Genetic Analysis of Fatty Acid Composition of Milk: Basis for Improvement of the Healthfulness of the U.S. Milk Supply

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Jon P. Schoonmaker, Rafael A. Nafikov, James M. Reecy, Diane E. Spurlock, Dept. of Animal Science; Jenny Minick-Bormann, Department of Animal Sciences and Industry, Kansas State University

Introduction

Saturated fatty acid content of the human diet continues to be of concern for health-conscious consumers, especially with regard to plasma cholesterol concentration. The reason for concern is that cardiovascular disease is the leading cause of death in the United States, killing nearly one million people per year from cardiovascular disease or stroke (American Heart Association, 1998). Nearly 60 million people in the United States (20% of the population) have at least one type of cardiovascular disease such as hypertension, coronary artery disease, stroke, or rheumatic heart disease (American Heart Association, 1998). Many of these deaths could be delayed or prevented by prudent changes in the diet.

Dairy products alone account for 17% of the total fat in the American diet (National Research Council, 1988). Animal products provide collectively 56% of total fat, 74% of saturated fatty acids, and 100% of the cholesterol consumed by humans (Rhee, 1992). Milk is a primary source of animal saturated fatty acids. The fatty acid composition of bovine milk is more dependent on the genetics of the animal and much less dependent on diet composition. Bacteria in the rumen of dairy cows hydrogenate a very high proportion of dietary unsaturated fatty acids, which results in a greater concentration of saturated fatty acids in milk and meat from ruminants compared with milk and meat from non-ruminants. The fatty acid composition of pork, for example, is easily changed by changing the fatty acid composition of the diet, but, in cattle, 85% of the double bonds in dietary linolenic acid (18:3), linoleic acid (18:2), and oleic acid (18:1) are reduced before the acids enter the small intestine for absorption, primarily as stearic acid (18:0) (Scott et al., 1969).

Decreasing the consumption of dietary fats is commonly recommended by human nutritionists as the best dietary means for decreasing risk of coronary disease. Replacing dietary saturated fatty acids with monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA), however, can have positive long-term effects on human health. In a 14-year study of more than 80,000 women, reduction of total fat intake had little effect on long-term coronary health, whereas replacement of saturated fatty acid with MUFA decreased relative risk of coronary disease significantly (Hu et al., 1997). By developing animal products with greater percentages of MUFA and PUFA, coronary disease may be decreased significantly. Decreasing the saturated fatty acid composition of milk by replacing these "less healthy" fatty acids with MUFA would truly lead to an improvement in healthfulness for the human diet and may prevent a decline in milk consumption. Because so many Americans drink milk and because milk is a primary source of saturated fatty acids and cholesterol, this research focuses on a critical target with respect to modifying fatty acid intake and cholesterol intake without drastically changing food choices.

We hypothesize that variation in fatty acid composition of milk fat lipids is heritable and that the differences will be associated with single nucleotide polymorphisms (SNP) in lipogenic enzymes.

Materials and Methods

Monthly milk samples were collected from Holstein, Brown Swiss, and Jersey cows at Iowa State University (Ames, IA) and at Kansas State University (Manhattan, KS). When combined, these herds consist of 400 + lactating cows. Data will only be presented for Holstein cows in the current paper. Our goal is to collect monthly milk samples from two complete lactations from as many cows as possible. To accomplish this goal, we will collect monthly milk samples for 2.5 years. Because of an estimated 25% culling rate, we will not be able to collect data from two lactations on all cows, but we are collecting samples from replacement animals and will utilize single lactation data as well. When finished, we estimate that we will have data on two lactations from 350 cows and single lactation data on 150 cows.

We have currently collected and will present 1 year (September 2006 to August 2007) of data from the Iowa State University herd and 5 months (November 2006 to March 2007) of data from the Kansas State University herd. All cows were used under the guidelines of the individual university or college animal use committees. Only data from healthy cows have been used for analyses. All cows within a herd have been managed similarly.

Sampling and analytical procedures.

Monthly milk samples were collected from cows at alternate AM and PM milkings (only cows starting their lactation after August 2006), frozen and stored at -20°C, and transported to Iowa State University for fatty acid analyses. Lipids were extracted from milk according to the procedures of Hara and Radin (1978) and fatty acids were derivatized as methyl esters according to the method of Christie (1982)

as modified by Chouinard et al. (1999). This procedure is commonly used in our laboratory. Briefly, milk is centrifuged at 10,000 x g for 30 minutes, lipid is removed and dissolved in hexane/isopropanol (3/2 vol/vol), and the hexane layer is removed following addition of a 6.7% solution of sodium sulfate. Four-hundred uL of sample (representing approximately 40 mg of lipid) was trans-esterified by addition of 40 uL of methyl acetate, 40 uL of 1 M sodium methoxide, and 60 uL of 3.33 % oxalic acid. A portion of the upper hexane layer was removed for analysis by gas chromatography.

The methyl esters of fatty acids from milk were quantified on a Varian 3800 gas chromatograph equipped with an automatic injector (Varian Inc, CA) to allow for 24-hour operation. A 100-meter SP-2560 column (Supelco Inc., Bellefonte, PA) was used so that all fatty acids, including conjugated 18:2, were resolved. Fatty acid percentage was calculated by dividing individual fatty acid peak areas by the total of all fatty acid peak areas. An atherogenic index (AI), as described by Ulbricht and Southgate (1991), was calculated for each milk sample. The AI described by these authors ranks mixtures of fatty acids according to their propensity to cause atherogenesis, as predicted from concentrations of individual fatty acids in the lipid. The AI is calculated as: $(12{:}0 + 4(14{:}0) + 16{:}0) \div (\Sigma MUFA + \Sigma PUFA)$. Cows were ranked according to the AI of their milk and split into three groups (low: AI < 1.83, medium: AI from 1.83 – 2.13, and high: AI > 2.13). Changes in individual fatty acid in the low, medium, and high groups were graphed over the entire lactation. We have only been collecting samples from cows after they start their lactation; thus, data for 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, and 10th months of lactation represents 140, 100, 64, 40, 28, 22, 12, 8, 6, 5 cows for the low AI group, 136, 126, 111, 89, 69, 57, 43, 34, 23, and 17 cows for the medium AI group, and 141, 136, 127, 118, 106, 100, 88, 78, 59, and 33 cows for the high AI group.

One goal of this research is to determine heritability values for the phenotypes we are collecting for use in breeding programs and determine a ranking of animals by different phenotypes for use in identifying single nucleotide polymorphisms (SNPs) in candidate genes for lipid synthesis and in performing association analysis in dairy cattle. A SNP is a DNA sequence variation occurring when a single nucleotide - A, T, C, or G - in the genome differs between members of a species. SNPs that result in an amino acid change, in the case of the lipogenic enzymes, can alter the efficiency with which the enzyme converts lipogenic precursors into specific lipids. The three genes that we have chosen to screen, initially, include fatty acid synthase (FAS), the multifunctional enzyme responsible for *de novo* fatty acid synthesis from acetyl-CoA, acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in fatty acid synthesis, and stearoyl-CoA desaturase (SCD), an enzyme that converts saturated long-chain fatty acids into MUFA. We have collected blood from all animals, isolated DNA, and are performing polymerase chain reaction – restriction fragment length P (PCR-RFLP) to screen for SNPs identified by our sequencing efforts or from publicly available EST data. The long-term outcome of our research is that the producers will have selection tools for producing milk that has fatty acid compositions that promote human health.

Results and Discussion

As expected, the trend was for high AI cows to produce milk fat with the highest percentage of saturated fatty acids (SFA), and to produce milk fat with the lowest percentage of MUFA and PUFA. High AI cows also produced milk fat with the highest percentage of short-chain fatty acids (SCFA) and the highest percentage of medium-chain fatty acids (MCFA). Low AI cows produced milk-fat with the lowest percentage of SFA, SCFA, and MCFA and highest percentage of MUFA, PUFA, and LCFA (Figures 4-7).

Of the saturated fatty acids, lauric (12:0), myristic (14:0), and palmitic (16:0) acids seem to have the most harmful cardiovascular effects (Keys et al., 1965, 1974), with 14:0 thought to be the most harmful; stearic (18:0) seems to be neutral (Bonanome and Grundy, 1988). Thus 14:0 concentration is multiplied by a factor of four in the AI, and 18:0 is left out. Mechanistically speaking, long-chain saturated fatty acids suppress hepatic low-density lipoprotein (LDL) receptor activity (Woollett et al., 1989), thereby decreasing uptake of LDL-cholesterol. LDL-cholesterol can accumulate as atherogenic plaques in arteries. Monounsaturated fatty acids and PUFA increase hepatic LDL receptor activity and thereby decrease circulating concentrations of LDL-cholesterol (Woollett et al., 1992).

The fatty acids that had the most influence on the AI (either an increase or a decrease) are presented in graph form. Myristic acid was present in concentrations ranging from 7 to 11 %, with high AI cows producing milk fat with the highest percentage of 14:0, and low AI cows producing milk fat with the lowest percentage of 14:0 (Figure 8). For all groups, 14:0 percentage was low at the beginning of lactation, peaked by 5 months of lactation, and then steadily declined. Palmitic acid stayed fairly constant for each group throughout lactation. Low AI cows produced milk with a 16:0 content ranging from 25 to 27 %, and high AI cows produced milk with a 16:0 content ranging from 29 to 31 % (Figure 10). Stearic acid content of milk did not differ much between treatments. Stearic acid content of milk fat is high in early lactation, reaches a low at about 6 months, and then increases in late lactation (Figure 12).

Fatty acids of milk are derived from uptake of preformed (diet and tissue storage) fatty acids from circulation and from *de novo* synthesis in mammary epithelial cells. One half of the mass of bovine milk fatty acids are derived from *de novo* fatty acid synthesis in the mammary gland (Bauman and Davis, 1974). Blood acetate

is the principal precursor for *de novo* fatty acid synthesis. ACC and FAS catalyze *de novo* fatty acid synthesis; thus, the activity and concentration of these enzymes regulate the amount of *de novo* synthesized fatty acids, providing a mechanism for genetic regulation of milk fat concentration. The major product of FAS, 16:0, is elongated by fatty acid elongase 6 (Elovl6) to yield 18:0. SCD catalyzes the introduction of *cis* double bonds at carbon number 9 of 16:0 and 18:0 for the synthesis of 16:1 and 18:1, the two major MUFA of bovine lipids.

Concentration of 14:1, 16:1, and 18:1 in milk fat from cows in the present study was highest for low AI cows and lowest for high AI cows, indicating the SCD activity is greater in the low AI cows (Figures 9, 11, 13). However, 18:1 concentration differed the most between treatments (2 – 6 percentage point difference between low, medium, and high AI groups at each individual time point throughout the lactation), indicating that fatty acids are more readily elongated in low AI cows, and that SNPs in Elovl6 may be a primary determinant of AI in milk.

Milk fat consists primarily of triacylglycerols (TAGs), phospholipids (PLs), and cholesterol. The TAGs and PLs of milk fat contain fatty acids that contribute more than 95% of the food calories of milk. The fatty acids of milk fat, like the fatty acids of other foods, differ in their contributions to flavor and textural properties of foods, in their degree of digestibility, and in their physiological effects once absorbed from the intestine. Milk fat has relatively high abundances of 14:0 and especially 16:0 and relatively low concentrations of 18:2 and 18:3. The AI of milk is high (~2.5) when compared with other sources of dietary fat (e.g., beef ~0.8, pork ~0.6, and corn oil ~0.4). Because milk fat contains cholesterol and 16:0, which are hypercholesterolemic when fed together, a shift away from 16:0 and toward a fatty acid such as 18:1 could decrease cholesterol concentrations in consumers of modified milk fat.

Conjugated linoleic acid (CLA), a group of positional isomers resulting from incomplete biohydrogenation of linoleic or linolenic acid to stearic acid in the rumen, has been reported to have a wide range of beneficial effects including anticarcinogenic (Parodi, 1994), antiatherogenic (Lee et al., 1994), and antiobesity activities (Pariza et al., 1996) as well as the ability to stimulate immune function (Miller et al., 1994). Ruminant meat, milk, and dairy products are the predominant sources of CLA in the human diet (Lawson et al., 2001). For these reasons, increasing the CLA content of milk has the potential to raise the nutritive and therapeutic values of dairy products. Endogenously, *cis*-9, *trans*-11 CLA (the primary isomer found in milk) is synthesized from *trans* vaccenic acid, another intermediate of ruminal biohydrogenation, via Δ^9-desaturase in tissues (Corl et al., 2001). In the present study, cis-9, trans-11 was consistently higher in milk from low AI cows, indicating that Δ^9-desaturase activity is higher in low AI cows.

Several reviews (Bauman and Griinari, 2003; Grummer, 1991; Jensen, 2002; Palmquist et al., 1993; Sutton, 1989) document that fat content and composition of milk can be affected markedly by diet. Feeding relatively high ratios of concentrates to roughage and feeding supplemental fish oils and plant oils decrease fat content of milk and increase the proportion of unsaturated fatty acids in milk fat (Bauman and Griinari, 2003). Although the effect is relatively small, milk fat from younger cows and from cows in the first few months of the lactation cycle contains a higher percentage of unsaturated fatty acids (Palmquist et al., 1993; Jensen, 2002). Hence, we collected samples from our experimental cows at all ages and throughout the lactation cycle to obtain data that is representative of the population.

Although the previously mentioned factors influence fatty acid composition, their influence is relative, depending on the genetic potential of the animal. To make consistent progress toward improving the fatty acid composition of milk, one first must identify animals with genes that favor a healthier fatty acid composition and then breed, feed, and manage those cattle to maximize a healthier fatty acid composition. Identifying genetic lines that have the potential to produce milk with a consistently desirable fatty acid composition is needed in the effort to enhance the healthfulness of milk.

Karijord et al. (1982) studied abundances of fatty acids in the milk of Norwegian cattle and found that heritabilities of the individual fatty acids ranged from 0.05 to 0.26. Furthermore, strong positive phenotypic and genetic correlations among short- and medium-chain fatty acids (6:0-14:0) and among 18-carbon, unsaturated fatty acids were observed. Data from our laboratory indicated that the heritability of Elovl6 and SCD is moderate ($h^2 \simeq 0.2$). Genetic selection, therefore, can be used over the long-term to improve composition of milk fat.
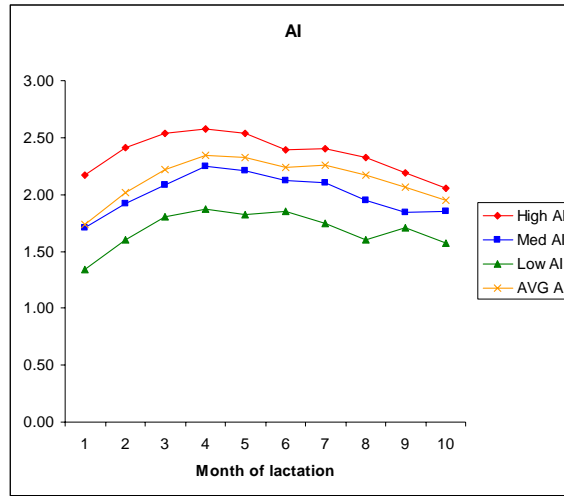
Figure 1. Atherogenic Index (AI) of milk collected from cows at Iowa State and Kansas State Universities.
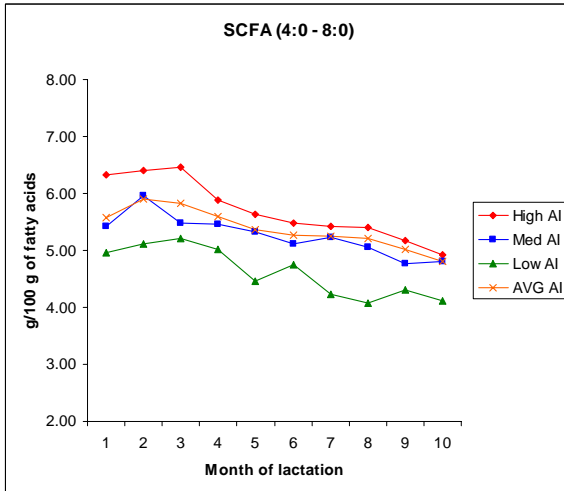

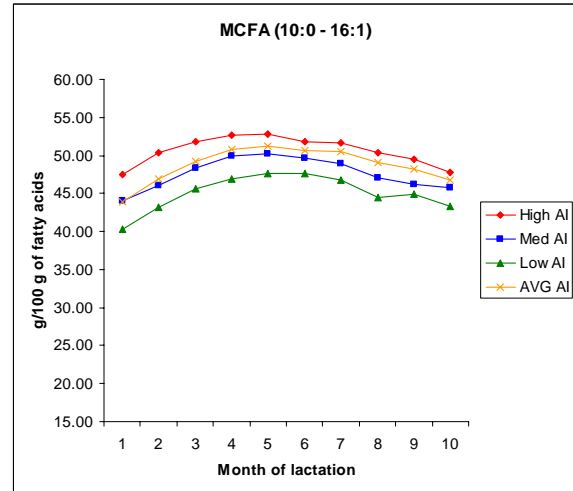**Figure 2. Short-chain fatty acid (SCFA) content of milk.**

Figure 3 . Medium-chain fatty acid (MCFA) content of milk.
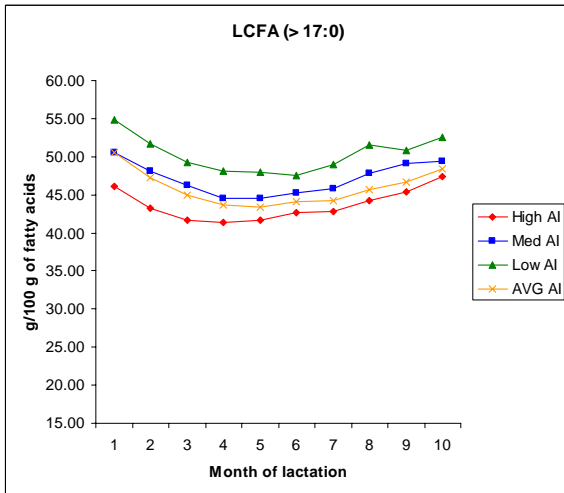

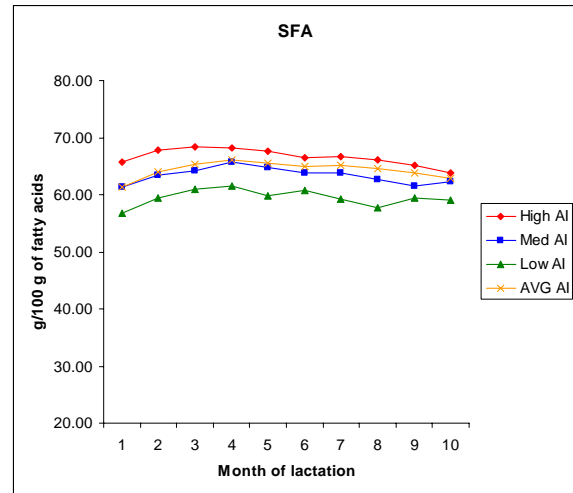**Figure 4. Long-chain fatty acid (LCFA) content of milk.**

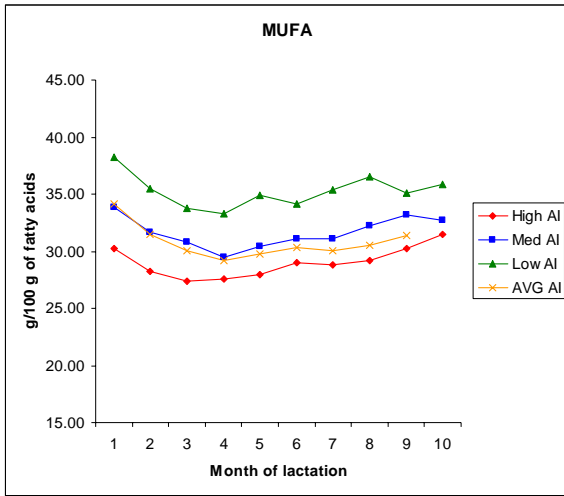**Figure 5. Saturated fatty acid (SFA) content of milk.**

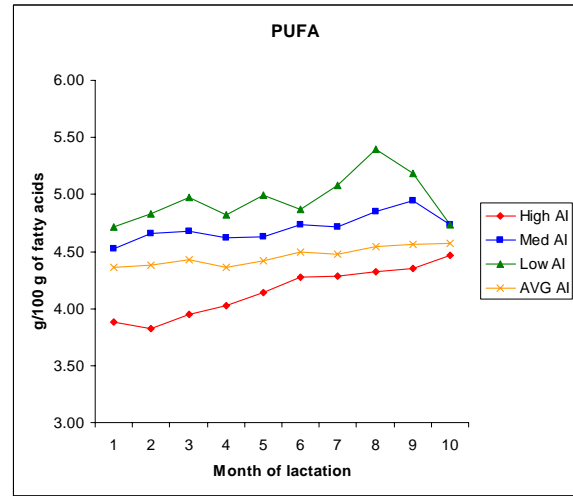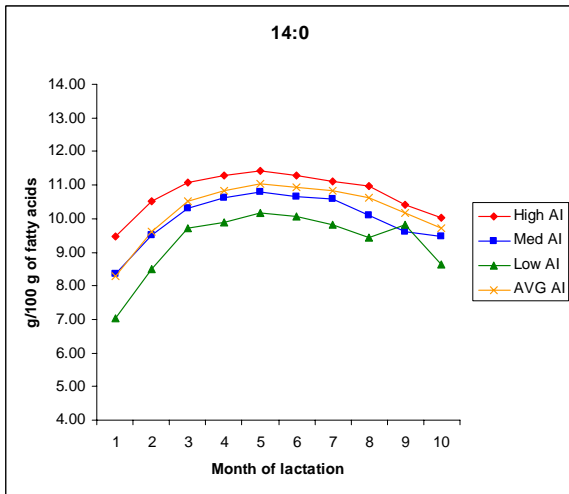**Figure 6. Monounsaturated fatty acid (MUFA) content of milk.**

Figure 7. Polyunsaturated fatty acid (PUFA) content of milk.

Figure 8. Myristic acid (14:0) content of milk.
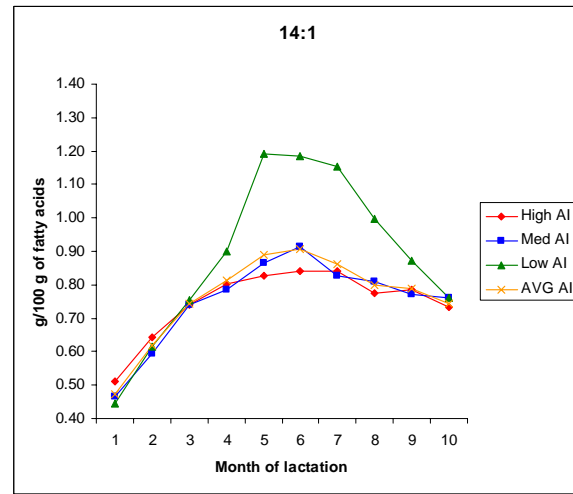
Figure 9. Myristoleic acid (14:1) content of milk.
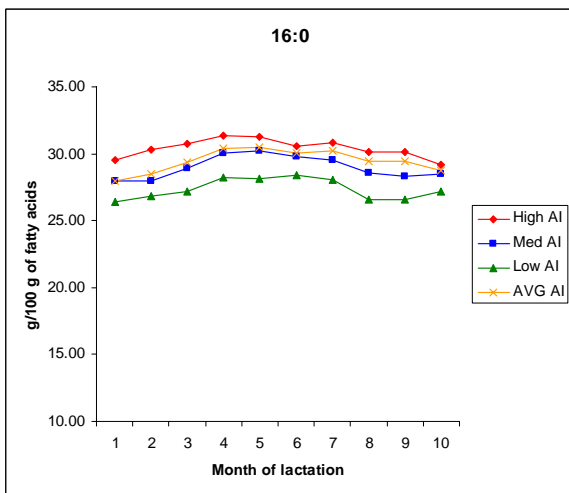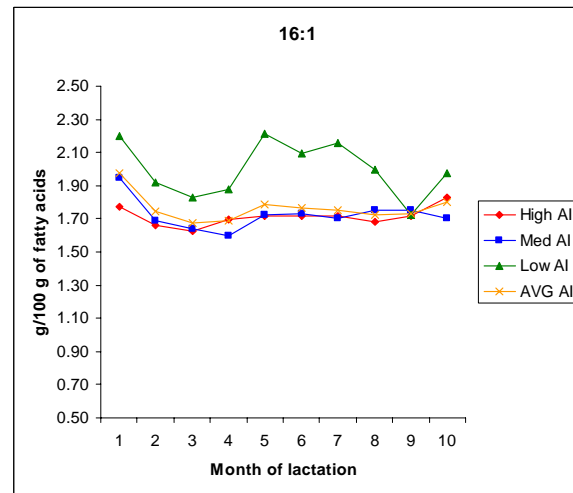

Figure 10. Palmitic acid content of milk.

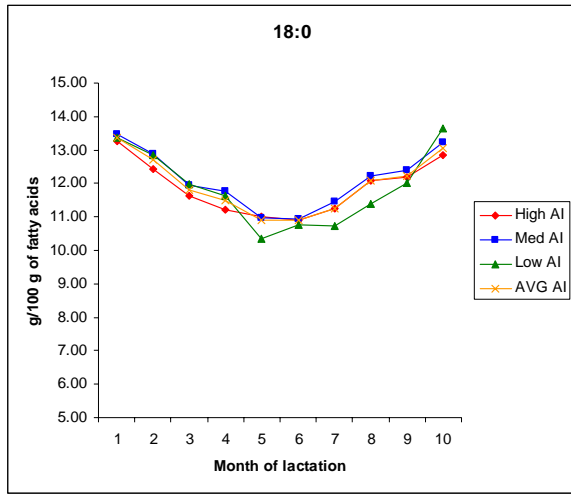**Figure 11. Palmitoleic acid (16:1) content of milk.**
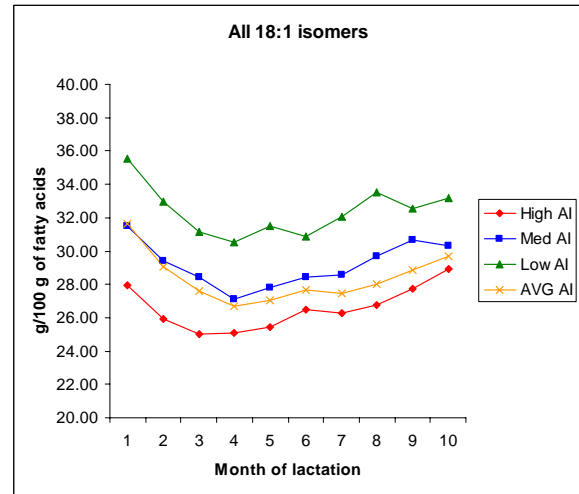
Figure 12. Stearic acid (18:0) content of milk.

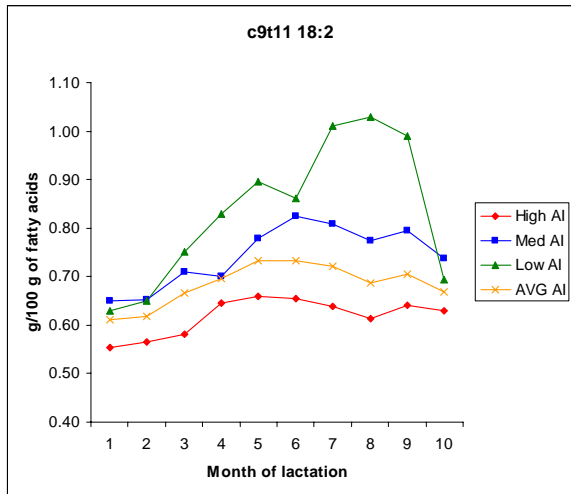**Figure 13. Oleic acid isomer (18:1) content of milk.**

Figure 14. *Cis*-**9,** *trans*-**11 conjugated linoleic acid (c9t11 18:2; CLA) content of milk.**