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Variation in mouse milk fatty acid profile across genetically diverse strains

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ABSTRACT

Fat is the most variable component of milk in both concentration and composition. Differences can be attributed to diet, environmental conditions, and genetics. The study of milk fat has important implications nutritionally and economically. Diets higher in saturated fatty acids increase the risk of cardiovascular disease. Conversely, diets higher in long-chain omega-3 fatty acids decrease the risk of heart disease. In the dairy industry, milk prices are based on the fat and protein yield, which makes milk fat a valuable component for producers. The objective of the present thesis was to determine if significant variation in milk lipid concentration exists between inbred mouse strains. Milk fatty acids (FA) from 325 mice of 31 different strains were extracted and methylated, then quantified via gas chromatography. Statistical analysis software was used to fit a model that included strain effect for each of the 22 fatty acids. Further genetic analysis was conducted for the following groups: FA with less than 16C, FA with 16C, FA with greater than 16C, and C14 and C16 desaturation indexes. Significant variation in fatty acid profile was shown between strains, particularly when comparing de novo synthesis in the mammary gland (FA less than 16C) and preformed fatty acids from the plasma (greater than 16C). Analysis of genetic variation identified SNPs associated with selected milk fatty acids. We can conclude that the variation in milk fatty acid profile between strains is due to specific genes, but further research is required to determine the functional important of the SNPs.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ACC	Acetyl-CoA Carboxylase
ADG	Average Daily Gain
ALA	α-Linolenic Acid
CoA	Coenzyme-A
DHA	Docosahexaenoic Acid
DPA	Docosapentaenoic Acid
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FABP	Fatty Acid Binding Protein
FAS	Fatty Acid Synthase
FATP	Fatty Acid Transport Protein
FID	Flame Ionization Detector
G3P	Glyceraldehyde-3-Phosphate
GC	Gas Chromatography
GLC	Gas-Liquid Chromatography
HDL	High Density Lipoprotein
IS	Internal Standard
LA	Linoleic Acid
LCFA	Long Chain Fatty Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acid
LDL	Low Density Lipoprotein
MCFA	Medium Chain Fatty Acid
MUFA	Monounsaturated Fatty Acid
NEFA	Non-Esterified Fatty Acid
PPAR	Peroxisome Proliferator-Activated Receptor
PUFA	Polyunsaturated Fatty Acid
QTL	Quantitative Trait Loci
SCD1	Stearoyl-CoA Desaturase
SCFA	Short Chain Fatty Acid
SNPs	Single Nucleotide Polymorphisms
SREBP-1	Sterol Response Element Binding Protein
TAG	Triacylglycerol
VLCFA	Very Long Chain Fatty Acid
VLDL	Very Low Density Lipoprotein

Chapter 1

INTRODUCTION

All mammals, by definition, produce milk to provide essential nutrients and energy to their offspring. Milk is comprised of six major components: water, carbohydrates, fat, protein, vitamins, and minerals. Fat is the most variable component of milk in both concentration and composition. Variability occurs between different species and within the same species. Over 95% of the lipids in milk fat are triacylglycerols (TAG). The remaining 5% consists of diacylglycerols, phospholipids, cholesterol, cholesterol esters, and non-esterified fatty acids (Barber et al., 1997).

Milk fat is the most energy dense part of milk and is important to its nutritive value. Milk fat is especially important for breastfeeding infants, as it provides energy for proper growth and development, contains essential fatty acids, and plays a key role in satiety. Milk fat is also economically valuable to dairy producers, as fat and protein content are the basis for milk pricing. Substantial research has been done to determine the optimal fatty acids for human health and to maximize the concentration of fat in milk production. The amount and composition of milk fat is variable depending on a number of factors, including diet, management, and genetics.

The genetic variation of milk fat has not been well studied, but inbred strains selected for divergent phenotypes provide an interesting model. Hadsell et al. (2012) characterized the milk macronutrient phenotype of 31 strains of inbred mice. The objective of the current thesis was to determine the variation in milk fatty acid (FA) profile of these strains. The hypothesis was that the FA profile of the strains differed in key components, including the proportion of de novo and

preformed FA and the concentration of very-long chain omega-3 FA. This data can also be used in future projects to identify genetic markers associated with milk fatty acid profile.

Chapter 2

LITERATURE REVIEW

Importance of Fatty Acids

Fatty acids are a primary component of lipids and essential to the functional characteristics and energy density of lipids. Fatty acids play a role in a number of biological functions, including the formation of cell membranes in the form of phospholipids. Lipids are necessary for the fluidity and functionality of cell membranes. Fatty acids also serve as energy substrates, which make up about 30% of total energy intake for humans. Excess fatty acids are stored as adipose tissue and provide an important long-term energy store. Chemically, the majority of lipids in the body are composed of fatty acids bound to organic alcohols and circulate the blood stream in combination with lipoproteins. Non-esterified fatty acids (NEFA), or free fatty acids, bind to plasma albumin to circulate (Tvrzicka et al., 2011).

Fatty acids differ in their chain length and number and orientation of double bonds. Saturated fatty acids contain only single bonds. Short chain fatty acids (SCFA; four or less C) can form as a result of fiber fermentation in the colon or in pregastric fermentation compartments in some species. Short chain fatty acids are rapidly absorbed and can be utilized in other areas of the body. Propionic acid (C3:0) is transported to the liver and used as a substrate for gluconeogenesis. Acetic acid (C2:0) is transported to all tissues of the body where it is used as an energy source and as a precursor for longer chain fatty acid synthesis. Dietary medium chain fatty acids (MCFA) can be absorbed in the small intestine and transported by the portal vein. Examples of MCFA include caproic acid (C6:0), caprylic acid (C8:0), and capric acid (C10:0)

and are not found in common plant foods. Medium and long chain fatty acids (LCFA), such as lauric acid (C12:0), myrisitic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0), are mostly consumed from the diet. Numerous plant-based oils and butters provide 80 to 90% of the total SFA from food intake (Tvrzicka et al., 2011). Animal sources, such as butter, lard, and beef tallow, also contain LCFA. Saturated LCFA can be detrimental to animal health by increasing cholesterol levels, specifically low density lipoprotein (LDL), resulting in coronary heart disease that can lead to death (Astrup et al., 2011). Some studies have shown that stearic acid actually decreases LDL-cholesterol and increases high density lipoprotein (HDL)-cholesterol, which lowers the risk of cardiovascular disease (Tholstrup et al., 1994). Very long chain fatty acids (VLCFA), containing between 20 and 30 carbon length chains, have been linked to heritable metabolic diseases.

Unsaturated fatty acids contain one or more double bond. Monounsaturated fatty acids (MUFA) in the *cis* formation have a double bond with two hydrogens on the same side of the molecule. Examples of such molecules include oleic acid (C18:1 cis-9) and palmitoleic acid (C16:1 cis-7). Similar to stearic acid, oleic acid has also been shown to increase the HDL-/LDL-cholesterol ratio and decrease grouping of clotting factors known as thrombocytes (Tvrzicka et al., 2011). Plant oils, such as olive oil, canola oil, and peanut oil are dietary sources of oleic acid. Monounsaturated fatty acids can be synthesized in the body from saturated fatty acids by the stearoyl-CoA desaturase (SCD1) enzyme. This enzyme is especially efficient at synthesis of oleic acid from stearic acid. MUFA can also be in the *trans* configuration with hydrogens on opposite sides of the double bond. *Trans* fats are consumed in the diet and their properties are similar to those of SFA, since the formation remains relatively linear. However, *trans* fats are

twice as active in raising LDL-cholesterol and decreasing HDL-cholesterol as SFA. Thus *trans* fats are assumed to have a greater atherogenic effect than SFA (Tvrzicka et al., 2011).

Polyunsaturated fatty acids (PUFA) contain two or more double bonds. Endogenous PUFA are synthesized more frequently in the absence of essential fatty acids, specifically linoleic acid (LA) and α-linolenic acid (ALA). LA is the parent fatty acid of the n-6 PUFA family. Metabolic products of LA include the following: γ-linolenic acid (C18:3n-6), dihomo-γ-linolenic acid (C20:3n-6), arachidonic acid (C20:4n-6), adrenic acid (C22:4n-6), and docosapentaenoic acid (C22:5n-6). High concentrations of n-6 PUFA can also be found in plant oils such as soybean oil, sunflower seed oil, and safflower oil, to name a few. The family of n-6 PUFA is activators of peroxisome proliferator-activated receptor (PPAR) transcription factors that play numerous roles in metabolism, including increased synthesis of cholesterol and up-regulation of LDL-receptors. ALA is the parent fatty acid of the n-3 PUFA family. Its metabolic products include: stearidonic acid (C18:4n-3), eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3), and docosapentaenoic acid (DPA, C22:5n-3). ALA can be consumed via seeds and leaves of plants such as soybeans, linseed, flaxseed, and blackcurrant seeds. EPA and DHA can also be consumed directly through oily fish or algal extracts. PUFA in the n-3 family are thought to decrease lipogenesis and very low density lipoprotein (VLDL) secretion by suppression of sterol response element binding protein (SREBP-1; Tvrzicka et al., 2011).

Milk Fat Synthesis

The mammary gland can utilize fatty acids from several sources, including preformed fatty acids taken up from the plasma that originate from dietary fat, liver, and adipose tissue, and

fatty acids synthesized de novo in the gland itself. The relative use of each source depends on the physiological and nutritional state of an individual animal. Determining the fatty acid composition of milk fat can provide insight as to the source of the fatty acids. De novo synthesis in the mammary gland, for example, can only produce fatty acids up to 16 carbons in length. Thus, an increased proportion of such fatty acids indicates a relative increase in the activity of the de novo synthesis pathways (Barber et al., 1997).

The major substrates for milk fat synthesis are glucose, glycerol, and free fatty acids, with the addition of acetate and β-hydroxybutyrate in ruminants. As shown in **Error! Not a valid bookmark self-reference.**, glucose has three potential fates once it enters the mammary gland. First, it can be converted to ribulose-5-phosphate via the pentose phosphate shunt, which also produces NADPH to be used for reduction to synthesize fatty acids. Second, glucose can be converted into pyruvates, which then enter the mitochondria and are converted to acetyl-coenzyme A (CoA). Via citrate synthesis, the glucose can ultimately become malonyl-CoA that provides carbon chains for fatty acid synthesis. Finally, glyceraldehyde-3-phosphate (G3P) can be produced from glucose via the glycolysis pathway. Glyceraldehyde-3-phosphate can be converted to glycerol-3-phosphate and used for TAG synthesis (Neville and Picciano, 1997).

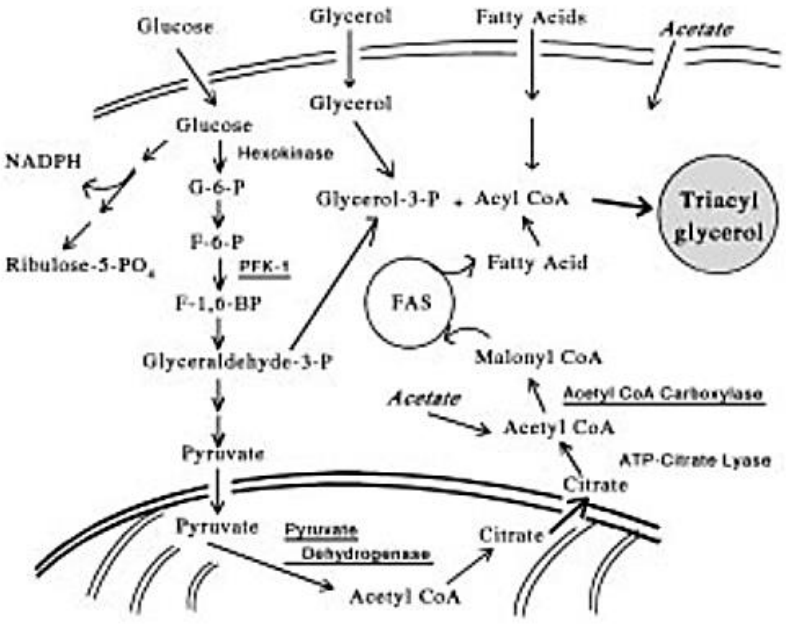

Figure 2.1: Key steps in glucose utilization and fatty acid synthesis in the mammary alveolar cell.

G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; PFK-1, phosphofructokinase-1; F-1,6-BP, fructose 1,6-biphosphate; FAS, fatty acid synthase; CoA, coenzyme A
(With permission from Neville and Picciano, 1997).

De novo synthesis of fatty acids in the mammary gland begins when acetyl-CoA is converted to malonyl-CoA. This rate-limiting reaction is catalyzed by the enzyme acetyl-CoA carboxylase, which is highly regulated at the levels of transcription, translation, and phosphorylation (Neville and Picciano, 1997). Next, the enzyme fatty acid synthase (FAS) catalyzes the condensation reaction of malonyl-CoA to eliminate a CO_2 molecule and produce an intermediate ketoacyl moiety. From here, the molecule undergoes a 3-step β-carbon reduction cycle that results in the addition of two carbons to the chain. The reduction cycle can be repeated an additional six times to generate fatty acids up to 16 carbons in length (Barber et al., 1997). Each cycle requires 2 NADPH molecules (Neville and Picciano, 1997); thus, 14 molecules of NADPH are required to produce one 16-carbon fatty acid.

Palmitic acid (C16:0) is the primary product of the FAS reaction in most eukaryotic cells for a number of reasons. First, an acyl-chain that has extended beyond four carbons cannot be readily released from FAS as a CoA ester due to the specificity of the 4'-phosphopantetheine prosthetic group of FAS for acetyl and butyryl moieties. Second, as the length of the carbon chain increases, the rate of condensation of the acyl-CoA increases as well. Consequently, once the condensation reaction is initiated, it will continue until the maximum length is reached. Finally, fatty acids with 16 or more carbons cannot be readily elongated in the mammary gland (Barber et al., 1997). Thioesterase I is a diacyclase enzyme that removes the completed fatty acid molecule from FAS and terminates further synthesis (Neville and Picciano, 1997). Thioesterase I has a very high specificity for C16:0 compared to C14:0 (Barber et al., 1997). Mammary epithelial cells in nonruminants contain the enzyme thiosterase II, which terminates fatty acid synthesis after 8 to 14 carbons have been added to produce medium-chain fatty acids (Neville and Picciano, 1997).

Long-chain fatty acids cannot be synthesized in the mammary gland; rather, they are extracted from the plasma. Very low density lipoprotein carries endogenous fatty acids from liver or adipose tissue. Lipoprotein lipase can release long-chain fatty acids from VLDL to use for milk production. Non-esterified fatty acids that are attached to albumin and circulate in the blood can also be imported. Long-chain fatty acids consumed in the diet are transported on chylomicrons in the plasma and can be released for utilization in the mammary gland (Neville and Picciano, 1997).

The exact mechanism by which fatty acids enter the alveolar cell is not fully understood, but is expected to involve cell surface proteins including CD36 and fatty acid transport proteins (FATP). Once imported into the cell, they may be bound to fatty acid-binding protein (FABP), and maintained in a readily available fatty acid pool. To be activated, fatty acids are joined with CoA and G3P by transacylase enzymes in the endoplasmic reticulum to form TAG. The TAG molecules are fused into microlipid droplets that merge into larger droplets. Ultimately, the droplets are secreted from the cell covered by a specialized plasma membrane called the milk fat globular membrane (Neville and Picciano, 1997). The milk fat globular membrane is essential to the secretion process and numerous proteins and phospholipids remain associated with the lipid droplet in milk.

Approximately half of the fatty acids in animals are unsaturated and contain between one and six double bonds. Figure 2.2 illustrates the numerous elongation and desaturation pathways stemming from palmitic acid (C16:0). Monounsaturated fatty acids are typically produced from the reaction catalyzed by the enzyme Δ9-desaturase at the Δ9 position. Desaturation of stearic acid (C18:0) results in oleic acid (C18:1), and desaturation of palmitic acid results in palmitoleic acid (C16:1). Elongation and desaturation of oleic acid produces MUFA of n-9 with 20 to 24

carbon atoms, as diagrammed in Figure 2.2. MUFA of n-11 are typically the result of elongation and desaturation of arachidic acid (C20:0). Endogenous desaturation in humans is only possible up to the Δ9 position due to the lack of necessary enzymes for further desaturation. Thus, long-chain unsaturated fatty acids, such as linoleic acid (C18:2(n-6)) and α- linolenic acid (C18:3(n-3)), must be consumed in the diet. Production of long chain polyunsaturated fatty acids (LC-PUFA) from these is possible in humans but not very efficient; therefore, dietary consumption is conditionally essential (Tvrzicka et al., 2011).

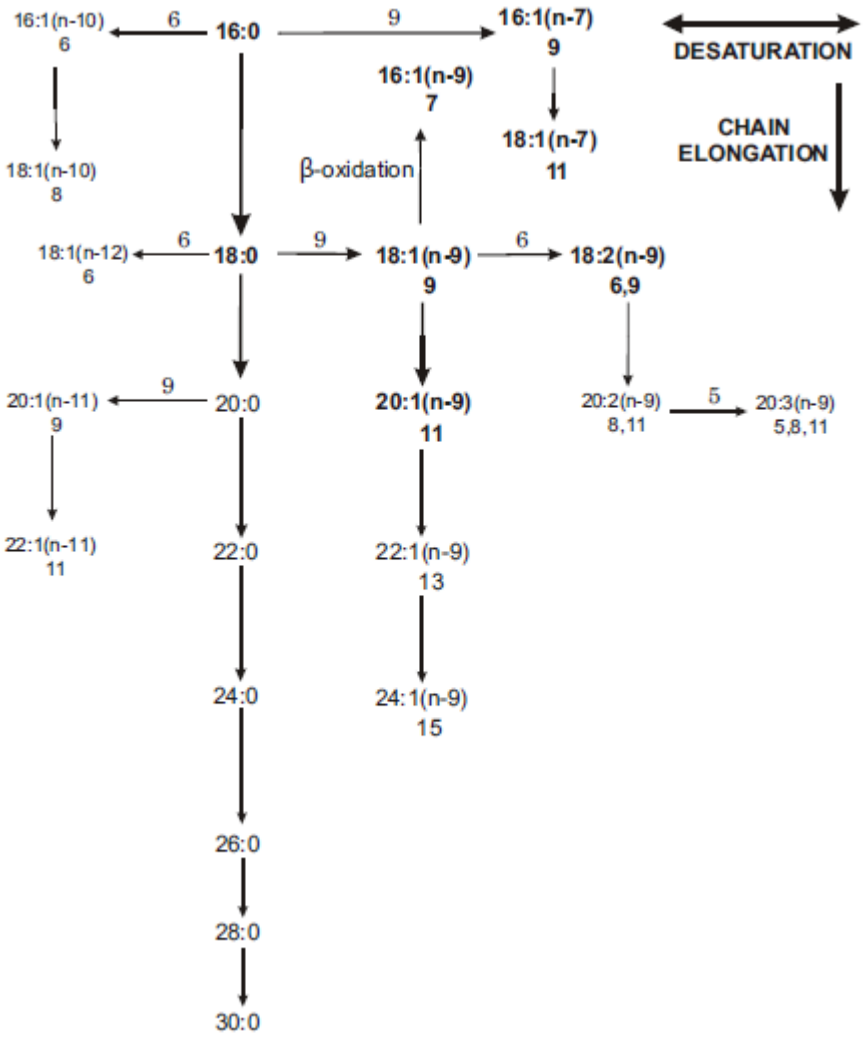

Figure 2.2: Elongation and desaturation of endogenous fatty acids (With permission from Tvrzicka et al., 2011).

Factors Affecting Concentration and Composition

The concentration and chemical composition of milk fat is dependent on a number of factors, many of which can be manipulated. Milk fat concentration and profile has been extensively studied in dairy cattle (See review by Heinrichs et al. 2005). Briefly, genetics account for more than half of the differences in milk fat content in dairy cows. Heritability estimates for the percent of fat in milk have been reported as 58% in Holstein cows and 55% in Jersey cows. Genetic approaches have also been used to determine quantitative trait loci (QTL) and single nucleotide polymorphisms (SNPs) associated with milk fat concentration and profile in dairy cows (e.g. Bouwman et al. 2011). These studies have primarily utilized a single breed of dairy cows and have identified variants of SCD1, ACC, DGAT1, and FASN associated with milk fat profile and yield.

The remainder of variation in milk fat not due to genetics is assumed to be due to environmental factors, which are also reviewed by Heinrichs et al. (2005). In short, milk fat varies depending on the stage of lactation. The highest amount is produced just after parturition in colostrum. Levels drop to their lowest point between 25 and 50 days in milk, and then increase to their highest point at 250 days in milk. Milk yield is inversely related to the percent of fat in milk. Greater milk production dilutes the amount of fat, thus producing a lower percentage. The age of an animal also affects milk fat. With each additional lactation cycle, the milk fat falls about 0.2% per year. The seasons are ever-changing and uncontrollable, and the climate changes affect milk fat production. Hotter and more humid summer months tend to reduce milk fat content, and there is a gradual increase through fall that peaks in the colder winter months. The milk fat content drops again in the spring as warmer weather arrives. The changes are likely

caused by differences in feed intake, which increases in colder weather and decreases in warmer weather.

The most variable environmental factor that has the greatest effect on milk fat production in dairy cows is nutrition. Changes in diet can alter the milk fat content considerably within 7 to 21 days in milk. Optimizing milk production requires a balanced diet. High energy and low fiber diets increase milk proteins but decrease milk fat levels. Consuming too many non-fiber carbohydrates can depress fiber digestibility, which leads to lower milk fat. Forage intake is important as well, since low forage intake also reduces milk fat content due to low fiber levels. Management situations that limit a cow's feed intake, such as overcrowding, age differences, and the physical forms of feed, will decrease milk production. Furthermore, the body condition of the animal affects milk components. Extremes on either end of the scale, whether very low body condition or very high body condition, can lead to low milk fat content. Minimal body stores deplete fat precursors, and excessive weight increases metabolic problems.

Importance in Humans and Industry

Milk fat content has a strong economic influence in the dairy industry. The majority of milk marketing orders in the United States are based on a multiple components pricing system (See review by Heinrichs et al. 2005). To summarize, the system pays producers based on pounds of milk fat and protein yield rather than pounds of milk. Therefore, milk component levels, particularly milk fat, directly affect the producer's income. Consequently, herd management is important for both the cows' health and the economic success of the farm. Milk fat production can greatly vary from herd to herd. A summary of the milk shipped in the Mideast federal order from 2000 to 2002, as described in the Heinrichs et al. (2005) review, showed that

the average milk fat of a herd ranged from 1.77% to 5.98% with an average of 3.76%. Differences in breeds account for much of the variation, but even when extremes are eliminated, a range of herd production differences remains.

Humans produce milk for the sole purpose of breastfeeding infants. Breast milk supplies LC-PUFA, such as DHA and arachidonic acid (AA, C20:4n-6), that are not traditionally found in infant formulas. A study by Agostoni et al. (2001) demonstrated that breastfeeding for at least 6 months significantly improves a child's mental development and psychomotor development. Additionally, the milk fat content was shown to be the only milk component at 6 months associated with mental development at 12 months. The absolute amount of milk fat was shown to have the greatest influence on development, as opposed to specific composition. However, the effect of specific fatty acids on development has not yet been studied. Mothers were shown to maintain milk fat levels between time points (1 day, 1 month, 3 months, 6 months, 9 months, and 12 months). While there was variation with the amount of milk fat produced between individual mothers, infants that were breastfed for at least 6 months and whose mothers produced high-fat milk benefitted in the short-term. An overview of the literature on long-term benefits of breast milk was included in the study. It has not been determined whether positive effects are based entirely on nutrition or other confounding variables, such as the maternal attitude toward breastfeeding and bonding with the breastfeeding mother.

Chapter 3

MATERIALS AND METHODS

Milk Samples

Mouse milk samples were collected in a previously published experiment and were obtained from Dr. Darryl Hadsell (Baylor College of Medicine, Houston, TX). A complete description of animals, sample collection, and milk macronutrient composition can be found in Hadsell et al. (2012) titled "In silica QTL mapping of maternal nurturing ability with the mouse diversity panel." Briefly, 31 inbred mouse strains were purchased from Jackson Laboratories (Bar Harbor, ME) as 6 to 10 week old breeding pairs. The panel of strains included 129SvIm/J, A/J, AKR/J, BALB/cByJ, BTBR T+ tf/J, BUB/BnJ, C3H/HeJ, C57BL/6J, C57L/J, C58/J, CBA/J, CE/J, CZECHII/EiJ, DBA/1J, DBA/2J, FVB/NJ, I/LnJ, KK/HlJ, LG/J, LP/J, MA/MyJ, NOD/LtJ, NZW/LacJ, PL/J, QSi5, RIIIS/J, SEA/GnJ, SJL/J, SM/J, and SWR/J. The QSi5 strain was not used in Hadsell et al. (2012) due to high fecundity and larger litters, but it was maintained as an inbred line. All animals were housed at an ambient temperature of 21°C and a light-dark cycle of 14h:10h and were maintained on a 2020X pelleted diet (Harlan Teklad, Indianapolis, IN) through day 15 of pregnancy. Individual matings were set up between males and females within each strain to allow all females to complete one lactation cycle. Females were mated a second time with CD-1 males (Charles River Laboratories, Wilmington, MA). During the second lactation, dams were switched to a powdered semi-purified diet AIN-93G (Harlan Teklad), which was dispensed in funnel feeders. Milk samples were collected on day 10

of the second lactation. Pup growth and milk fat, protein, and lactose concentration was reported by Hadsell et al. (2012). A 20 uL aliquot of milk was used for fatty acid analysis as described below.

Fatty Acid Extraction, Methylation, and Quantification

For each of the 347 milk samples, the milk was first transferred from a plastic microcentrifuge tube to a glass extraction tube, and the tube washed with saline. Lipids were then extracted with hexane: isopropanol according to Hara and Radin (1978), base transmethylated as described by Christie (1982), and modified by Chouinard et al. (1999) using sodium methoxide in the methanol. Fatty acid methyl esters were quantified by gas chromatography (GC; Agilent 6890A, Agilent Technologies, Palo Alto, CA) equipped with a fused-silica capillary column [SP-2560; 100 m x 0.25 mm (i.d.) with 0.2-μm film thickness; Supelco, Bellefonte, PA], and a flame ionization detector (FID). The temperature program was 70°C for 4 min, 8°C/min to 110°C, 5°C/min to 170°C and held 10 min, and 4°C/min to 215°C and held for 23 min. Gas constant flows held hydrogen carrier at 1 ml/min and detector hydrogen at 25 ml/min, airflow at 400 ml/min, and nitrogen plus carrier at 40 ml/min. Peaks were identified using pure methyl ester standards (GLC 780; NuChek Prep Inc., Elysian, MN) and recoveries of individual FA determined using an equal weight reference standard (GLC 461; NuChek Prep Inc.). Total FA were estimated using C19:0 as internal standard (IS; NuChek Prep Inc.). Peak areas were corrected for deletion of the methyl ester and efficiency of recovery of individual fatty acids based on the equal weight standard.

Statistical Analysis

JMP 9.0.2 (SAS Institute Inc., 2010) software was used to analyze milk fatty acid data. First, a model that included the effect of strain was fit for each of the 22 fatty acids. Data points with Studentized Residuals outside of ± 3.5 were considered outliers and excluded. Secondly, the distribution of each fatty acid for each of the 31 strains was determined. The mean, standard deviation, and upper and lower 95% confidence intervals for each fatty acid were determined for each strain. The sum of all fatty acids less than C16:0 (predominantly *de novo* synthesized), the sum of C16:0 and C16:1, and the sum of all fatty acids greater than C16:0 (predominantly preformed from plasma) were also analyzed as described above. The desaturation indexes for C14 and C16 were calculated as the substrate to precursor ratio of the delta-9 desaturase enzyme as shown below and analyzed as described above.

C14 index = C14:1 / (C14:0 + C14:1)

C16 index = C16:1 / (C16:0 + C16:1)

Individual SNP Association

To determine specific genetic variations in individual SNPs, we used the UCLA EMMA webserver (http://mouse.cs.ecla.edu/emmaserver/). The genome-wide significance threshold for this analysis was $P <= 10^{-5}$. The analysis was used for the following data sets: FA with less than 16C, FA with 16C, FA with greater than 16C, and C14 and C16 desaturation indexes.

Chapter 4

RESULTS

Milk fat extraction, methylation, and quantification were successful in determining milk fat variation by strain in 325 of 347 samples. Of the 22 samples eliminated prior to analysis, some samples failed to result in a quantifiable concentration of methylated fatty acids, as shown by a total peak area below 500 units (1000 to 2000 units is normal range). Also, unknown peak area in some samples was very high, presumably due to a failed methylation. Lastly, a high amount of isopropanol residue was found in some samples, which inhibited efficient methylation. The concentration of C18:3(n-3) and C20:1 was not consistently separated with the analysis conditions used and are reported together.

Individual fatty acids were eliminated from the remaining 325 samples during statistical analysis if they were outside of ± 3.5 standard deviations from the mean. No more than 8 samples for a fatty acid were removed, with the average removed per fatty acid being 3.45. All data points for the 325 samples were included when calculating mean values for less than C16:0, sum of C16:0 + C16:1, greater than C16:0, and desaturation indexes of C14:0 and C16:0, but outliers were excluded for this variable in a similar way during analysis. For all parameters, strains are plotted from the lowest to highest based on the mean. Additionally, the upper and lower 95% confidence interval is shown in bars from the mean.

Concentration of milk fatty acids less than 16 carbons for all 31 mouse strains is shown in Figure 4.1 and represents the proportion of fatty acids synthesized by the mammary gland. The

means ranged from 18.8 to 36.6% of FA. Looking across strains, the first four strains (SJL/J, CZECHII/EiJ, MA/MyJ, and 129SvIm/J) were significantly lower than the highest 21 strains, which showed a gradual linear increase in FA less than 16 carbons across strains. Specifically, SJL/J was significantly lower than all but two strains (CZECHII/EiJ and MA/MyJ). In addition, SJL/J was one of the lowest strains for C8:0 (Figure 4.6) and was the lowest for C10:0, C12:0, and C14:0 (Figure 4.7, 4.8, and 4.9, respectively). On the other hand, SJL/J was the highest for C14:1 (Figure 4.10). On the opposite end of milk fatty acids less than 16 carbons, SEA/GnJ was the highest strain and was significantly higher than 20 other strains (Figure 4.1). Specifically, SEA/GnJ had the highest milk fat concentration of C8:0, C10:0, and C12:0 (Figure 4.6, 4.7, and 4.8, respectively). In addition, SEA/GnJ was nearly the highest for C14:0 (Figure 4.9), but was one of the lowest for C14:1 concentration (Figure 4.10).

Milk fat concentrations of 16 carbon fatty acids (C16:0 and C16:1) for all 31 mouse strains is shown in Figure 4.2, and the means ranged from 22.8 to 37.3% of fatty acids. These fatty acids arise from both de novo synthesis and preformed fatty acid uptake by the mammary gland. The strain means rise slightly in the first few points then linearly until the last two points, which are significantly higher than 26 of the other strains. The strain SEA/GnJ had the lowest 16 C fatty acid concentration, and the strain BUB/BnJ had the second lowest concentration. SEA/GnJ and BUB/BnJ were also the two lowest for C16:0 and C16:1 concentration (Figure 4.11 and 4.12). On the opposite end of C16 FA concentration, KK/HIJ had the highest mean and was significantly greater than all 30 other strains. KK/HIJ was also the highest for C16:0 and was significantly higher than all 30 other strains (Figure 4.11). However, KK/HIJ C16:1 concentration ranked near the middle of all the strains (Figure 4.12).

Milk fat concentrations of fatty acids greater than 16 carbons is shown in Figure 4.3, and represents the proportion of fatty acids taken up from the plasma by the mammary gland. The means range from 34.0 to 49.6% of FA. The trend across strains was rather linear with one low strain and a few higher strains. KK/HIJ had the lowest mean and was significantly lower than 25 other strains. Specifically, KK/HIJ was also the lowest for C18:1c9, C20:3, and C22:6 concentrations (Figure 4.14, 4.20, and 4.27, respectively). KK/HIJ was also one of the lowest strains for the concentration of C18:2, C18:3(n-6), C20:4, C20:5, and C22:5 (Figure 4.16, 4.17, 4.21, 4.22, and 4.26, respectively). On the opposite end of fatty acids greater than 16 carbons, SJL/J had the highest mean and was significantly greater than all other strains with the exception of CZECHII/EiJ, which has the second highest. SJL/J had the highest concentration of C18:1c9, C18:1t13, C18:2, and 18:3(n-3)+C20:1, and was one of the highest for C20:2 and C22:6. Lastly, CZECHII/EiJ had the highest mean for C18:0 and the second highest mean for C18:1c9, C18:2, and 18:3(n-3)+20:1.

Milk fat C14 and C16 desaturation index for all 31 strains of mice is shown in Figure 4.4 and 4.5, and represents the activity of the stearoyl-CoA desaturase enzyme. The means ranged from 0.012 to 0.030 for the C14 index and 0.068 and 0.151 for the C16 index. The trend for C14 desaturation indexes (Figure 4.4) is mostly linear with a few strains significantly lower or higher than the majority of the other strains. SEA/GnJ has the lowest C14 index but is only significantly lower than 16 other strains. Specifically, SEA/GnJ was the third highest for C14:0 and the third lowest for C14:1 (Figure 4.9 and 4.10). The trend for C16 desaturation indexes (Figure 4.5) had more of a gradual incline with a few higher points. KK/HIJ was the lowest for C16 desaturation index, but was only lower than 13 other strains. Specifically, KK/HIJ was the highest for C16:0 and was significantly higher than every other strain, but its C16:1 concentration ranked towards

the middle of the strains (Figure 4.11 and 4.12). SJL/J had the highest C14 and C16 desaturation indexes, which were significantly greater than 27 and 30 other strains, respectively. SJL/J had the lowest mean for C14:0 and the highest for C14:1 (Figure 4.9 and 4.10). SJL/J ranked near the middle of the strains for C16:0 concentration, but SJL/J had the highest C16:1 concentration.

Milk fat concentrations of C20:4, an important long chain n-6 fatty acid, are shown in Figure 4.21 and range from 0.309 to 1.15% of FA. SM/J had the lowest concentration and was significantly lower than 20 other strains. The strain means gradually increase and remain relatively linear until the last two points, which are significantly higher. CE/J had the highest mean and was significantly higher than all other strains. BALB/cByJ had the second highest and was significantly higher than 23 other strains.

Milk fat concentrations for C20:5, an important n-3 fatty acid, are shown in Figure 4.22 and range from 0.074 to 0.334% of FA. SJL/J had the lowest mean but was only significantly lower than 19 other strains. The means gradually increase until the last four strains, which are much higher. CE/J had the highest mean and was significantly higher than all other strains. CBA/J has the second highest mean and was significantly higher than 27 lower strains. BALB/cByJ and C58/J had the third and fourth highest strains, respectively, and were significantly higher than 24 other strains.

Milk fat concentrations for C22:6, another important n-3 fatty acid, are shown in Figure 4.27. The means range from 0.100 to 0.255% of FA. KK/HIJ had the lowest mean and was significantly lower than 26 other strains. DBA/2J and SM/J had the second and third lowest mean, respectively, and were significantly lower than 23 other strains. The means are relatively linear until the last few higher strains. CE/J had the highest mean and was significantly higher than 23 other strains.

Manhattan plots (Figure 4.28, 4.29, 4.30, 4.31, and 4.32) show the significant SNPs associated with variation between strains. The peaks represent the negative logarithm of the association *P*-value for each SNP displayed on the x-axis, and the chromosomal location is indicated on the y-axis. Consequently, taller peaks illustrate a lower *P*-value and indicate a stronger association. The SNPs associated with variation in fatty acids with less than 16 carbons are shown in Figure 4.28. Most of the significant SNPs were clustered on the same chromosome. There were thirty significant SNPs on chromosome 2 and only two significant SNPs on chromosome 18. Figure 4.29 shows the associations for the sum of 16 carbon fatty acids. The significant SNPs were distributed more across different chromosomes. There were ten significant SNPs on chromosome 14 and three significant SNPs on chromosome 2. Chromosomes 4, 5, 9, and 18 each had one significant SNP. The concentration of fatty acids greater than 16C had the greatest number of significant SNPs and is shown in Figure 4.30. Twenty-seven of the significant SNPs are clustered on chromosome 11 and eleven are on chromosome 2. The rest of the significant SNPs are distributed across the genome.

The desaturation indexes did not have as many significant SNPs as determined by the genome analysis. Figure 4.31 shows associations for the C14 desaturation indexes. Chromosome 10 had the most significant SNPs with six. Chromosome 9 had three significant SNPs, and chromosome 2 had two significant SNPs. Chromosomes 3, 6, 7, and 16 each had one significant SNPs. Figure 4.32 shows associations for the C16 desaturation indexes and has the fewest number of significant SNPs. There were six significant SNPs on chromosome 17, two significant SNPs on chromosome 2, and one significant SNP on chromosome 11.

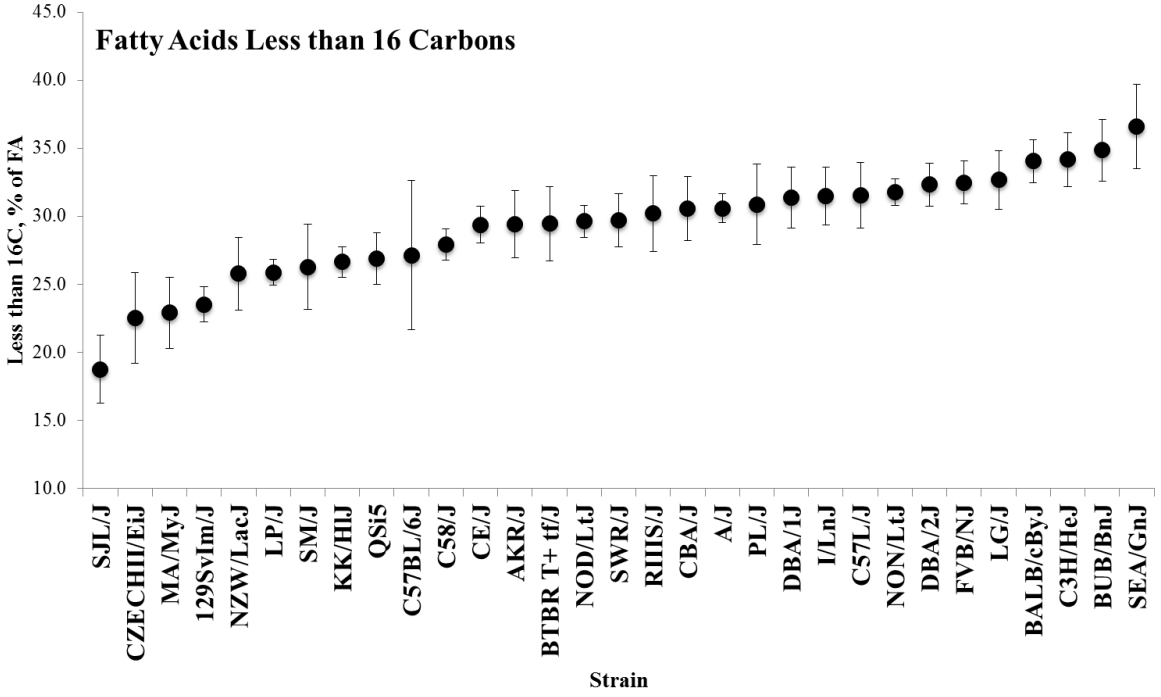

Figure 4.1: Concentration of milk fatty acids with less than 16 carbons in 31 mouse strains.

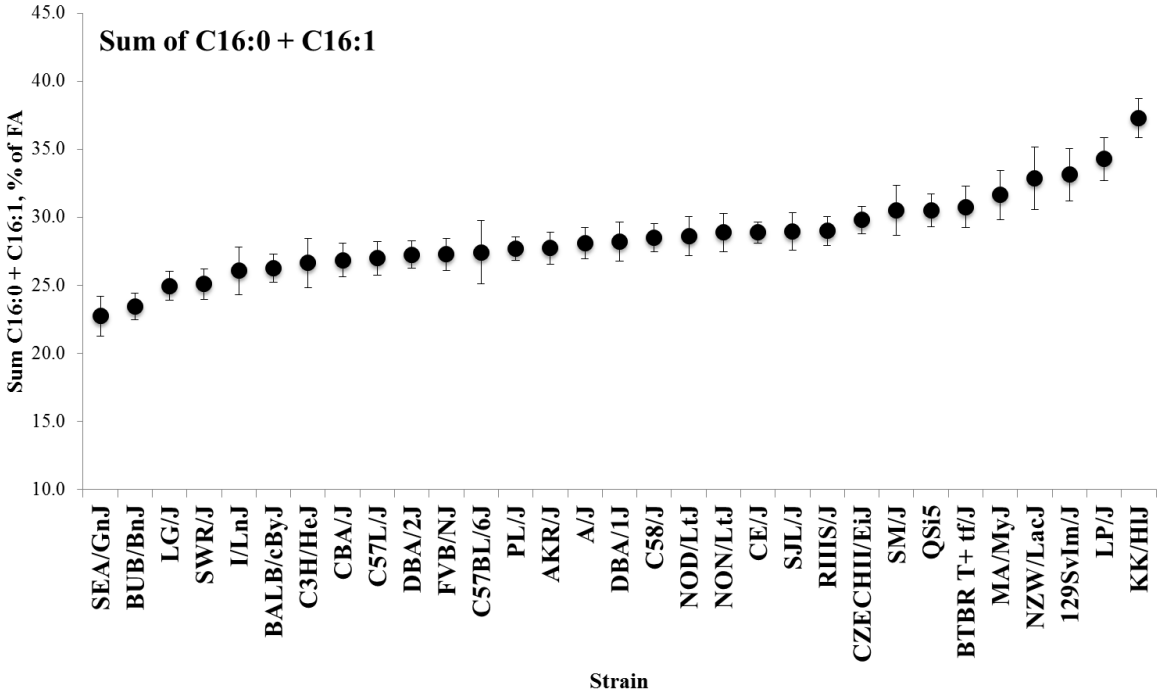

Figure 4.2: Concentration of C16:0 and C16:1 milk fatty acids in 31 mouse strains.

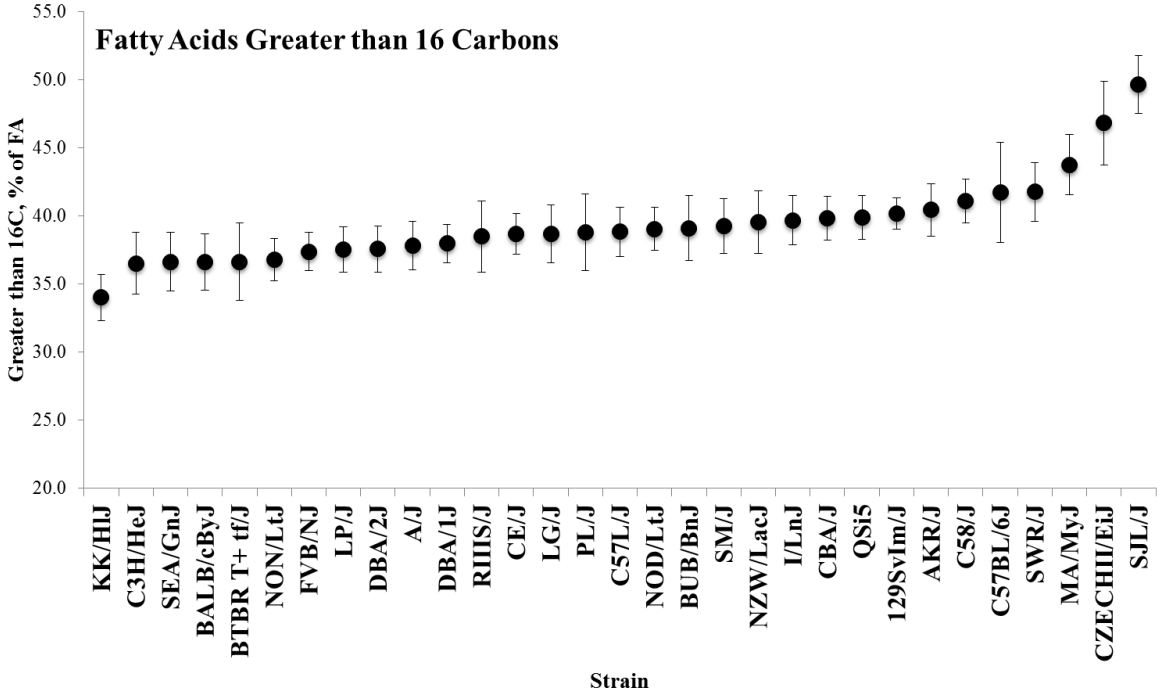

Figure 4.3: Concentration of milk fatty acids with greater than 16 carbons in 31 mouse strains.

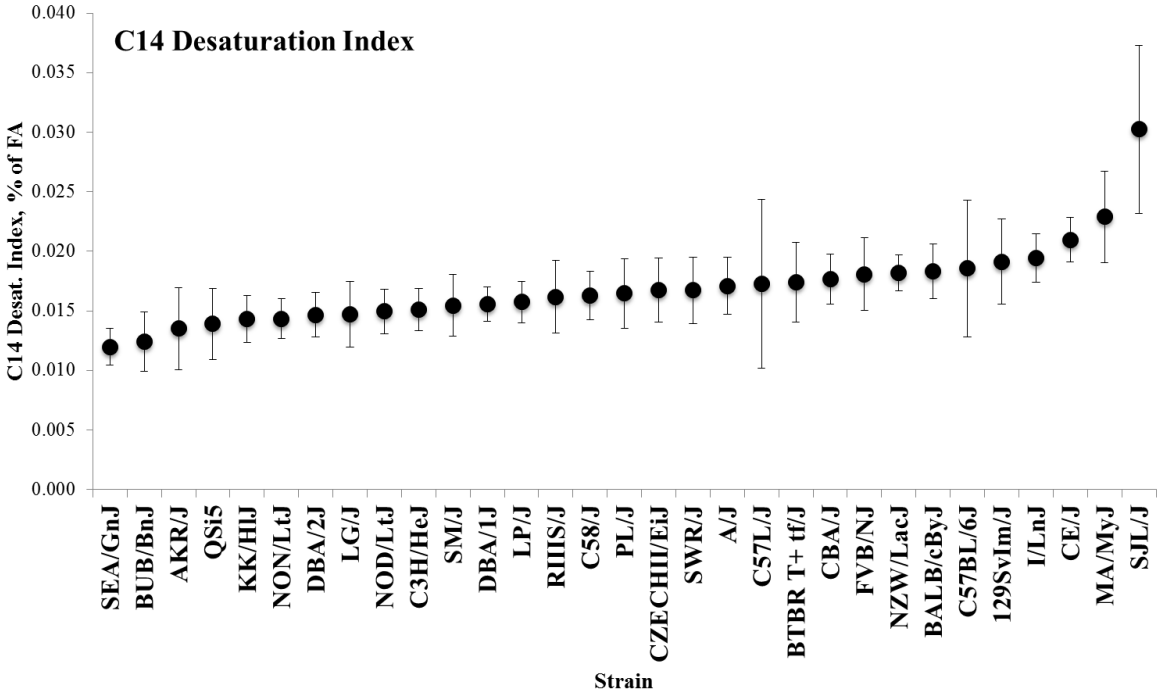

Figure 4.4: Desaturation index for C14 in 31 mouse strains.

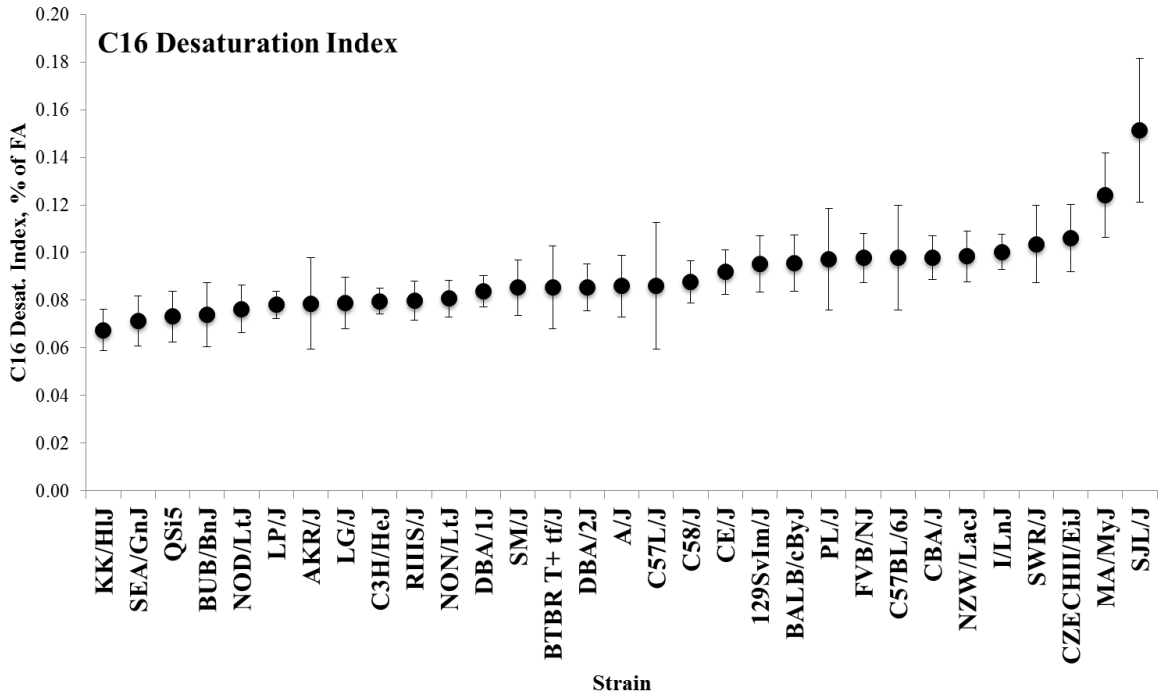

Figure 4.5: Desaturation index for C16 in 31 mouse strains.

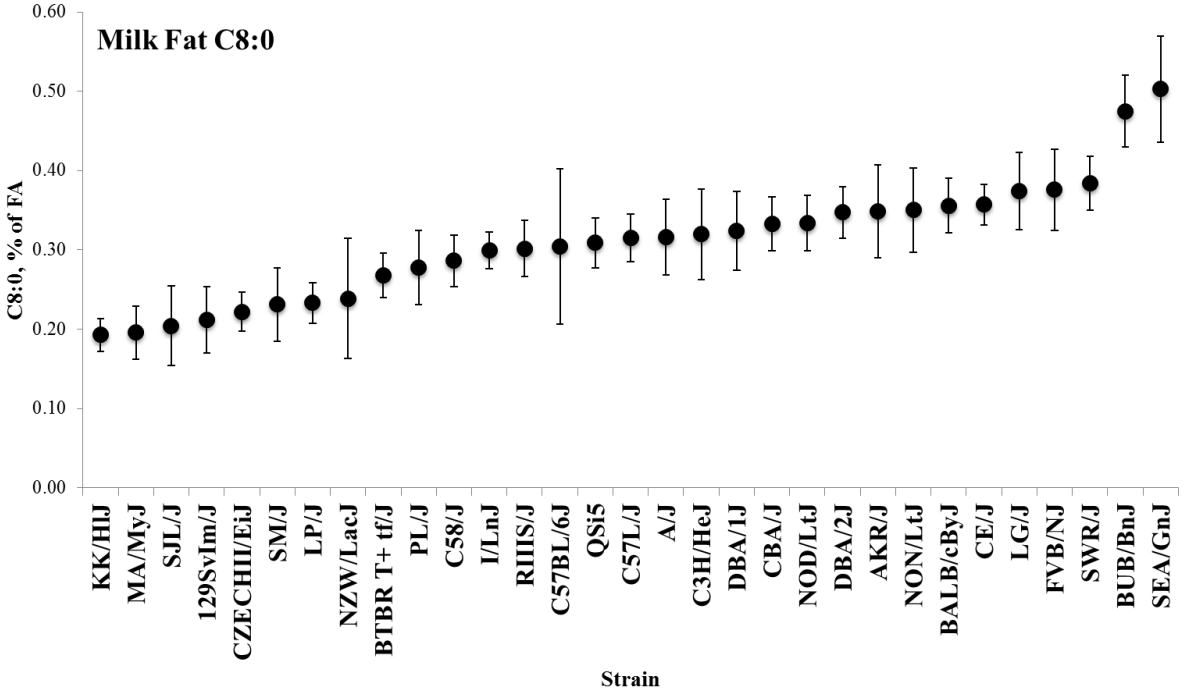

Figure 4.6: Concentration of C8:0 in milk fatty acids of 31 mouse strains.

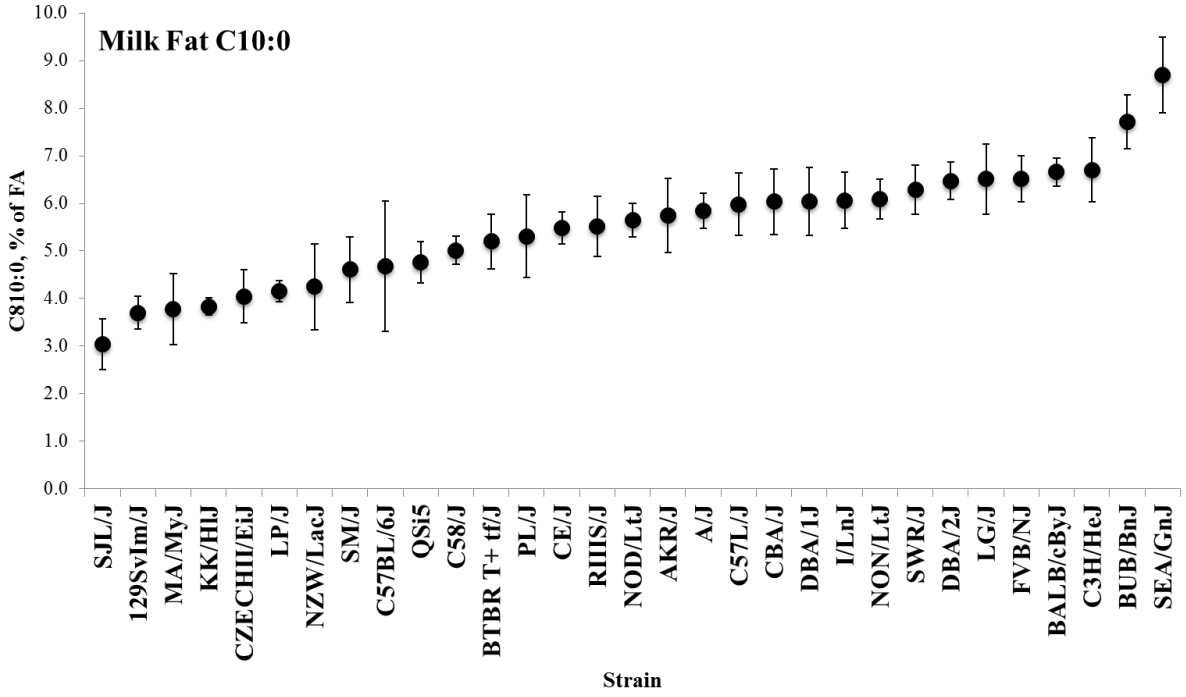

Figure 4.7: Concentration of C10:0 in milk fatty acids of 31 mouse strains.

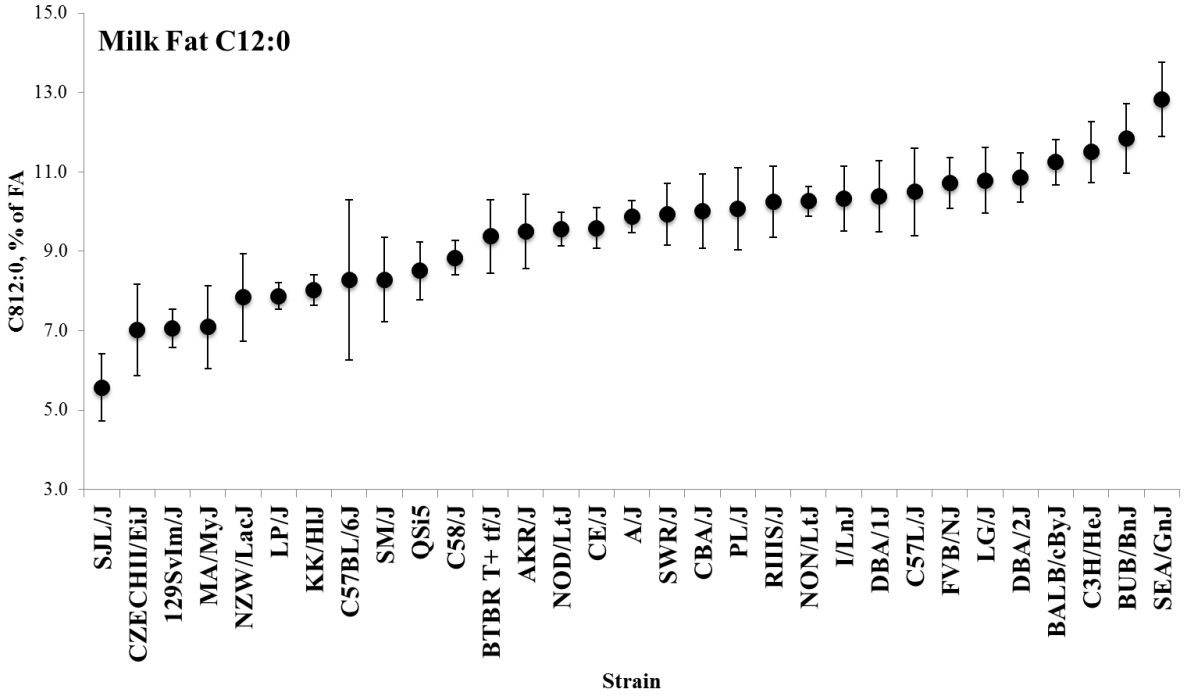

Figure 4.8: Concentration of C12:0 in milk fatty acids of 31 mouse strains.

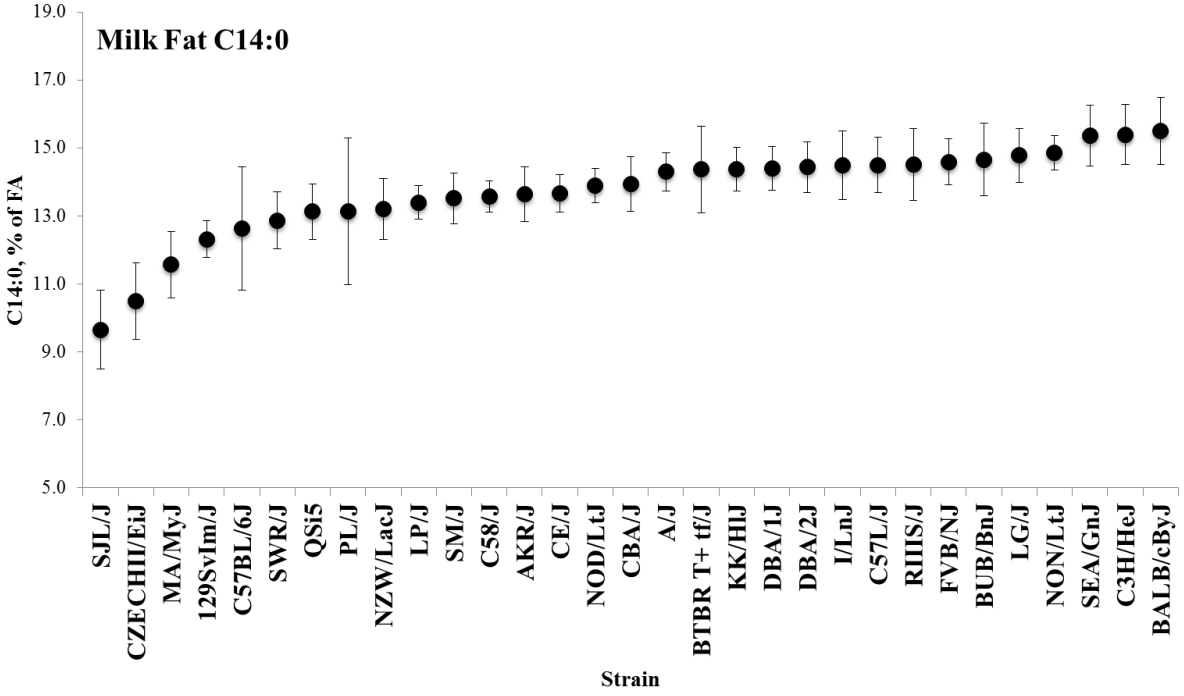

Figure 4.9: Concentration of C14:0 in milk fatty acids of 31 mouse strains.

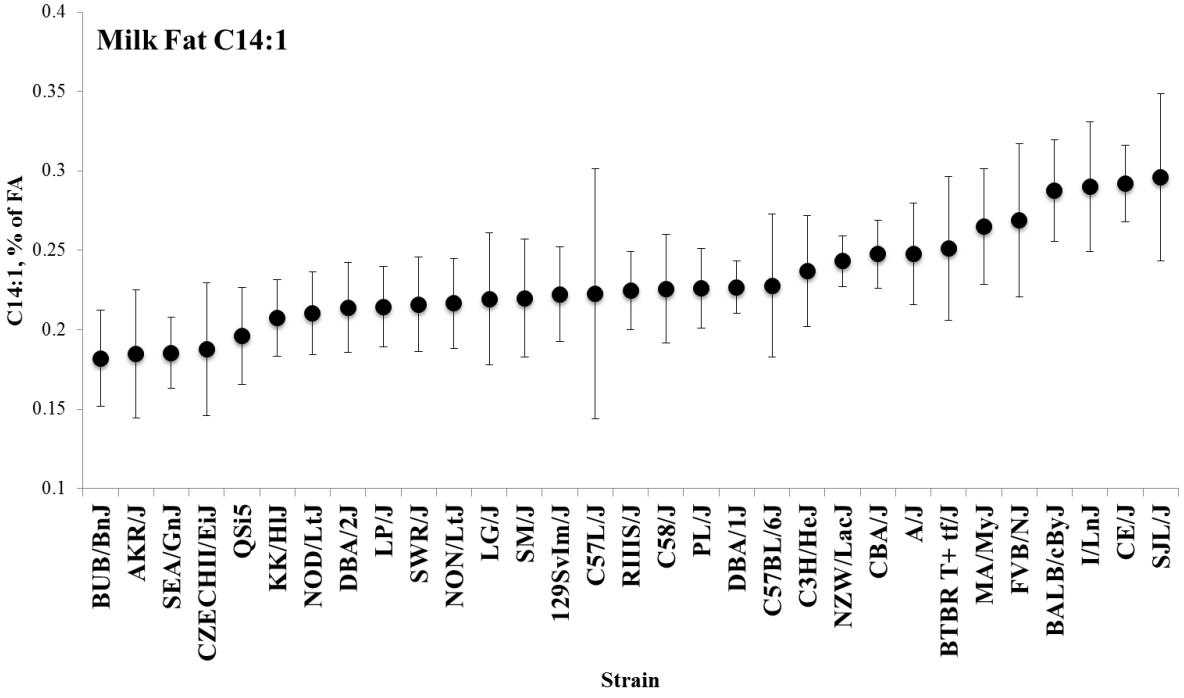

Figure 4.10: Concentration of C14:1 in milk fatty acids of 31 mouse strains.

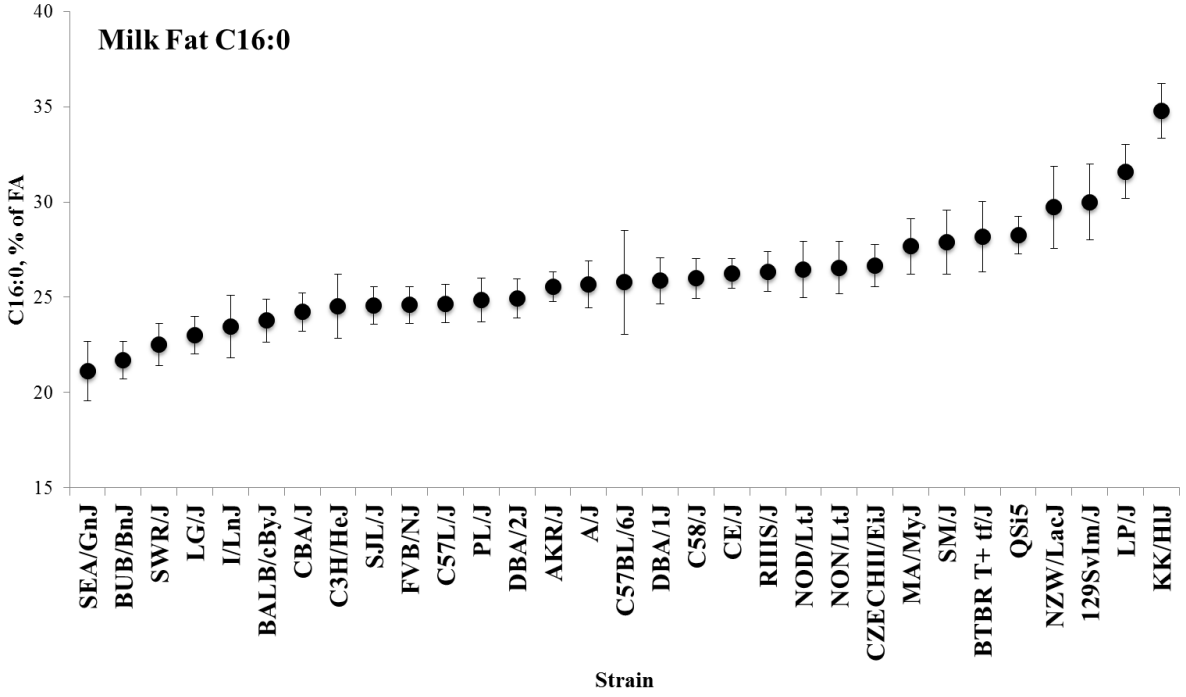

Figure 4.11: Concentration of C16:0 in milk fatty acids of 31 mouse strains.

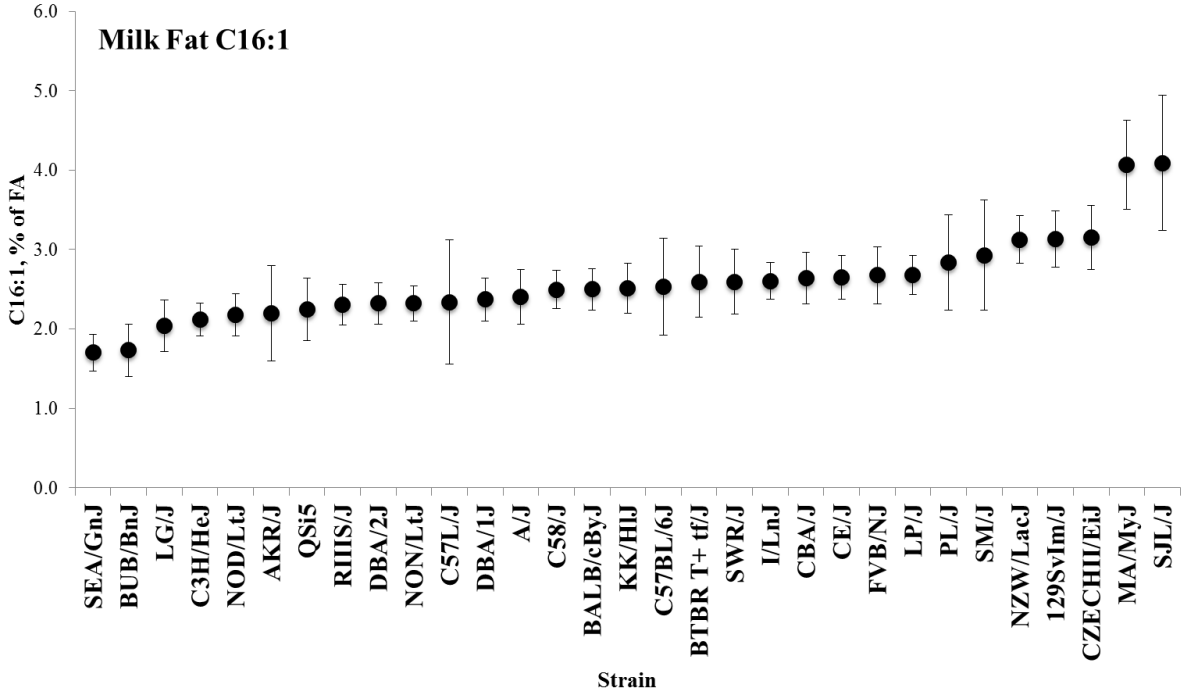

Figure 4.12: Concentration of C16:1 in milk fatty acids of 31 mouse strains.

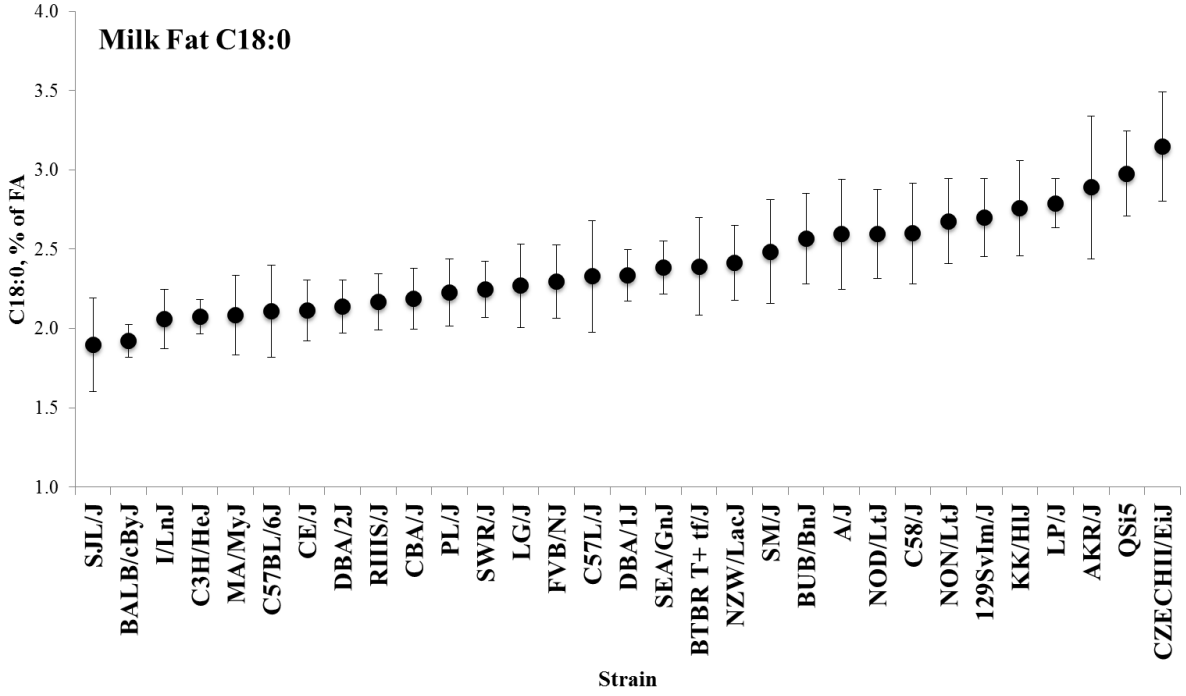

Figure 4.13: Concentration of C18:0 in milk fatty acids of 31 mouse strains.

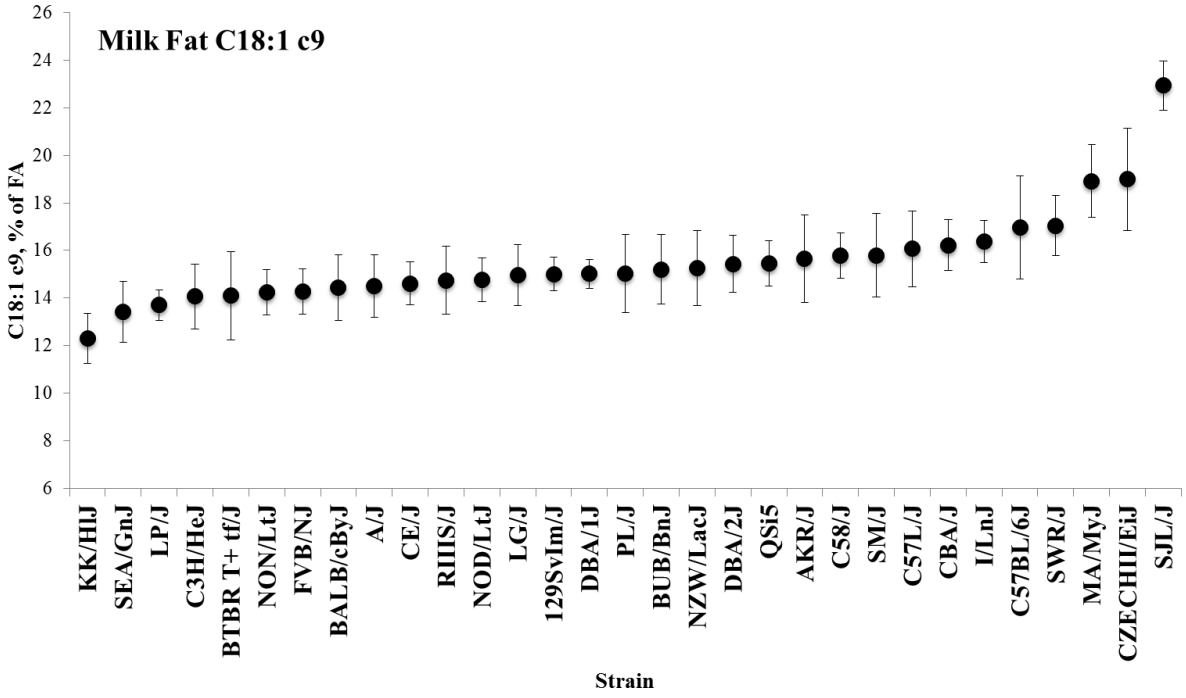

Figure 4.14: Concentration of C18:1c9 in milk fatty acids of 31 mouse strains.

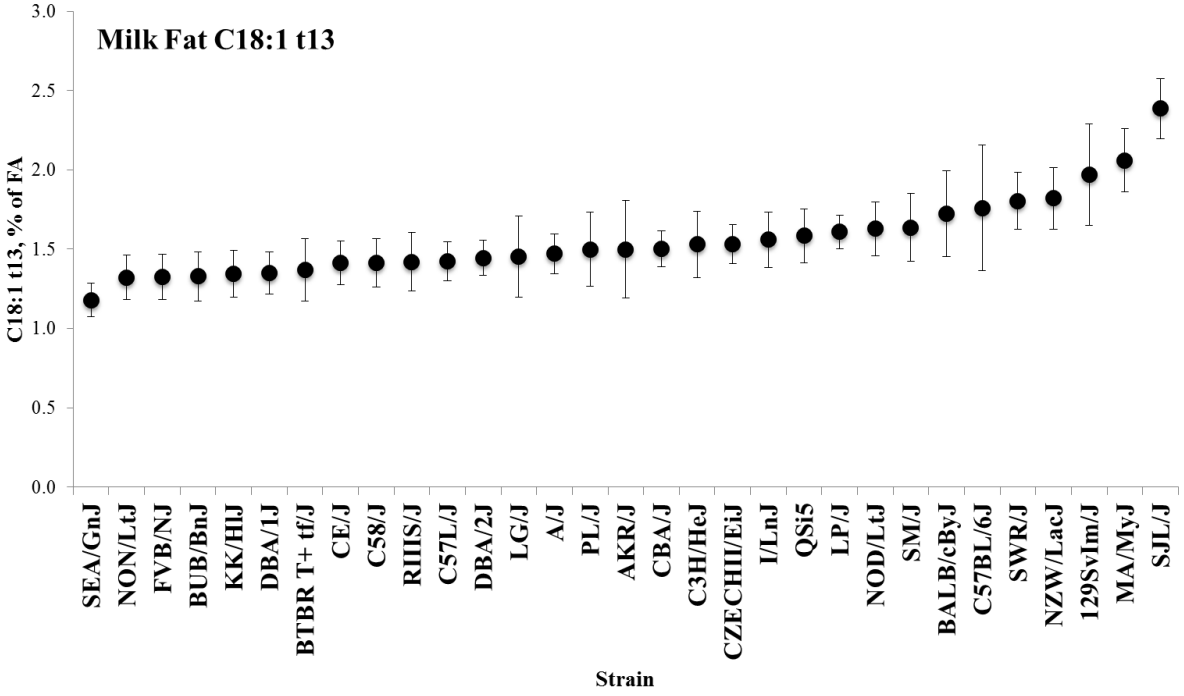

Figure 4.15: Concentration of C18:1t13 in milk fatty acids of 31 mouse strains.

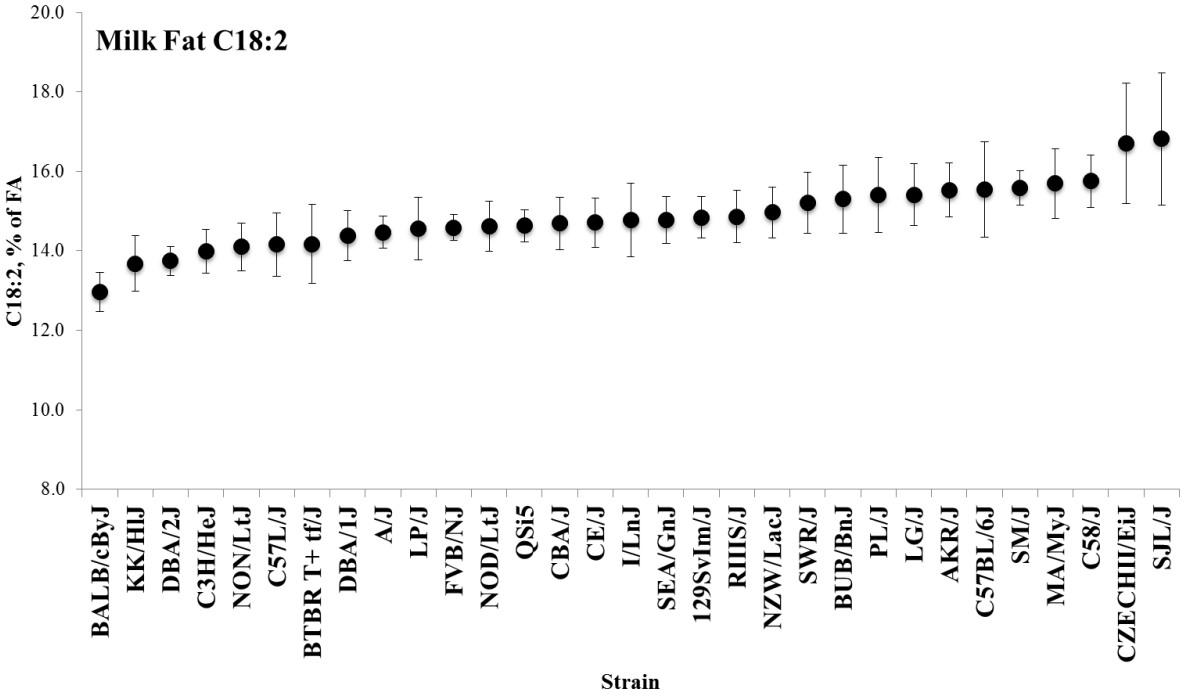

Figure 4.16: Concentration of C18:2 in milk fatty acids of 31 mouse strains.

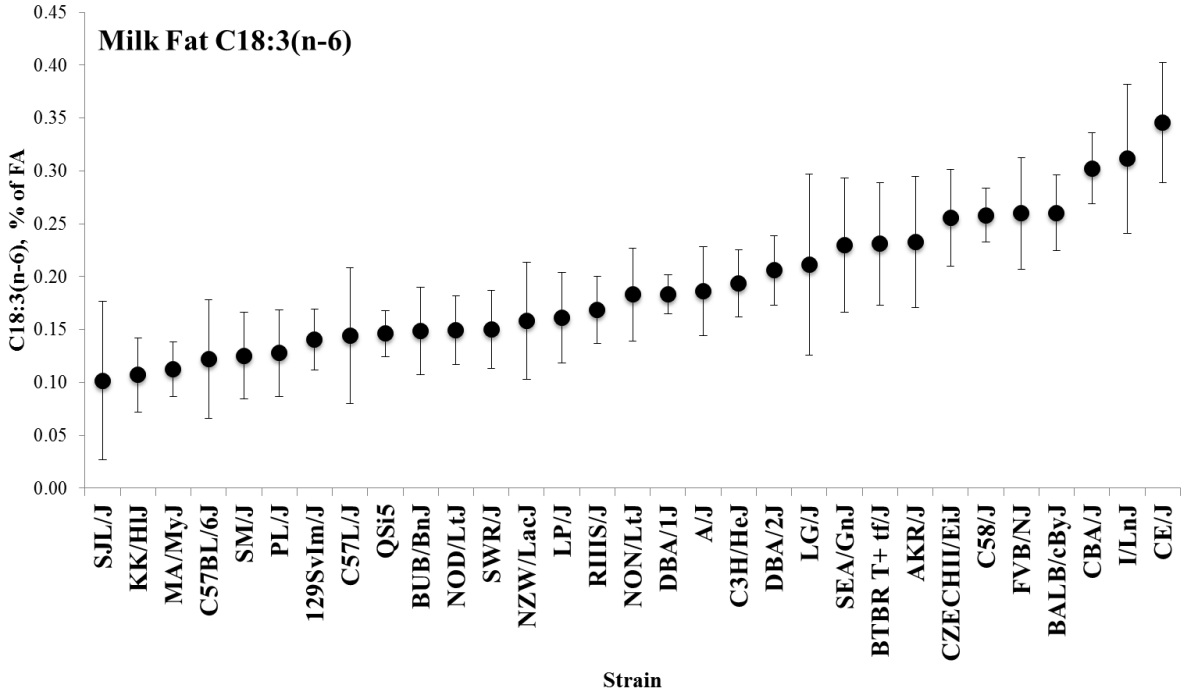

Figure 4.17: Concentration of C18:3(n-6) in milk fatty acids of 31 mouse strains.

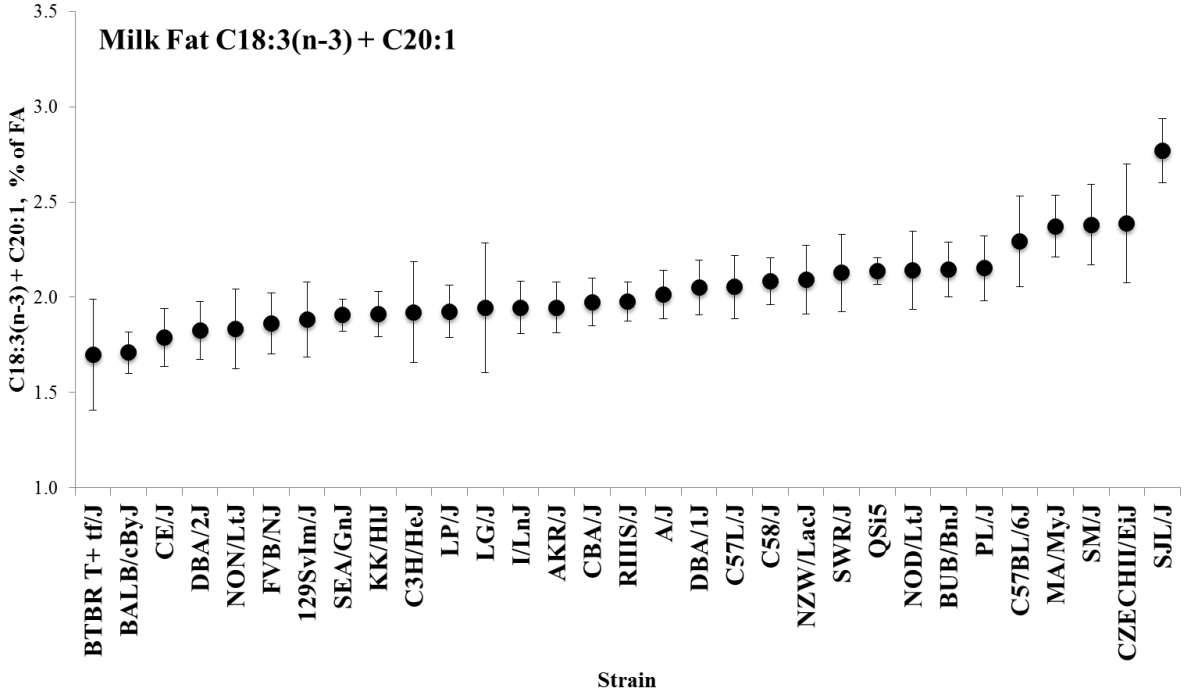

Figure 4.18: Concentration of C18:3(n-3) + C20:1 in milk fatty acids of 31 mouse strains.

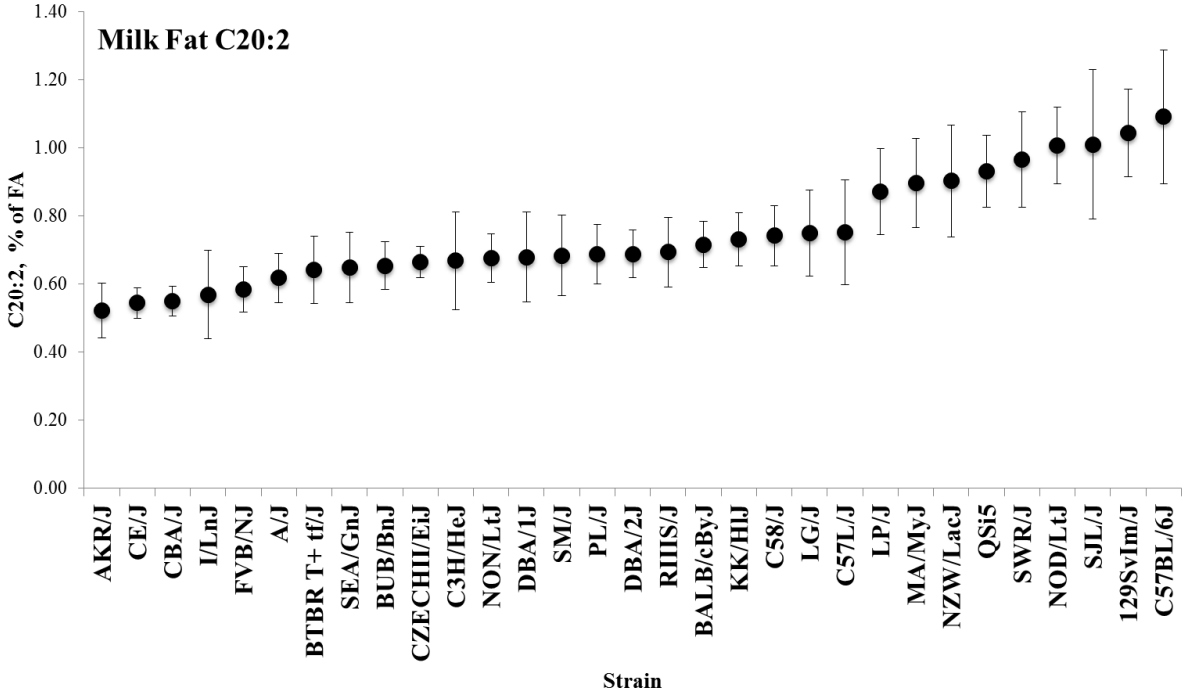

Figure 4.19: Concentration of C20:2 in milk fatty acids of 31 mouse strains.

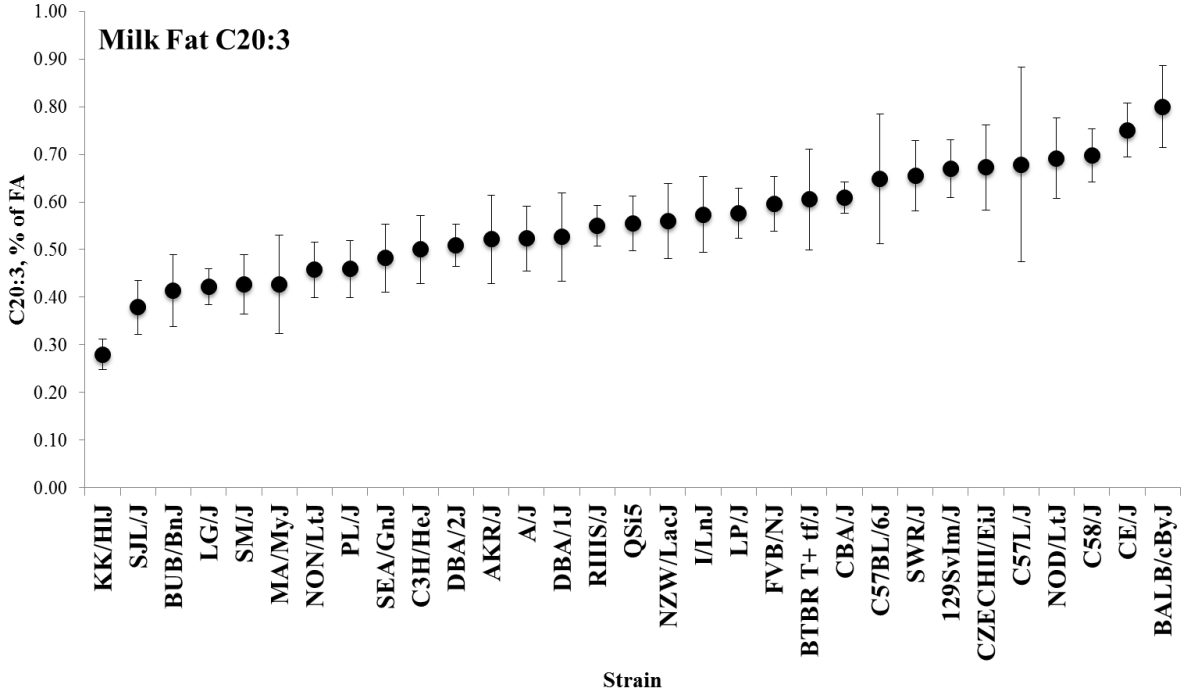

Figure 4.20: Concentration of C20:3 in milk fatty acids of 31 mouse strains.

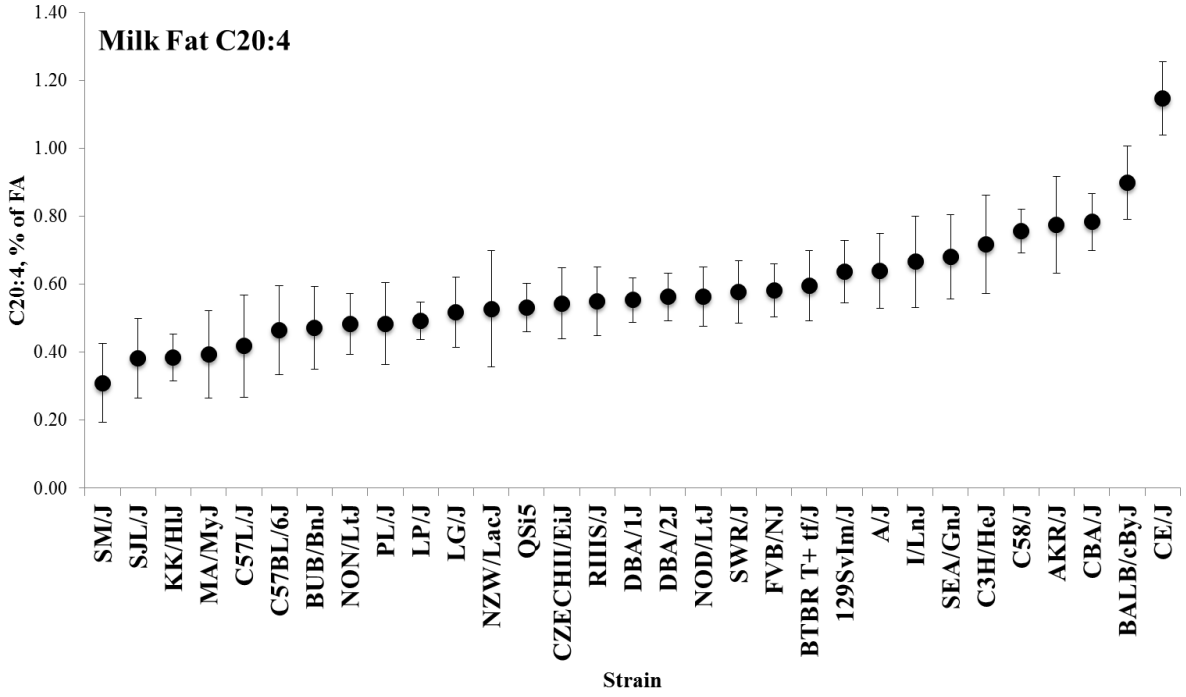

Figure 4.21: Concentration of C20:4 in milk fatty acids of 31 mouse strains.

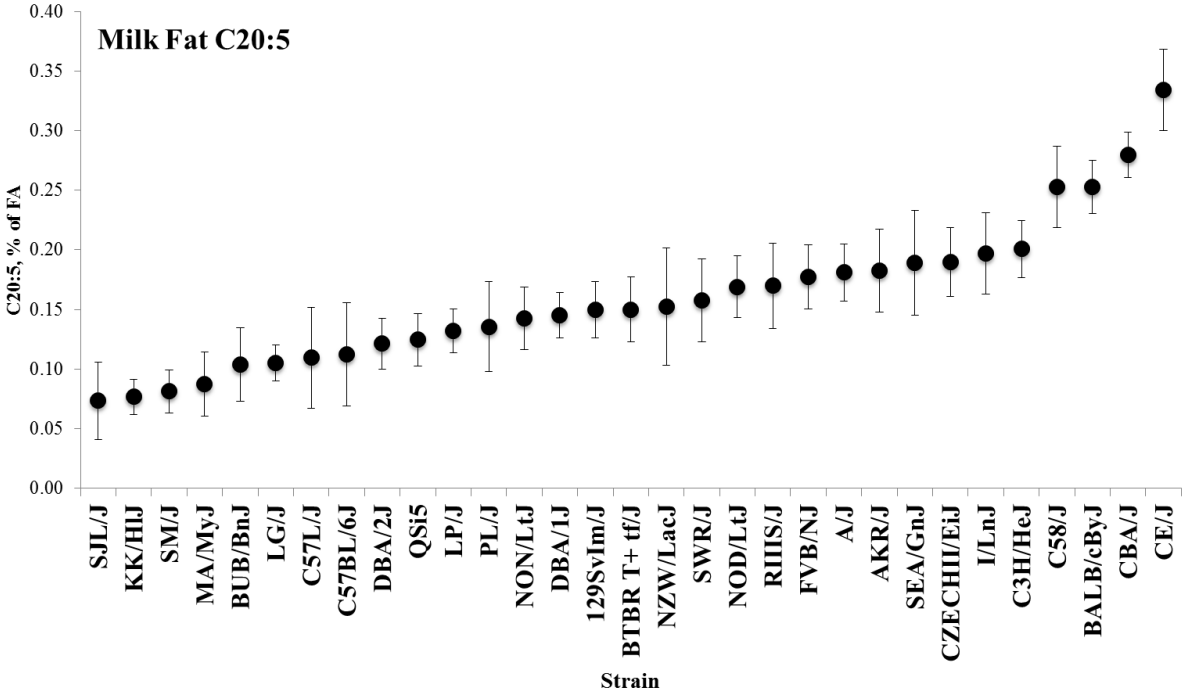

Figure 4.22: Concentration of C20:5 in milk fatty acids of 31 mouse strains.

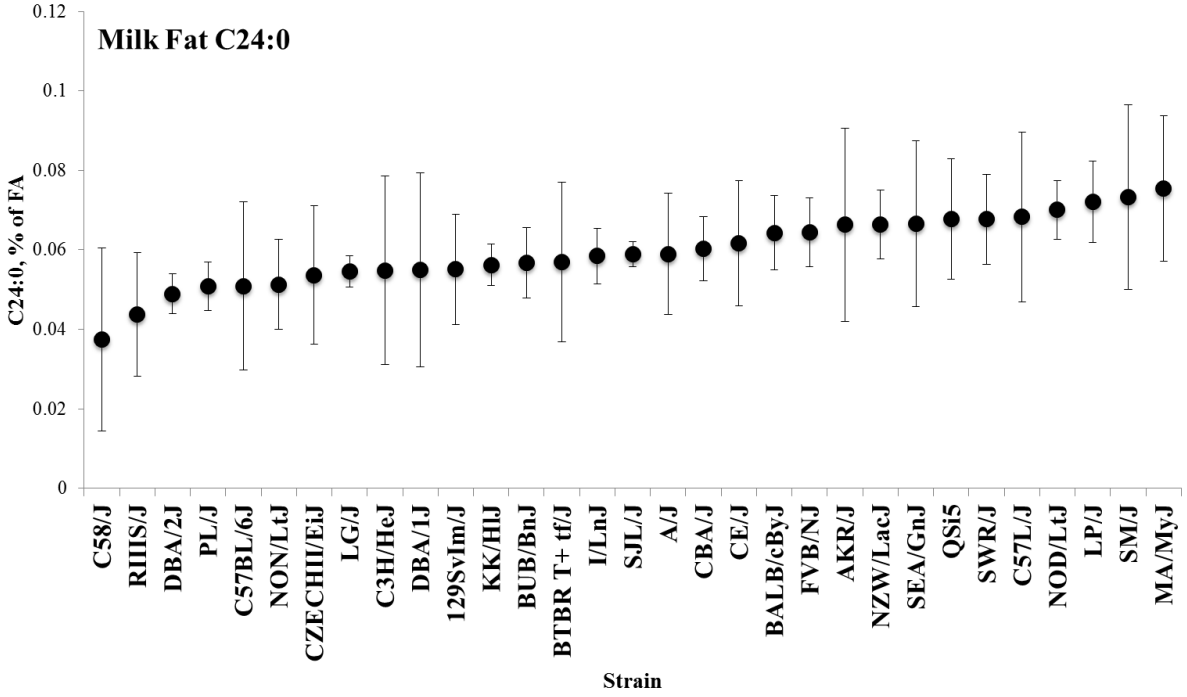

Figure 4.23: Concentration of C24:0 in milk fatty acids of 31 mouse strains.

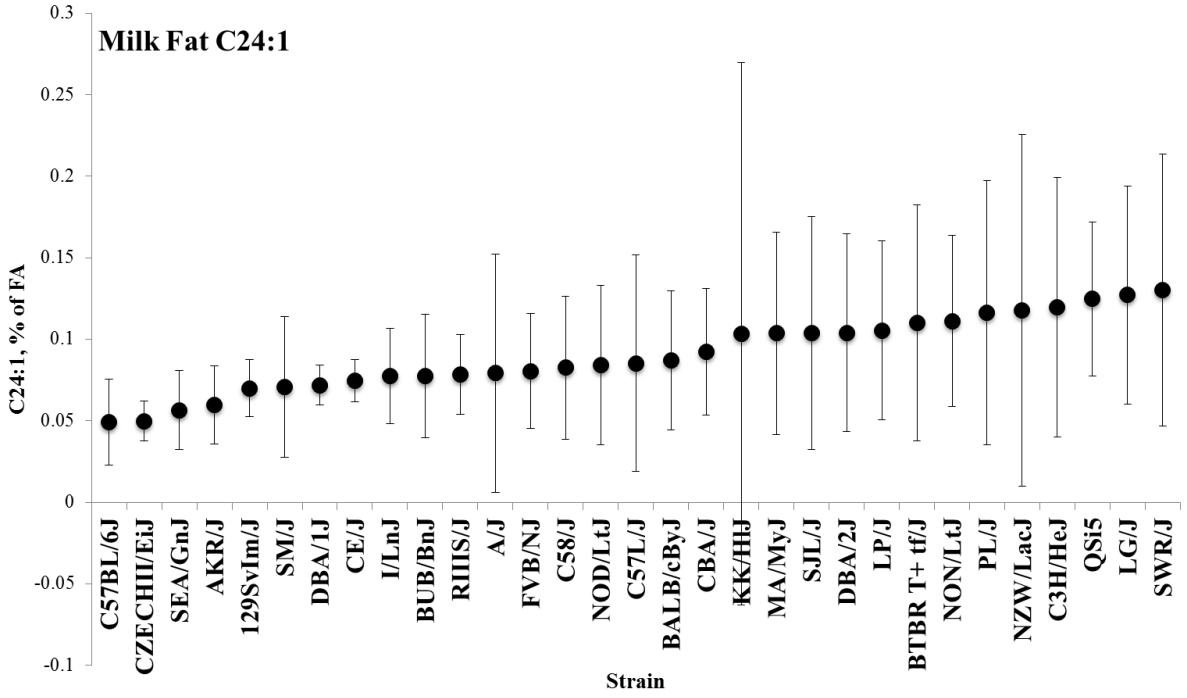

Figure 4.24: Concentration of C24:1 in milk fatty acids of 31 mouse strains.

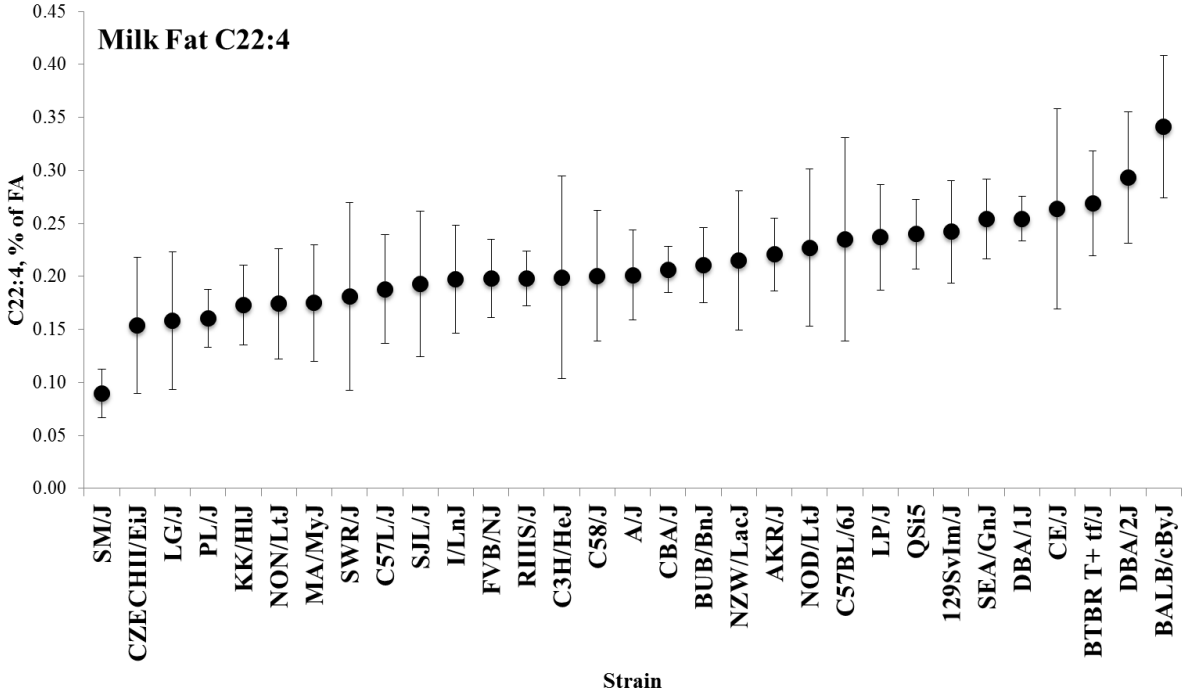

Figure 4.25: Concentration of C22:4 in milk fatty acids of 31 mouse strains.

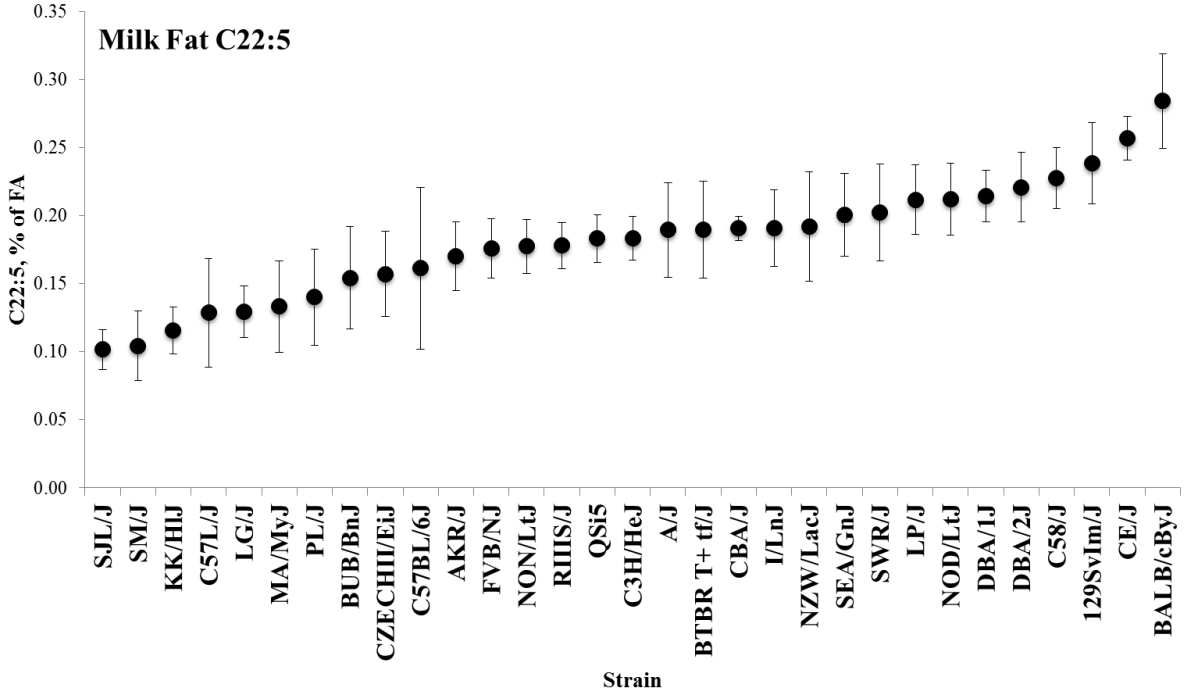

Figure 4.26: Concentration of C22:5 in milk fatty acids of 31 mouse strains.

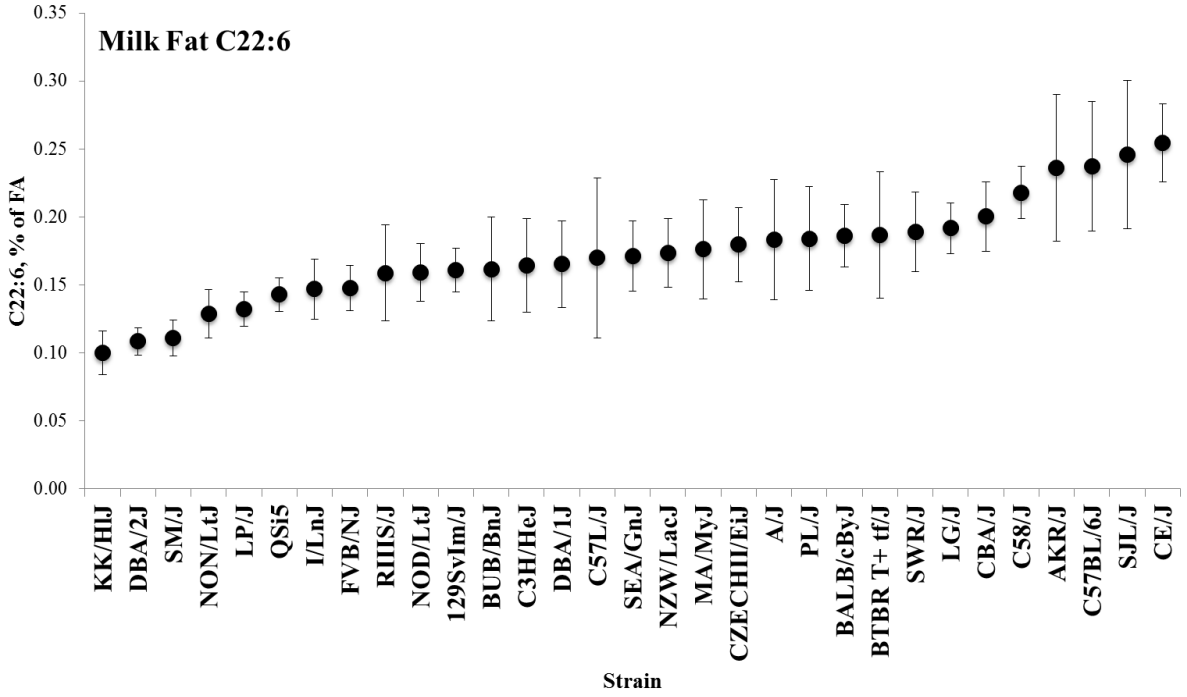

Figure 4.27: Concentration of C22:6 in milk fatty acids of 31 mouse strains.

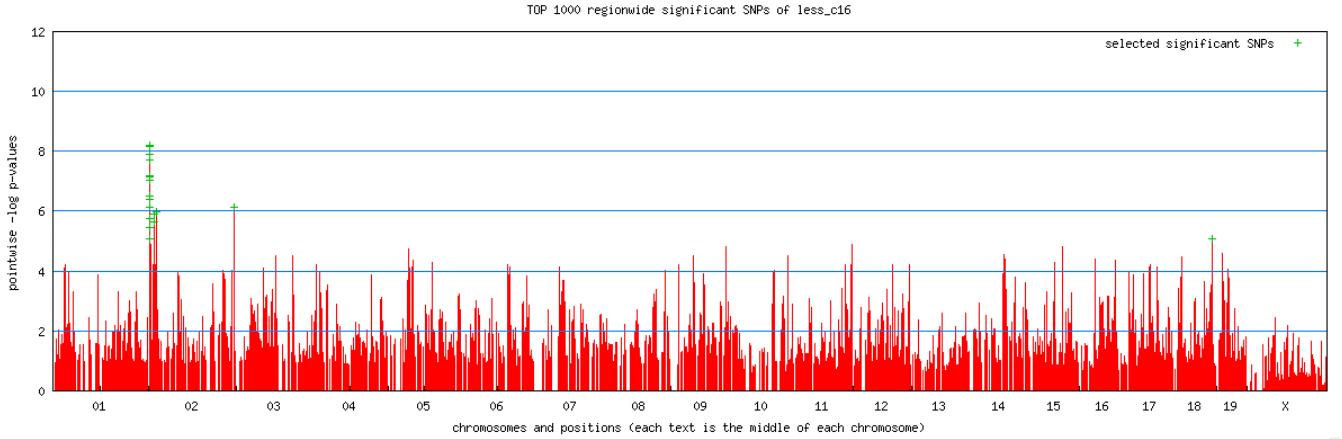

Figure 4.28: Manhattan plot illustrating significant genome-wide SNPs for milk FA less than 16C.

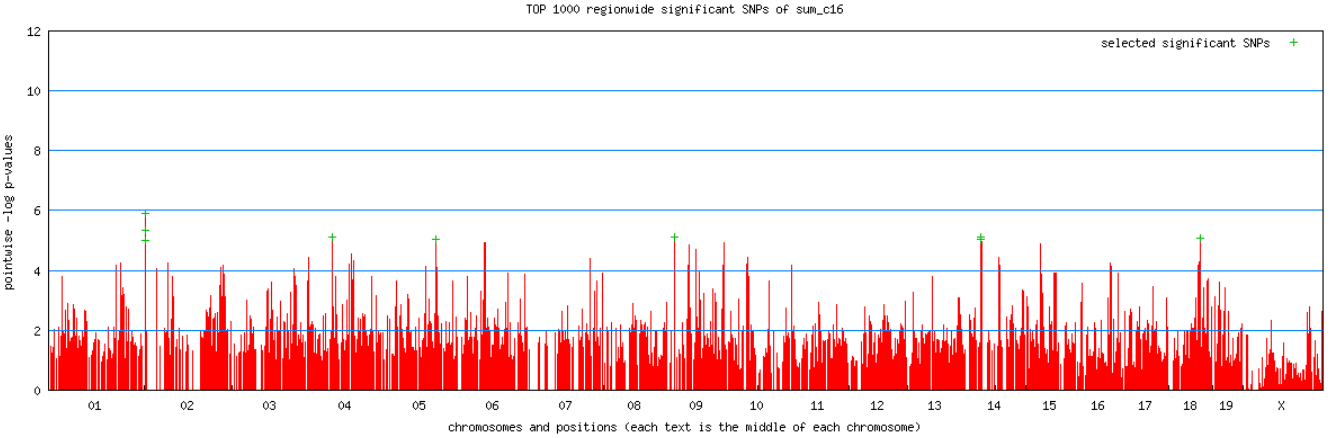

Figure 4.29: Manhattan plot illustrating significant genome-wide SNPs for milk FA with 16C.

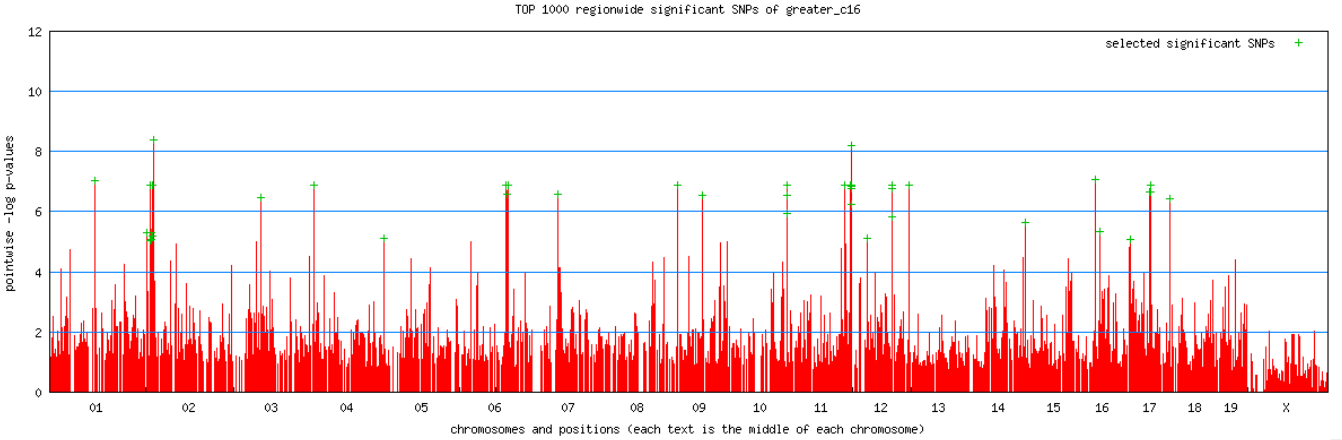

Figure 4.30: Manhattan plot illustrating significant genome-wide SNPs for milk FA greater than 16C.

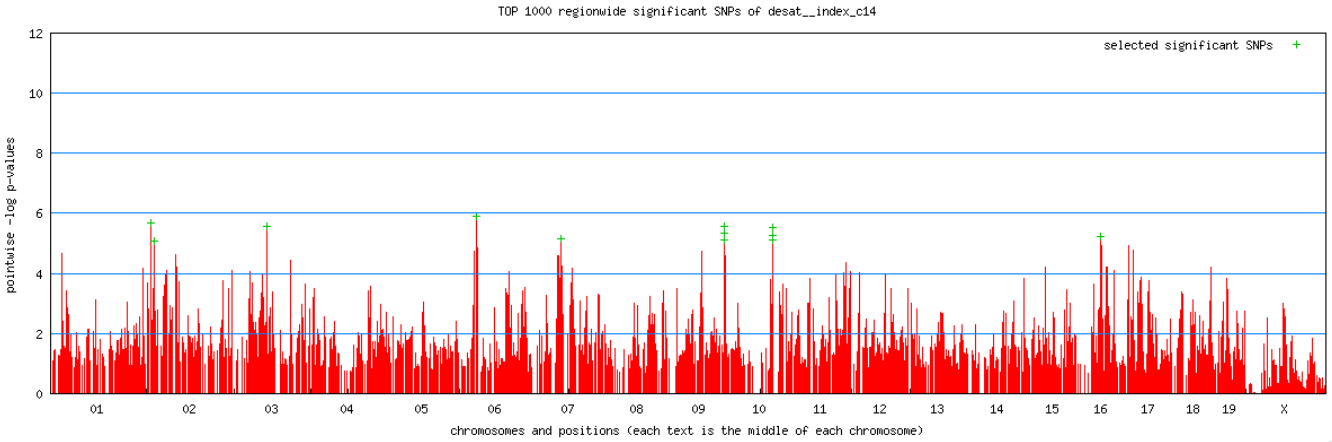

Figure 4.31: Manhattan plot illustrating significant genome-wide SNPs for C14 desaturation indexes.

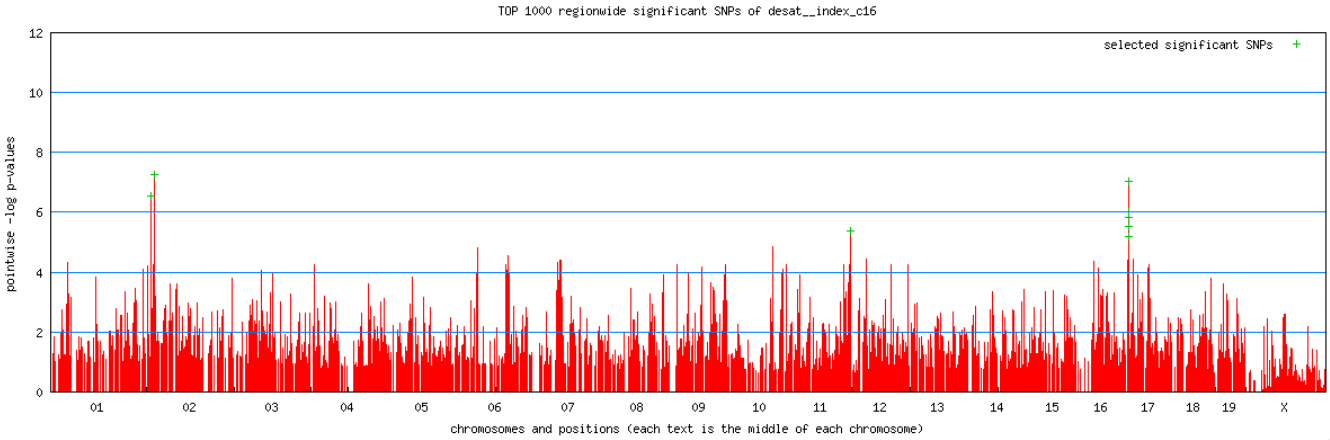

Figure 4.32: Manhattan plot illustrating significant genome-wide SNPs for C16 desaturation indexes.

Chapter 5

DISCUSSION

The data show a reasonable amount of variation in fatty acid profile among different strains of mice. The SEA/GnJ and BUB/BnJ strains had the highest concentrations of fatty acids with less than 16 carbons, including C8:0, C10:0, and C12:0. This indicates that these particular strains produce more fatty acids via de novo synthesis. We can speculate that strains with higher proportions of synthesized fatty acids have greater activity of key lipogenic enzymes, such as ACC or FAS, or have more efficient enzyme activity (Chapter 1). According to the study by Hadsell et al. (2012), the SEA/GnJ strain mice had one of the greatest average daily gains (ADG), but the ADG of the BUB/BnJ strain mice was near the average of the other strains.

There was greater variation among strains for fatty acids with less than 16C than 16C fatty acids or greater than 16C fatty acids, which were relatively linear. The SJL/J strain had the lowest concentration of fatty acids with less than 16 carbons, but the highest concentration of fatty acids greater than 16 carbons. Specifically, SJL/J had the highest concentration of C18:1c9, C18:1t13, C18:2, and C18:3(n-3)+C20:1. This indicates that SJL/J strains utilize more preformed fatty acids taken up from the plasma by the mammary gland than de novo synthesis pathways. In the study by Hadsell et al. (2012), the SJL/J strain had one of the lower ADG.

SEA/GnJ and BUB/BnJ had two of the highest concentrations of C14:0, but two of the lowest concentrations of C14:1. Thus, they had the two lowest C14 desaturation indexes. They also had two of the lowest C16 desaturation indexes, though they had the lowest C16:0 to start with. Low C14 and C16 desaturation indexes indicate that SEA/GnJ and BUB/BnJ had poor

SCD1 activity, which is an enzyme that converts SFA to MUFA. SJL/J, on the other hand, had the highest C14 and C16 desaturation indexes, which indicates high SCD1 enzyme activity. The C14 and C16 desaturation indexes are reliable indicators of SCD1 activity because C14:1 and C16:1 are not found in substantial levels in the diet. Therefore, we can assume high levels of either MUFA are the result of SCD1 enzyme activity.

Each mouse strain used in this study was inbred and assumed to have the same genetic composition within a strain. All mice were fed the same diet and exposed to the same environmental conditions (Chapter 3). Furthermore, the strains used were not known to have any specific fatty acid variation prior to the study. Therefore, we can assume that fatty acid variation among strains is due to differences in genetic composition. Genome analysis showed significant SNPs associated with specific fatty acids. Further study is needed to determine the functional or regulatory importance of the individual SNPs.

Chapter 6

CONCLUSION

The objective of determining the variation in milk fatty acid profiles of different mouse strains was accomplished. The data support the hypothesis that the FA profile of the strains differed in key components, specifically the proportion of de novo and preformed FA. The variation in strains can be attributed to individual SNPs in the genome. Further research projects can look more closely at the SNPs to identify specific genetic markers associated with milk fatty acid profile.

Chapter 7

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Honors and Awards

Pennsylvania State University Scholarships

- Schreyer Honors College Academic Excellence Scholarship *2009, 2010, 2011, 2012*
- C. Channa and Usharani Reddy Trustee Scholarship *2012*
- Kenton D. Rexford Honors Scholarship *2012*
- *Shigley* Memorial Pre-Veterinary Scholarship *2010*
- Society of Distinguished Alumni Trustee Scholarship *2009, 2010, 2012*
- Robert E. Hayes and Mary Brueilly Hayes Trustee Scholarship *2009, 2010, 2011*

Association Memberships/Activities

Pre-Vet Club

- Membership Chair: 2012-13
- Fundraising Chair: 2011-12
- Member: 2009-10, 2010-11

Tri-STATE THON

- Fundraising Chair: 2012-13
- Member: 2010-11, 2011-12