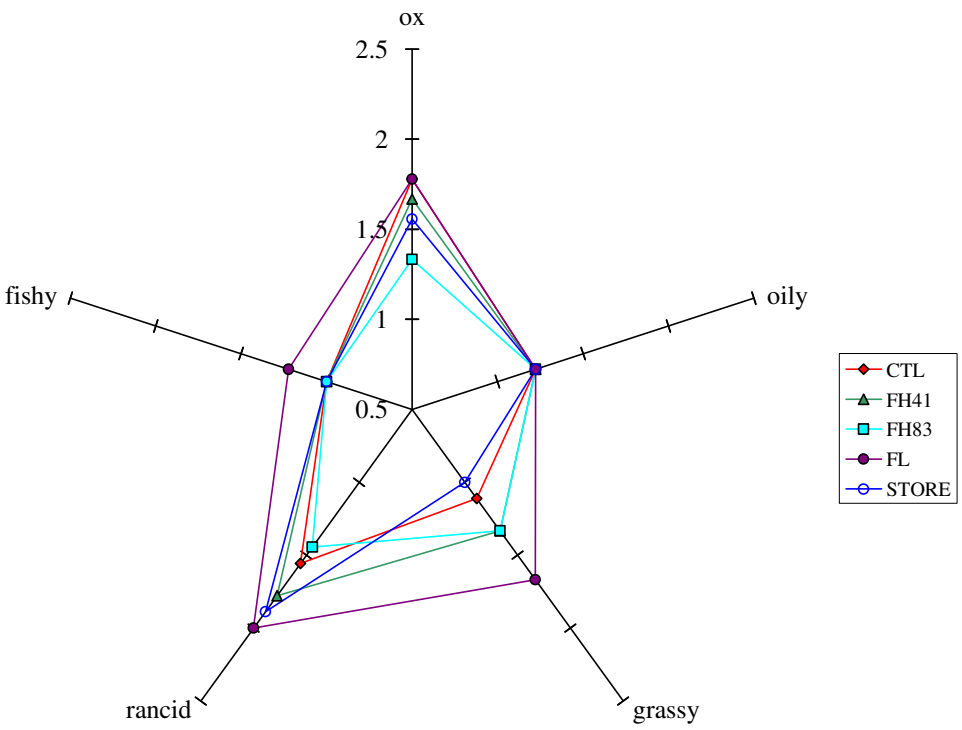

Figure 16. Descriptive analysis of milk for week 5, 3 days of storage**.** 1 = no flavor, 2 = slight, 3 = moderate, 4 = strong, and 5 = extremely strong. Treatment effect was *P* = 0.329, 0.785, 0.093, 0.245, and 0.674 for oxidized, oily, grassy, rancid, and fishy, respectively.

Table 7. Average intensities of off-flavors between treatments detected in milk from cows fed calcium salts of fish oil (FO) and EnerGII at varying levels during certain periods of sampling and storage

	Off-flavor[1]				
Time Period	Oxidized[3]	Fishy[4]	Grassy	Rancid	Oily
Week 5					
3 days[2]	1.62 (0.10)[5]	1.04 (0.10)	1.29 (0.07)	1.73 (0.13)	1.22 (0.06)
10 days	1.45 (0.11)	1.00 (0.11)	1.15 (0.07)	1.73 (0.14)	1.15 (0.06)
Average	1.54 (0.07)	1.03 (0.07)	1.22 (0.05)	1.73 (0.10)	1.19 (0.04)
Week 8					
3 days	1.36 (0.10)	1.33 (0.10)	1.20 (0.07)	1.84 (0.13)	1.27 (0.06)
10 days	1.60 (0.11)	1.30 (0.11)	1.14 (0.08)	2.00 (0.14)	1.10 (0.06)
Average	1.47 (0.07)	1.32 (0.07)	1.18 (0.05)	1.92 (0.10)	1.19 (0.04)

[1]Values represent means of intensities of treatments combined for each off-flavor. Evaluated on a 5-point scale where 1 = no flavor, 2 = slight, 3 = moderate, 4 = strong, and 5 = extremely strong.
[2]Days milk was stored before evaluated.
[3]Off-flavor that yielded significant difference for week and storage day interaction ($P = 0.044$).
[4]Off-flavor that yielded significant difference between weeks ($P = 0.005$).
[5]Values in parentheses equal standard error of least square means (SEM).

oxidized and rancid off-flavors that were above an intensity of 1.5. Nothing in the production variables or FA composition clearly indicated why only these 2 diet treatments, versus FH83 and CTL, had intensities close to barely perceptible for only oxidized and rancid after 3 d of storage during wk 5. The mean intensities for oxidation, rancid, and oily during this week and day of storage, as well as the other off-flavors for both weeks and both days of storage, are presented in Table 7.

In the evaluation 7 d later (10 d of storage) of the same milk (wk 5), CTL and the STORE seemed to have a lower intensity of grassy off-flavor flavor when compared to the means of the other samples (see Figure 17). The slightly more intense grassy off-flavor in the other treatments could be attributed to the CLA in the EnerGII or the CLA synthesized endogenously from the FA supplied by EnerGII, as well as the enhancement of CLA production by the FO. This is supported by the fact that FH41, FH83, and FL all had more total CLA that CTL, which had no FO supplement. Again, treatments FH41, FL, CTL, and STORE had intensities above 1.5 for rancid off-flavor. As with 3 d of storage, this cannot be explained by production variables or FA composition. Additionally, CTL was the only treatment to rate above 1.5 for oxidized off-flavor with no production variable or FA composition indicating any possible discrepancies among treatments.

The first evaluations show that there is little to no perceptibility of off-flavors, specifically fishy, when cows have been fed varying levels of FO supplement through their diet for 5 wk. This holds true for the same milk that has been stored for an additional 7 d (simulation of shelf life of consumer's milk). The average intensities for both days of storage during wk 5 for oxidation, rancid, oily, fishy, and grassy were 1.54, 1.73, 1.19, 1.03, and 1.22, respectively. Note that although some come close, no off-flavor reached an intensity of 2 (barely perceptible); therefore, there was essentially no off-flavor detected. It should also be noted, however, that all the milk, even STORE, contained some rancid off-flavor.

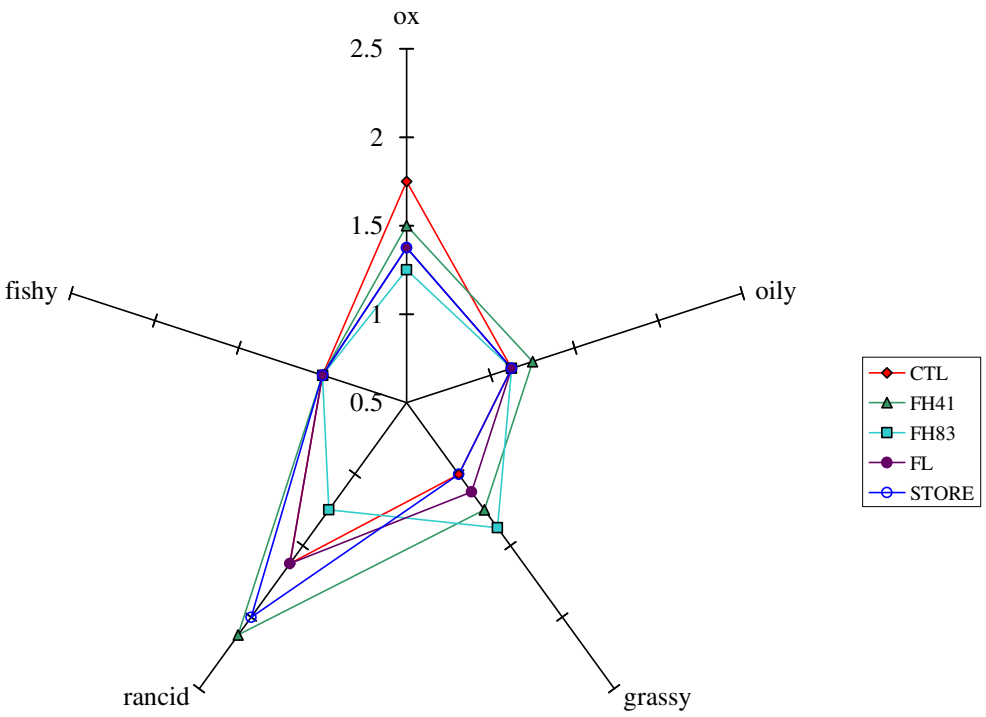

Figure 17. Descriptive analysis of milk for week 5, 10 days of storage**.** 1 = no flavor, 2 = slight, 3 = moderate, 4 = strong, and 5 = extremely strong. Treatment effect was *P* = 0.329, 0.785, 0.093, 0.245, and 0.674 for oxidized, oily, grassy, rancid, and fishy, respectively.

Sensory evaluations for wk 8 milk demonstrated more detectable off-flavors than for wk 5. The 3 d storage evaluation showed that FL and FH41 had noteworthy rancid flavor when compared to the other samples (both at 2.22; see Figure 18). Additionally, FL and CTL had intensities of rancid off-flavor above 1.5. The fact that all of the diet treatments had at least slight perceptibly of rancid off-flavor may be due to increased levels of unsaturated FA compared to STORE. The other off-flavors yielded similar intensities among treatments.

After 7 d of storage, greater intensities of off-flavors were present, although none above 2.5. Despite the lack of overall effect of treatment, STORE seemed to have a more intense oxidative flavor (2.13) when compared to the other treatments (1.38 for CTL, FH41, FH83 and 1.75 for FL). This may be due to packaging, as it was in a clear plastic jug. CTL also seemed to have a more intense oily flavor (1.38) when compared to the other treatments (1.13 for FH41 and 1 for FH83, FL, and STORE). Although all treatments displayed intensities of rancid off-flavor, FL showed the most intense at 2.36, although not statistically significant (see Figure 19).

The second evaluations show that FL seems to yield low-intensity rancid flavors after cows have been fed FO for 8 wk and the milk has been stored for 3 d. This off-flavor in FL seems to intensify after an additional 7 d of storage. This is interesting since FL contained the lowest amount of FO and was the most ruminally inert. These off-flavors, however, cannot be atttributed to simply the amount of EnerGII because FH83 contained the same amount of EnerGII as FL; nor can it be attributed to FO

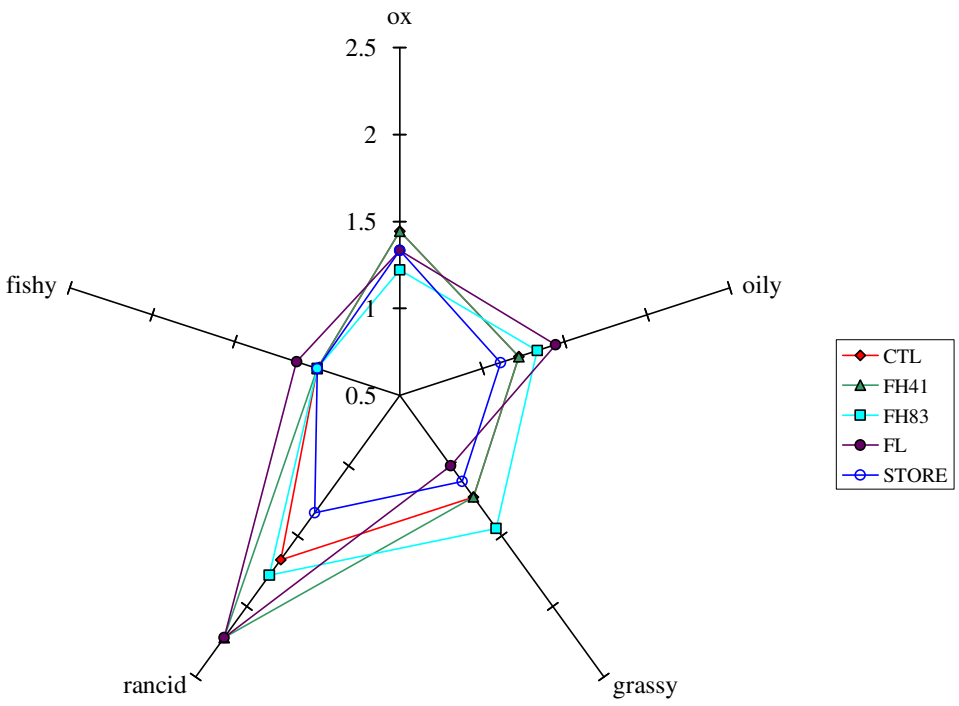

Figure 18. Descriptive analysis of milk for week 8, 3 days of storage**.** 1 = no flavor, 2 = slight, 3 = moderate, 4 = strong, and 5 = extremely strong. Treatment effect was *P* = 0.329, 0.785, 0.093, 0.245, and 0.674 for oxidized, oily, grassy, rancid, and fishy, respectively.

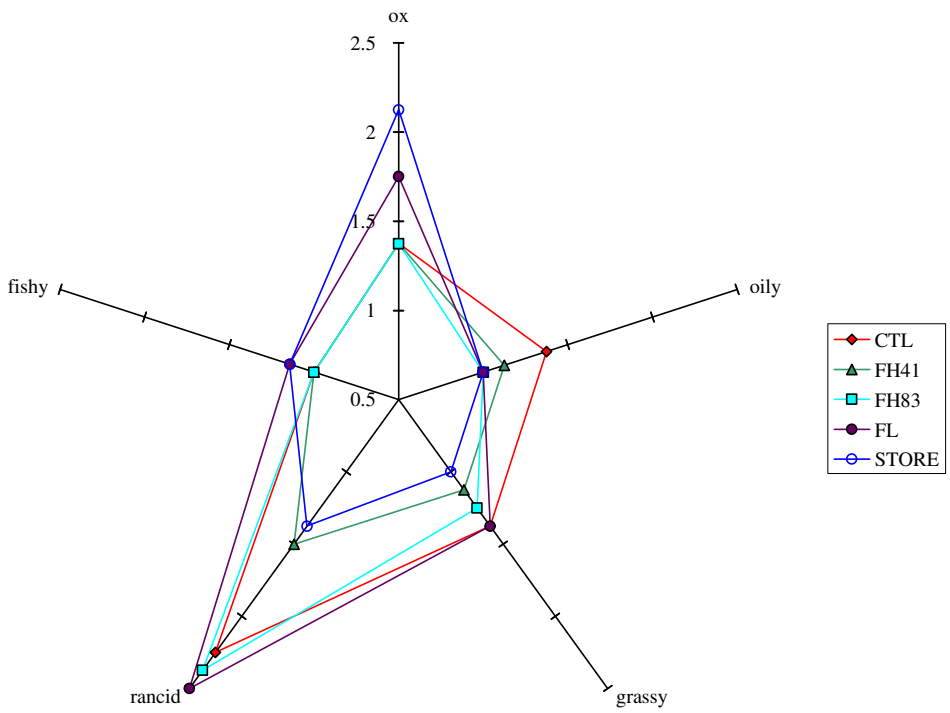

Figure 19. Descriptive analysis of milk for week 8, 10 days of storage**.** 1 = no flavor, 2 = slight, 3 = moderate, 4 = strong, and 5 = extremely strong. Treatment effect was *P* = 0.329, 0.785, 0.093, 0.245, and 0.674 for oxidized, oily, grassy, rancid, and fishy, respectively.

supplementation because STORE had higher intensities of rancid off-flavor in wk 5 than in wk 8.

While FL seemed to remain slightly rancid after 8 wk, regardless of storage, other treatments (CTL and FH83) displayed the rancid off-flavor after the additional 7 d of storage. In contrast, rancid off-flavor for FH41 actually went down from 3 d to 10 d (from 2.2 to 1.5). As in wk 5, these findings cannot be correlated to FA composition or any production variables. Interestingly, only CTL was found to have gained an oily off-flavor after 8 wk of FO and 10 d of storage. The oily off-flavor detected in CTL is probably due to the fact that this treatment contained no FO; therefore there was less milk fat depression, which FO tends to cause, thus creating excess fat after 8 wk and 10 d of storage to the point of detection. This seems to be supported by the fact that CTL contained the highest percentage of fat (3.41% versus 3.28%, 2.63%, and 3.25% for FH41, FH83, and FL, respectively, although not statistically significant), on average, when compared to the other treatments over the length of the experiment. Additionally, CTL had the lowest amounts of all omega-3 FA analyzed, as well as total omega-3 FA. The reason for the off-flavors detected in STORE is unknown. The source of this milk (dairy, type of cows, diet, etc.) is unknown and the possibilities for these off-flavors cannot be determined.

While it was expected that there may be some off-flavors due to FA supplementation, especially FO, the treatments that actually yielded these off-flavors do not coincide with initial predictions. Fish oil, in general, tends to yield fishy off-flavor,

especially when supplemented at high levels. Conjugated linoleic acid tends to yield a grassy off-flavor. It is also noted that oxidation of polyunsaturated FA can cause rancid and oxidative off-flavors, especially when unprotected, but the other off-flavors tested, in addition to oily, were not predicted to be caused specifically due to project supplementation. First, STORE, our sensory evaluation control, was often found to have detectable off-flavors, albeit slight. Again, this may be due to packaging and exposure to light. Lacasse et al. (2002), however, noted that their control (milk from cows fed no FO) also yielded a slight off-flavor. Second, CTL yielded slight oily and rancid off-flavors. While the oily flavor seems to be explained by the lack of FO, the rancid flavor may be explained by spoilage since rancid off-flavor increased from 3 d to 10 d of storage during wk 8. This may be due to increased lactose since it was at its highest level at this point. Lastly, both FL and FH41 consistently yielded off-flavor intensities between 1.5 and 2 (barely perceptible) for rancid. It was expected that perhaps FH83 would yield these results, especially fishy, since it had the most FO (71%) per DM (0.83%), but it generally tested favorably (no significant off-flavors). Jones et al. (2005) noted that off-flavor in milk from cows fed only 45 g/kg of FO and sunflower oil was detected the majority of the time when compared to milk from cows feds either FO or sunflower oil alone. A study by Ramaswamy et al. (2001) showed that milk from cows fed a diet with just FO supplemented yielded lower scores (less off-flavor) for detected oxidized flavor than for those fed only extruded soybeans. They also noted a higher score for cows fed both FO and extruded soybeans after 3 d of storage when compared to FO alone, but lower when compared to just extruded soybeans. Attributes Sensory Evaluations in these studies show

that even low levels of FO are detectable, these low levels can be affected by another FA source, and may explain why FL in our experiment contained a low-intensity rancid off-flavor in wk 8. Although not statistically significant, grassy off-flavor showed a trend towards treatment ($P = 0.093$) with STORE rating lower than the other treatments. Additionally, a trend in storage day for oily off-flavor (P = 0.051) showed that intensities lessened from d 3 (1.2 $\pm$ 0.06) to d 10 (1.1 $\pm$ 0.06). Our results showed that a fishy off-flavor is of least concern, while low-intensity off-flavors of mainly rancid is of the most.

While there were off-flavors slightly detected in all the evaluated milk, especially FL overall and many of the other treatments during both evaluations in wk 8, none of these were rated above an intensity of 2.5 (2 = slight and 3 = moderate). Although a Consumer Acceptability Sensory Evaluation was not conducted, it could be possible that milk from cows fed these diets would be found generally acceptable and fit for sale. This seems to be supported by the fact that the store-bought milk in our experiment seemed to often possess an off-flavor. This shows that despite the presence of low-intensity off-flavors, the quality and taste of milk would be comparable to that currently sold in the store, if not better, and any off-flavors detected in the milk from cows fed the diets in this experiment cannot necessarily be attributed to FO, EnerGII, or their combination.

CONCLUSION

There was no week × treatment interaction for any production variable. Treatment only affected DMI and subsequent NE_L, but this cannot be conclusively attributed to diet. Week significantly affected milk yield and all milk components except for protein percent. Milk yield, protein yield, fat yield, fat percent, and DMI generally decreased over time. Lactose and solids shared a similar slight net increase with variability between weeks. Milk urea demonstrated no general trend over time, but was highly variable between weeks. Somatic cell count was also variable, but showed a slight net increase. There was a significant spike in wk 7 and 9 for SCC. Significant compositional differences in experimental milk may be due to heat stress, increasing DIM or the confounding of the two, but cannot necessarily be attributed to experimental diets.

Short-chain FA concentrations did not differ among treatments and only 2 medium-chain FA were affected by treatment, demonstrating a decrease. The majority of the long-chain FA concentrations were increased when compared to the control. EPA and DHA levels were significantly improved over the control. Additionally, CLA levels were increased, the omega-3-to-omega-6 ratio improved, and AI and TI maintained.

There were no detectable differences between milk samples from each of the treatments when looking at treatment as an effect alone. Only low intensity off-flavors (no higher than 2.5, where 1 = no off-flavor, 2 = barely perceptible, and 3 = moderate) were detected in any of the milk samples from any of the weeks and days of storage. Rancid, not fishy, off-flavors were the most detected and was the only off-flavor to contain a week × treatment interaction, with it being the greatest for FL in wk 8. FL, the

treatment with the lowest level of FO in the diet, contained most of the detected off-flavors. Week only significantly affected fishy off-flavor, with it being slightly more intense for wk 8. Oxidized was the only off-flavor to contain a week × storage day interaction, with it increasing from d 3 to d 10 in wk 8. The store-bought control often had detectable off-flavors and, therefore, any detectable off-flavors in the experimental milk cannot necessarily be attributed to FO, EnerGII or their combination.

Cows fed varying levels of FO and EnerGII displayed no negative effects on their health or overall milk composition due to experimental diets. Concentrations of EPA, DHA, and CLA were all significantly enhanced when compared to the control. Milk from cows fed these FA supplements generally yielded little to no off-flavors. Treatment FL, when considering health of the cow, DMI, milk yield, production variables, and sensory evaluations, seems to be the most viable diet for actual use in production. Enhanced milk like that created in this experiment would prove to be an important factor and benefit to a balanced and healthy diet.

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APPENDIX

Table 8. Altered fatty acid composition of milk from cows fed varying amounts of calcium salts of fish oil (FO) and EnerGII due to response variables

Fatty Acid[2]	Treatment[1] CTL	FH41	FH83	FL	P^3	SEM[4]
	--------- g/100 g of fatty acids reported -------					
$C_{4:0}$[5]	3.85	4.01	2.96	3.74	0.20	0.18
$C_{6:0}$	1.82	1.81	1.35	1.79	0.32	0.13
$C_{8:0}$	0.99	0.94	0.74	0.98	0.42	0.08
$C_{10:0}$	2.02	1.89	1.52	2.05	0.37	0.18
$C_{12:0}$	2.30	2.18	1.95	2.36	0.44	0.16
$C_{14:0}$	9.43	9.16	9.13	9.65	0.86	0.45
$C_{14:1}$	0.86^{ab}	0.65^{a}	1.13^{b}	0.87^{ab}	**	0.07
$C_{15:0}$	0.91	0.85	0.92	0.92	0.23	0.02
$C_{16:0}$	33.0^{ab}	31.6^{a}	33.6^{b}	32.3^{ab}	*	0.45
$C_{16:1}$	1.38	1.39	2.14	1.62	0.10	0.21
$C_{17:0}$	0.44	0.47	0.47	0.46	0.26	0.01
$C_{18:0}$	12.3^{ab}	12.9^{a}	9.46^{b}	11.4^{ab}	*	0.72
$C_{18:1}$ *t*-11 (VA)	1.13^{a}	1.64^{a}	2.48^{b}	1.82^{ab}	**	0.18
$C_{18:1}$ *c*-9	25.2^{a}	25.1^{ab}	23.1^{b}	23.7^{ab}	*	0.50
$C_{18:2}$	2.75^{a}	3.25^{b}	2.89^{ab}	3.16^{b}	**	0.08
$C_{18:3}$ *c*-6,9,12	0.04^{a}	0.02^{ac}	0.01^{b}	0.02^{bc}	***	0.003
$C_{18:3}$ *c*-9,12,15	0.44	0.52	0.48	0.52	0.05	0.02
CLA *c*-9,*t*-11	0.52^{a}	0.67^{ab}	1.15^{c}	0.80^{b}	***	0.08
CLA *t*-10,*c*-12	0.02^{ab}	0.00^{a}	0.02^{b}	0.01^{ab}	*	0.30
Total CLA	0.54^{a}	0.68^{a}	1.18^{b}	0.82^{a}	***	0.08
$C_{20:2}$	0.03	0.03	0.04	0.04	0.06	0.002
$C_{20:3}$ *c*-8,11,14	0.13^{ab}	0.11^{b}	0.06^{c}	0.09^{abc}	**	0.11
$C_{20:3}$ *c*-11,14,17	0.01^{a}	0.01^{ab}	0.04^{b}	0.02^{ab}	***	0.12
$C_{20:4}$	0.12^{a}	0.11^{ab}	0.08^{b}	0.09^{ab}	**	0.006
$C_{20:5}$ (EPA)	0.03^{a}	0.07^{b}	0.08^{b}	0.08^{b}	*	0.01
$C_{22:4}$	0.02	0.02	0.02	0.02	0.09	0.001
$C_{22:5}$	0.05^{a}	0.09^{b}	0.11^{b}	0.10^{b}	**	0.01
$C_{22:6}$ (DHA)	0.02^{a}	0.09^{b}	0.12^{b}	0.11^{b}	**	0.02
Total n-3[6]	0.49^{a}	0.69^{b}	0.73^{b}	0.72^{b}	**	0.04
Total n-6[7]	3.06^{a}	3.51^{b}	3.05^{a}	3.38^{ab}	*	0.10
n-3:n-6	0.16^{a}	0.20^{b}	0.24^{c}	0.21^{bc}	***	0.01
Saturated fatty acids	67.1	65.9	62.2	65.6	0.17	1.23
Unsaturated fatty acids	31.9	33.1	32.8	32.2	0.70	0.68

[a,b,c]Means in the same row with different superscripts differ significantly for treatment effect with *P* value as mentioned in column for significance.

[1]Cows in the 4 treatments were fed either a control diet of 57% forage and 43% concentrate mix with EnerGII fat supplement at 1.65% of diet DM (**CTL**) or EnerGII in basal diet was partially replaced with (a) 0.21% partially ruminally inert calcium salts (Ca-salts) of 71% fish oil (Ca-FO71) given at 0.41% DM (**FH41**); (b) 0.41% inert Ca-FO71 given at 0.83% DM (**FH83**); or (c) 0.83% inert Ca-FO 43% fish oil (Ca-FO43) given at 0.83% DM (**FL**).

[2]Expressed as number of carbons: number of double bonds; *c* = *cis*, *t* = *trans.*

[3]Significance of effects of treatments.

[4]SEM = standard error of least square means.

[5]$C_{4:0}$ to $C_{15:0}$ may have significant response factors to convert relative area to relative percent. $C_{16:0}$ to $C_{22:6}$ are generally close to 1 and do not statistically differ from recorded values. See appendix for transformed values.

[6]Sum of $C_{18:3}$ *c*-9,12,15; $C_{20:3}$ *c*-11,14,17; $C_{20:5}$ (EPA); and $C_{22:6}$ (DHA).

[7]Sum of $C_{18:2}$; $C_{18:3}$ *c*-6,9,12; $C_{20:3}$ *c*-8,11,14; $C_{20:4}$; and $C_{22:4}$.

*P <.05; **P <0.01; ***P <0.001.