EFFECTS OF GENETIC SELECTION

ON COMPOSITION AND BIOSYNTHESIS OF MILK FATS

IN EARLY LACTATION OF HOLSTEIN COWS

A THESIS

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ABSTRACT

Genetic selection in dairy cattle has greatly improved the milk yield in the past decades. Contemporary Holsteins (CH) that were selected and maintained by the University of Minnesota produced more than 4,500 kg milk in 305 days than its counterpart unselected Holsteins (UH), which has stable milk yield since 1964. However, the influences of genetic selection on the chemical composition of cow milk were not well characterized. In this study, cows from UH and CH genotypes (CH) (n = 12/genotype) were co-housed and fed the same diet *ad libitum* 5 weeks prepartum. Weekly milk samples were obtained from the milking on each Tuesday night in the first 9 weeks of lactation. Analysis of macronutrients in milk indicated that CH and UH had comparable levels of lactose (4.75% vs. 4.69%, P=0.39). However, CH had a slight lower level of proteins (2.98% vs. 3.13%, $P<0.05$) and much higher level of fats than UH (4.33% vs. 3.55%, $P<0.01$). Lipidomic analysis through high-resolution liquid chromatography-mass spectrometry (LC-MS) analysis, multivariate data analysis (MDA), and MSMS fragmentation further revealed that the TAG profiles of UH and CH milk differed greatly in early weeks of lactation, but became more comparable by week 9. Hierarchical clustering analysis (HCA) of TAGs markers indicated that CH milk were more enriched with preformed fatty acids (FAs) while UH milk had higher abundance of FAs

originated from *de novo* synthesis. This conclusion was further confirmed by quantitative analysis of FAs and organic acid precursors. Overall, these observations suggested that genetic selection increased the contribution of preformed FAs from blood lipids to the biosynthesis of milk TAGs, especially in the early phase of lactation.

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Literature Review

[1]**KEYWORDS:** milk, triacylglycerol, fatty acids, lipidomics, metabolomics

ABBREVIATIONS: **ACACA**, acetyl-coenzyme A carboxylase alpha; **ACC**, acetyl-CoA carboxylase; **ACSL1**, acyl-CoA synthetase long-chain family member 1; **ACSS2**, acyl-CoA synthetase short-chain family member 2; **AGPAT6**, 1-acylglycerol-3-phosphate O-acyltransferase 6; **BDH1**, 3-hydroxybutyrate dehydrogenase, type 1; **BHBA**, β-hydroxybutyrate; **BTN1A1**, butyrophilin, subfamily 1, member A1; **CD36**, cluster of differentiation 36; **CH**, contemporary Holstein; **CLA**, conjugated linoleic acid; **CLSM**, confocal laser scanning microscopy; **DAGs**, diacylglycerols; **DHA**, docosahexaenoic acid; **EBV**, estimated breeding value; **EPA**, eicosapentaenoic acid; **ER**, endoplasmic reticulum; **FABP3**, fatty acid-binding protein; **FADS1**, fatty acid desaturase 1; **FAs**, fatty acids; **FASN**, fatty acid synthetase; **GA**, Golgi apparatus; **GC**, gas chromatography; **GC-MS**, gas chromatography-mass spectrometry; **GH**, growth hormone; **GPAM**, glycerolglycerol-3-phosphate acyltransferase; **GT**, galactosyltransferase; **HCA**, hierarchical clustering analysis; **HMDB**, Human Metabolome Database; **HPLC**, high-performance liquid chromatography; **HPLC-MS**, high-performance liquid chromatography-mass spectrometry; **ICA**, independent component; **INSIG1**, insulin induced gene 1; **KEGG**, kyoto encyclopedia of genes and genomes; **α-LA**, α-lactalbumin; **LIR**, lactational incidence rates; **LPIN1**, lipin 1; **LPL**, lipoprotein lipase; **MAGs**, monoacylglycerols; **NADPH**, nicotinamide adenine dinucleotide hydrogen

phosphate; **NEFA**, nonesterifired FA; **NMR**, nuclear magnetic resonance; **OPLS**, orthogonal partial least squares; **PCA**, principal component analysis; **PLA-DA**, partial least squares-discriminant analysis; **PLS**, principal least square; **PPARG**, peroxisome proliferator-activated receptor gamma; **PPARGC1A**, PPAR gamma, coactivator 1 alpha; **SCD**, stearoyl-CoA desaturase; **SEM**, scanning electron microscopy; **SREBP1**, sterol response element binding protein 1; **TAGs**, triacylglycerols; **TEM**, transmission electron microscopy; **TLC**, thin-layer chromatography; **UH**, unselected Holstein; **UPLC**, ultra-performance liquid chromatography; **USDA-NASS**, united states department of agriculture, national agriculture statistics service; **VLDL**, very low density lipoproteins; **XDH**, xanthine dehydrogenase.

1.1 COW MILK

Cattle, buffaloes, goats, sheep and camels are the major dairy animals in the world. Yaks, horse, reindeers and donkeys are also used as dairy animals in specific regions and ethnic groups [1]. Among these dairy animals, cattle are responsible for about 83.1% world milk production, followed by 13.1% from buffaloes. More specifically, cow milk represents 61% milk production in Asia, 75% in Africa, 97% in Europe, 99.7% in Americas, and almost 100% in Oceania [2]. Therefore, cow milk is the most produced and consumed milk in the world. In 2012, average annual fluid milk consumption in the United States was 76.8 kilograms per capita [3], while the consumption of dairy products in the United States was 278.1 kilograms per capita [4], making milk and dairy products as one of the most important sources of energy and nutrients in human diet.

1.1.1 Milk composition and nutritional value

As one of the most consumed food, cow milk provides all of nutrients that calves need and majority of nutrients that humans need. Cow milk contains carbohydrates, fats, proteins, water, vitamins, minerals and other micronutrients (Table 1.1). Among these nutrients, water is the most abundant constituent of cow milk. On average, cow milk contains 87.7% water, 4.9% lactose, 3.4% fat, 3.3% protein, and 0.7% minerals [5].

Lactose, as a disaccharide, is the dominant carbohydrate in cow milk, while trace amounts of monosaccharides and oligosaccharides also exist in milk. Lactose is composed of D-glucose and D-galactose through a β-1→4 glycosidic linkage. It is synthesized by galactosyltransferase (GT) with its regulatory subunit, α-lactalbumin (α-LA) on the Golgi apparatus (GA) membrane of mammary epithelial cells [6]. Since it is only produced by mammary epithelial cells, lactose is a milk specific metabolite [7]. Its level is very stable in cow milk, potentially due to its role as the most important osmole [8].

Milk proteins are complete proteins since milk contain all nine essential amino acids for humans. About 3,000 different proteins have been identified in milk [9]. In cow milk, about 82% of proteins are caseins. The other 18% are serum proteins or whey proteins, of which β-lactoglobulin and α-lactalbumin are the most abundant ones [10, 11]. More importantly, caseins, β-lactoglobulin and α-lactalbumin are unique milk proteins only synthesized in mammary gland [12]. Caseins are a group of phosphoproteins, including αS1-casein, αS2-casein, ß-casein and κ-casein. Caseins commonly aggregate in milk, together with calcium phosphate to form a multi-molecular and granular structure called a casein micelle [13, 14]. The unique structure of the casein micelle allows not only the safe secretion of high concentrations of calcium, phosphate and casein protein through mammary gland, but also the retention of casein micelle in the stomach of calves so that nutrients can be fully digested and absorbed [15, 16].

Compared to carbohydrates and proteins, lipids are the most variable macronutrient in cow milk. Many non-nutritional and nutritional factors have influences on milk lipid production and composition (detailed in **1.1.2**) [17]. Diverse lipid species, including triacylglycerols (TAGs), phospholipids, cholesterols, sterols, and free fatty acids (FAs), are present in cow milk (detailed in **1.1.2**) [18]. Saturated and monounsaturated fatty acids, such as palmitic acid, stearic acid and oleic acid are the most abundant moieties in milk lipids with high energy density. Polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) [19, 20] and conjugated linoleic acid (CLA) [21, 22], are minor moieties in cow milk but possess the bioactivities that can affect human metabolism, health and disease [23, 24]. Among these diverse lipid species, CLAs and odd chain fatty acids are unique in ruminant milk [25]. CLA is an intermediate of biohydrogenation, a conversion of unsaturated FAs to saturated FAs by rumen microorganisms [26].

Calcium, phosphorous, and potassium are the most abundant minerals in cow milk (Table 1.1). As the building blocks of bone and soft tissues, calcium and phosphorous are required in large quantities in rapidly growing calves. In milk, they are mainly stored in the form of salts inside casein micelles [27]. Potassium is the primary electrolyte for maintaining fluid balance [28], while other minor minerals, such as copper, iron, cobalt, zinc, manganese are utilized as the cofactors of enzymes and regulatory molecules [29].

Cow milk contains all the vitamins required by calves. Fat soluble vitamins A, D, E, and K are distributed in milk fat while vitamins B1 (thiamin), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B12 (cobalamin), vitamin C, and folate are dissolved in the aqueous phase of milk [30].

1.1.2 Milk lipids

1.1.2.1 Composition of milk lipids

Milk lipids are primarily in the form of TAGs, which count for about 98% of total milk lipids [31]. Other milk lipids include diacylglycerols (DAGs), monoacylglycerols (MAGs), phospholipids, cholesterols, glycolipids and free FAs [32]. TAGs, as the most abundant milk lipids, are composed of three FAs and one glycerol moiety. Because the three FAs of a TAG could have different carbon lengths and unsaturation levels, diverse TAG species exist in milk.

1.1.2.2 Sources of milk fatty acids

Preformed FAs from the breakdown of blood lipids and the FAs from de novo synthesis in mammary gland are the two sources of FAs for synthesizing milk TAGs (Figure 1.1) [33]. The majority of FAs shorter than 16 carbons as well as some of palmitic acid (C16:0) are from de novo synthesis in the cytoplasma of

mammary epithelial cell, utilizing acetate and β-hydroxybutyrate (BHBA) from the rumen as the main carbon sources [34, 35]. Acetate and BHBA contribute equally for the first four carbon of FA synthesis. Acetate is transformed to acetyl-CoA and used as a carbon source for chain elongation via malonyl-CoA pathway, whilst BHBA can enter the synthesis cycle directly (Figure 1.2) [36]. Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, whereas fatty acid synthetase (FAS) is responsible for the condensation cycles of acetyl-CoA or butyryl-CoA [37]. The reducing agent used in FA synthesis pathway is NADPH (nicotinamide adenine dinucleotide hydrogen phosphate, reduced form) [36].

Blood lipids, such as very low density lipoproteins (VLDL), chylomicrons, and plasma albumin bound nonesterified FAs (NEFA) are the main sources of FAs longer than 16 carbons and most palmitic acid (C16:0) [38]. In the lactation phase of ruminants, lipolysis of adipose tissues and lipogenesis in the liver are major contributors of these preformed FAs [39]. TAGs in the VLDL and chylomicrons are hydrolyzed in the mammary capillaries by lipoprotein lipase (LPL). The LPL can remove one, two or all three fatty acids from glycerol, releasing free fatty acids, glycerol, MAGs, and DAGs for reuse in mammary gland.

1.1.2.3 Synthesis of TAGs

Milk TAGs are synthesized in the mammary epithelial cells. Precursors of TAGs synthesis including acetate, BHBA, preformed fatty acids, glycerol, and monoacylglycerols are taken up at the basolateral membrane of epithelial cells [40]. After being synthesized on the smooth endoplasmic reticulum (ER) of epithelial cells, TAGs form micro lipid droplets coated with lipid monolayer derived from ER [41]. Small lipid droplets can be secreted directly from the mammary epithelial cells as very small milk fat globule (less than 0.2 μm in diameter) [42]. Alternatively, multiple small lipid droplets can fuse together to form large lipid droplets, then move to the apical membrane of mammary epithelial cells as large fat globule (greater than 15 μm in diameter) [43]. When milk fat globules move out of cell membrane, they are enveloped again by the apical membrane of the cell to form a phospholipid trilayer (Figure 1.3) [44]. The trilayer phospholipid membrane stabilizes milk fat globules as emulsions in the aqueous environment of milk.

1.1.2.4 Factors influencing milk fat synthesis and composition

Nutritional and dietary factors can readily affect the yield and composition of milk fat [45]. Inadequate dietary fat intake can depress fat content in cow milk while appropriate supplementation of dietary fat can enrich specific fatty acids and alter milk fat composition [46, 47]. However, excessive unsaturated fat intake can cause

a milk fat depression when rumen microorganisms produce CLA in excess [48-50]. CLA-induced milk fat depression is through decreasing the expression of mammary lipogenic genes by sterol response element binding protein 1 (SREBP1), a central regulator of lipid synthesis [51].

Besides nutritional and dietary factors, many other factors including genetics [52], lactation stage [53], season [54, 55], circadian rhythm [56], and physiological state [47, 57] also influence the composition of milk fat. A network of genes and proteins associated with milk fat synthesis have already been identified [58]. Among them, lipoprotein lipase (LPL) and cluster of differentiation 36 (CD36, thrombospondin receptor) protein are responsible for mammary FA uptake from blood; fatty acid-binding protein (FABP3, heart) for intracellular FA trafficking; acyl-CoA synthetase long-chain family member 1 (ACSL1) and acyl-CoA synthetase short-chain family member 2 (ACSS2) for intracellular activation of long-chain and short-chain FAs; acetyl-coenzyme A carboxylase alpha (ACACA) and fatty acid synthetase (FASN) for de novo FA synthesis; stearoyl-CoA desaturase (SCD, delta9-desaturase) and fatty acid desaturase 1 (FADS1, delta5-desaturase) for desaturation; 1-acylglycerol-3-phosphate O-acyltransferase 6 (AGPAT6), glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) , lipin 1 (LPIN1) for TAG synthesis; butyrophilin, subfamily 1, member A1 (BTN1A1) and xanthine dehydrogenase (XDH) for lipid droplet formation; 3-hydroxybutyrate dehydrogenase, type 1 (BDH1) for ketone body utilization; and insulin induced

gene 1 (INSIG1), peroxisome proliferator-activated receptor gamma (PPARG), and PPAR gamma, coactivator 1 alpha (PPARGC1A) for transcriptional regulation [58]. During the lactation, the highest percentage of milk fats are usually found in colostrum and then decrease in the first 2 months of lactation, followed by a slow increase afterwards [59]. Seasonal and circadian rhythm effects also contribute to the variations of milk fat. For example, milk fat is commonly in its highest level during winter and night time [60, 61]. Furthermore, mastitis a common disease in lactating dairy cows, can significantly reduce milk fat and lead to a compositional change [62, 63].

1.1.2.5 Current methods for the evaluation of milk lipids

Lipid content in milk is evaluated by diverse analytical approaches. Each individual analytical method usually targets one specific property of milk lipids, such as fat globules, concentration, composition, etc.

Various microscopic techniques, such as optical microscopy, fluorescence microscopy, confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and holographic video microscopy can be used to determine the size and morphology of milk fat globules [64]. Light scattering techniques [65] and scanning flow cytometry [66] are also used to characterize milk fat globules.

The analytical techniques used to determine the total milk fat content can be categorized into three types based on their different principles: solvent extraction, non-solvent extraction, and instrumental methods. Solvent extraction is based on the principle that lipids are highly soluble in organic solvent, but poorly soluble in water. Non-solvent extraction use other chemicals, instead of organic solvents, to separate lipids with other components. Well known methods, such as Babcock Method, Gerber Method and Detergent Method are widely used in determining milk fat content [67, 68]. A variety of instrumental methods are available for determining the total fat concentration. These methods are mainly based on the physicochemical properties of milk fat, including density, ultrasonic velocity, UV-visible absorbance, infrared absorbance, and nuclear magnetic resonance (NMR) [69].

For determining lipid composition, chromatography is commonly used for the separation of lipids and it also facilitates the identification of interested lipids when combined with mass spectrometry or NMR. In practice, thin-layer chromatography (TLC) can conveniently classify milk lipids into TAGs, DAGs, MAGs, phospholipids, cholesterol, and cholesterol ester [18]. Gas chromatography (GC) is widely used to quantify fatty acids in milk through analyzing their methyl esters [70]. As for TAG analysis, liquid chromatography (LC), including high-performance LC (HPLC) and ultra-performance LC (UPLC) offers significant advantages over GC and TLC in analyzing intact individual TAG species [71].

1.2. GENETIC SELECTION OF DAIRY CATTLE

1.2.1 Genetic selection of dairy cattle in the United States

Farm animals have been artificially selected for manageability and productivity at the very beginning of domestication [72]. Contemporary practice still place heavy emphasis on production but include some other important performance traits including reproduction and health [73]. With the advent of statistical genetics, offspring with better phenotype are selected mainly based on the phenotypes of dairy cattle [74]. More importantly, the average of all genetic loci contributing to a trait as transmitted by the individual is reported as an estimated breeding value (EBV) [75]. Despite being targeted to enhance multiple traits, the genetic improvement of dairy cattle since mid-20th century was largely unbalanced with more emphasis on the production traits. As a result, although the annual milk production in the United States has increased more than 5,500 kg per cow in the past 50 years (Figure 1.4) [76], negative influences on other performances, such as energy balance, fertility, maintenance, movement, immune defense and hormones levels, have been observed [77-80]. Declined fertility indicated by daughter pregnancy rate [76]is the main adverse outcome of selection for high milk yield [81], resulted from the disruptions in estrous cycling, embryo development and

uterine conditions [78, 82]. However, this problem has been recognized and the increased emphasis on reproduction traits have reversed this trend [76].

1.2.2 University of Minnesota dairy cattle selection study

Dr. Charles Young at the University of Minnesota started a selection study of dairy cattle in 1964 to demonstrate the merit of selecting for increased milk yield [83]. His efforts resulted in a group of high-merit contemporary Holstein (CH) and a group of low-merit unselected Holstein (UH). The selection of CH and the maintaining of UH occurred at the Southern Research and Outreach Center, Waseca, Minnesota [84]. After nearly 50 year artificial selection of these unselected and contemporary cows differ by 4,500 kg per lactation. Accompanied with the increase in milk yield, CH cows have a taller, more angular shape and better undder conformation than UH cows. However, lower reproduction rate has also been observed in CH cows [84]. Growth hormone (GH) levels and milk compositions in these two lines of cows have been compared [83]. In these studies, significant differences in GH levels were found between CH and UH cow [83], but no clear differences on the composition of milk fat, protein level and lactose level were observed [53]. However, CH milk tended to contain less monounsaturated fatty acids content than UH milk, especially in early lactation [53].

1.3. METABOLOMICS AND ITS APPLICATION IN MILK RESEARCH

1.3.1 Metabolomics

As the indispensable components of any biological system, metabolites have essential functions within living organisms for wellbeing and survival. Metabolites originate from external and internal sources. External sources include nutrients and non-nutrients in the diet as well as xenobiotics from environmental exposure, while internal sources are intermediary and endogenous metabolites generated for energy and homeostasis. The collection of all these metabolites in a biological system, such as a cell, tissue, organ or organism, is the metabolome of that system. Correspondingly, metabolomics is the study of the metabolome through investigating the status of metabolites and their changes within a biological system. The idea that abnormal changes of metabolites can reflect pathophysiological states has long been known. In ancient China and Europe, doctors evaluated the sweetness (glucose level) of urine for diabetes diagnosis [85, 86]. In the past decade, metabolomics underwent rapid developments. Due to its capabilities in conducting comprehensive profiling and detecting subtle changes in a metabolic system, metabolomics has been adopted in diverse research fields and scientific disciplines. The growth in the applications of metabolomics is accompanied by enhanced knowledge on human, animal, plant, and microbial

metabolomes. For example, METLIN, a major metabolomics web database, contained 10,000 metabolites in 2005 [87]. However, in 2015, the database grew to 240,000 metabolites. The Human Metabolome Project completed the first draft of the human metabolome with approximately 2,180 metabolites in 2007, but this number has increased to 41,993 in 2016 [88-90]. Overall, metabolomics has become a powerful technical platform for biomarker discovery [91, 92], as well as the prevention and therapy of certain diseases [93-96].

1.3.2 Technical platforms of metabolomics

The procedure of metabolomic analysis can be generally divided into four main steps: sample preparation, data acquisition, statistical analysis, metabolite and pathway analysis (Figure 1.5) [97].

The quality of sample preparation is critical to the result of metabolomics analysis. It serves many purposes. The main aim is to extract metabolites and analytes from complex biological samples, such as urine, plasma, serum, blood, plant or animal tissue, cell pellets, and to make them compatible with intended method of metabolite analysis. Widely used extraction techniques include liquid–liquid extraction, solid-phase extraction, supercritical fluid extraction, microwave-assisted extraction, protein precipitation, and dialysis. Their applications in sample preparation are largely determined by chemical and physical properties of samples

[98]. Besides extraction, addition preparations are needed to enhance the compatibility of specific metabolites with analytical platforms. Chemical derivatization is an effective approach in MS-based analysis to improve separation, detectability and sensitivity in the analysis of metabolites with specific functional groups. For example, dansyl chloride is commonly used for derivatization of amino acids [99]. The detection of organic acids is attributed to the esterification of carboxyl group with amines, hydrazines, or alcohol, while detection of aldehydes and ketones is assisted by the formation of Schiff bases after derivatization reactions [100].

Data acquisition is the most important step for the quality of metabolomics analysis since this step is highly depend on the stage of current technologies. In data acquisition platforms, NMR, GC-MS, HPLC-MS are the most prevalent techniques used at present [101]. Each of these techniques has its own advantages and limitations. Nuclear magnetic resonance (NMR) is based on a physical phenomenon in which nuclei absorb and re-emit electromagnetic radiation in a magnetic field [102]. NMR is a nondestructive technique that can identify and quantify metabolites with sample preparation [103]. Since NMR is based on physical features of nuclei, it can provide unbiased information on compounds. NMR can provide not only detailed structural information of specific metabolites but also a whole view of mixed metabolites [104]. However, the major

disadvantage of NMR is its relatively low sensitivity, which limit its application on high-abundance metabolites [105].

Gas chromatography-mass spectrometry (GC-MS) is a combination of the separating feature of GC and the identifying feature of MS based on the fact that different molecular has different mass to charge ratios. Due to the advantage of high sensitivity and a wider range of covered food metabolites, GC-MS has become a key technique in nutritional metabolomics [106]. However, derivatization is required to make compounds volatile in sample preparation of GC-MS, which partly limits its applications in metabolomics [101]. One of the most widely used application of GC-MS is to detect fatty acids as methyl ester derivatives [107].

High-performance liquid chromatography-mass spectrometry (HPLC-MS) is similar to GC-MS but different in that the compounds to be detected are seperated liquid phase rather than a gas phase. HPLC-MS is a powerful technique in nutritional metabolomics, which can provide a wider range of covered metabolites. Compared to GC, HPLC separations are more suitable for nonvolatile polar or nonpolar compounds without derivatization [101]. In addition, HPLC-MS can provide a list of m/z values, retention time and relative abundances of detected but unidentified metabolites [108]. The introductions of UPLC and Q-TOF-MS results in a more rapid analysis and a more precise mass measurement without loss of resolution [101].

NMR data comprise chemical shift and signal intensity, while HPLC-MS and GC-MS data comprise retention time (RT), mass-to-charge ratio (m/z), and signal intensity [98, 109]. Different statistical methods should be selected according to the different aims of the particular study, for statistical analysis. If the aim is sample classification, unsupervised methods such as hierarchical clustering analysis (HCA), principal component analysis (PCA), or independent component analysis (ICA) can be used. If the aim is discover characteristic biomarkers, supervised methods such as principal least square (PLS) or orthogonal partial least squares (OPLS) or partial least squares-discriminant analysis (PLA-DA) can be used [110].

Subsequently, for NMR-based metabolomics, identification of high-abundance metabolites can be achieved directly by structure elucidation and configuration determination. For MS-based metabolomics, metabolites can be determined by accurate mass measurement, elemental composition analysis, MS/MS fragmentation and database search [98]. Compared to genomics and proteomics, metabolomics faces challenges in metabolite identification and pathway mapping due to its complexity and limited databases. However, a database search is still the first chose to identify unknown metabolites and map pathways. Widely used mass spectrometry based metabolomics databases includes: Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg), Human Metabolome Database (http://www.hmdb.ca), Lipid Maps (http://www.lipidmaps.org),

METLIN database (http://metlin.scripps.edu), BioCyc (http://biocyc.org), Spectral Database for organic compounds (http://sdbs.riodb.aist.go.jp) [98].

1.3.3 Application of metabolomics in milk research

Milk, as the end product of a major metabolic process, is an idea subject for metabolomics analysis since metabolites and nutrients carry its most of the important biological information and significance. Both NMR and MS-based metabolomics have been performed in milk analysis.

1.3.3.1 NMR analysis of milk metabolome

With its capability in structural elucidation, NMR analysis has been used in examining diverse milk components including caseins [111], casein micelles [112], whey protein [113], TAGs [114] and phosphorylated compounds [115]. For instance, 25 metabolites were identified by the NMR analysis when comparing cow milk collected at early and late stages of lactation [103]. A similar approach was also adopted for identifying the biomarkers corresponding to the cows in different metabolic status [116]. In these two studies, BHBA, phosphocholine, and glycerophosphocholine, especially the ratio between phosphocholine and

glycerophosphocholine, were identified as reliable prognostic biomarker for risk of ketosis [103, 116].

Compared to many analytical techniques, NMR is less selective in untargeted analysis and therefore allow comprehensive detection of many compounds in a single run [117]. This feature of NMR has led to its applications in milk authentication [118, 119], traceability of geographical origin [120, 121], and quality control of milk [122-125].

1.3.3.2 MS analysis of milk metabolome

GC and LC are two commonly used separation methods in MS-based metabolomics. The higher sensitivity of MS-based metabolomics allows the detection of more metabolites than NMR-based metabolomics in milk analysis. For example, 223 metabolites including amino acid, short peptides, lipids, carbohydrate, nucleotides, vitamins and intermediary metabolites were identified in cow milk by GC-MS and LC-MS-based metabolomics analysis [126]. Multivariate analysis based on these detected metabolites further revealed a clear separation of different milk varieties [126].

Compared to GC-MS, LC-MS has a broader coverage of metabolites, and has been widely applied to the characterization and quantitation of carbohydrates, proteins, fat and other milk metabolites. The capabilities of LC-MS platform have been

demonstrated in the analysis of carbohydrates including monosaccharides [127], disaccharides [128], and oligosaccharides [129] in milk; the analysis of major milk proteins like κ-casein, αS1-casein, β-casein, β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin [130, 131]; and the analysis of milk lipids, including TAGs [132] and phospholipids [133]. Moreover, LC-MS is also capable of analyzing amino acids [134], fatty acids, ketones, aldehydes, carboxylic acids [100] and low molecular weight metabolites [135, 136]. Therefore, more applications of LC-MS analysis are expected in future milk studies.

Table 1.1 Nutrient composition of milk. The table is modified based on Website Milk Facts (http://www.milkfacts.info/Milk%20Composition).

Nutrients	Compositions
Carbohydrate	Monosaccharides: glucose, galactose; Disaccharides: Lactose (major); Oligosaccharides.
Protein	Caseins (major): αS1-casein, αS2-casein, ß-casein and κ-casein; Whey proteins: β-lactoglobulin, α-lactalbumin, albumin, immunoglobulin, lactoferrin, transferrin.
Lipid	TAGs (major), diacylglycerols, monoacylglycerols, phospholipids, cholesterols, glycolipids, free fatty acids.
Vitamins	Fat soluble vitamins: Vitamin A, Vitamin D, Vitamin E, Vitamin K. Water soluble vitamins: vitamins B1 (thiamin), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B12 (cobalamin), vitamin C, folate.
Minerals	Calcium (major), phosphorous (major), potassium (major), sodium, magnesium, chlorine, sulfur, selenium, copper, iron, cobalt, zinc, manganese.

Figure 1.1 Sources of fatty acids for TAG synthesis in mammary epithelial cells. Milk TAGs are synthesized in the mammary epithelial cells. Fatty acids in milk TAGs mainly come from two sources, i.e. blood lipids and de novo synthesis. Blood lipids from the VLDL synthesized in the liver and the chylomicrons synthesized in intestine account for about 40 to 60% of the fatty acids in milk TAGs. TAGs in the VLDL are hydrolyzed by LPL prior to the transport into mammary epithelial cells. De novo synthesis of fatty acids occurs in the cytoplasm of the mammary epithelial cell. In ruminants, the carbon sources used for FA synthesis are acetate and BHBA.

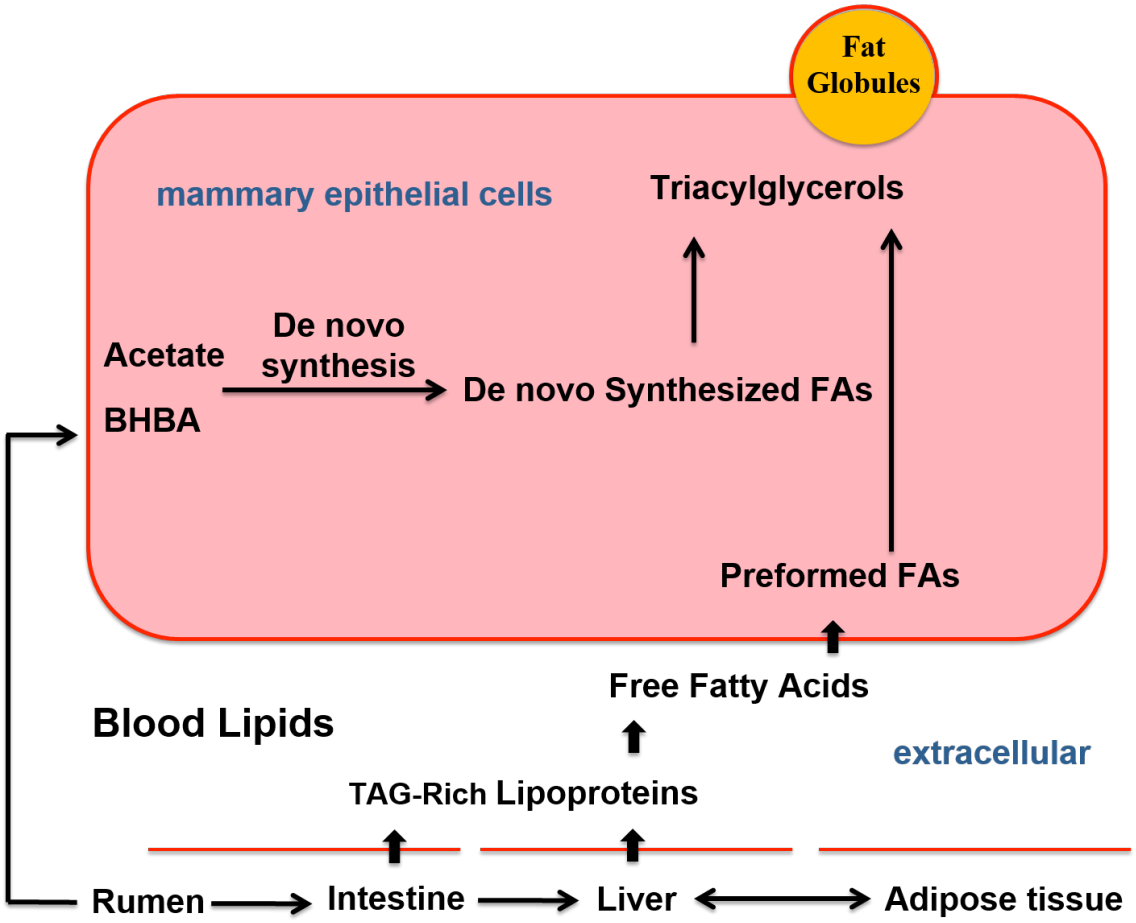

Fat Globules
mammary epithelial cells
Triacylglycerols
Acetate
BHBA
De novo synthesis
De novo Synthesized FAs
Preformed FAs
Free Fatty Acids
Blood Lipids
extracellular
TAG-Rich Lipoproteins
Rumen
Intestine
Liver
Adipose tissue

Figure 1.2 De novo synthesis of fatty acids in mammary epithelial cells. Acetate and BHBA from rumen fermentation are the starting metabolites for the de novo synthesis of fatty acids. In mammary epithelial cells, acetate is transformed to acetyl-CoA and malonyl-CoA before being utilized for FA synthesis, whilst BHBA enters the cycle directly. Fatty acid synthetase is responsible for the condensation cycles of acetyl-CoA or butyryl-CoA. The reducing agent used in FA synthesis pathway is NADPH. This figure is modified based on Website Milk Composition and Synthesis Resource Library (http://ansci.illinois.edu/static/ansc438/Milkcompsynth).

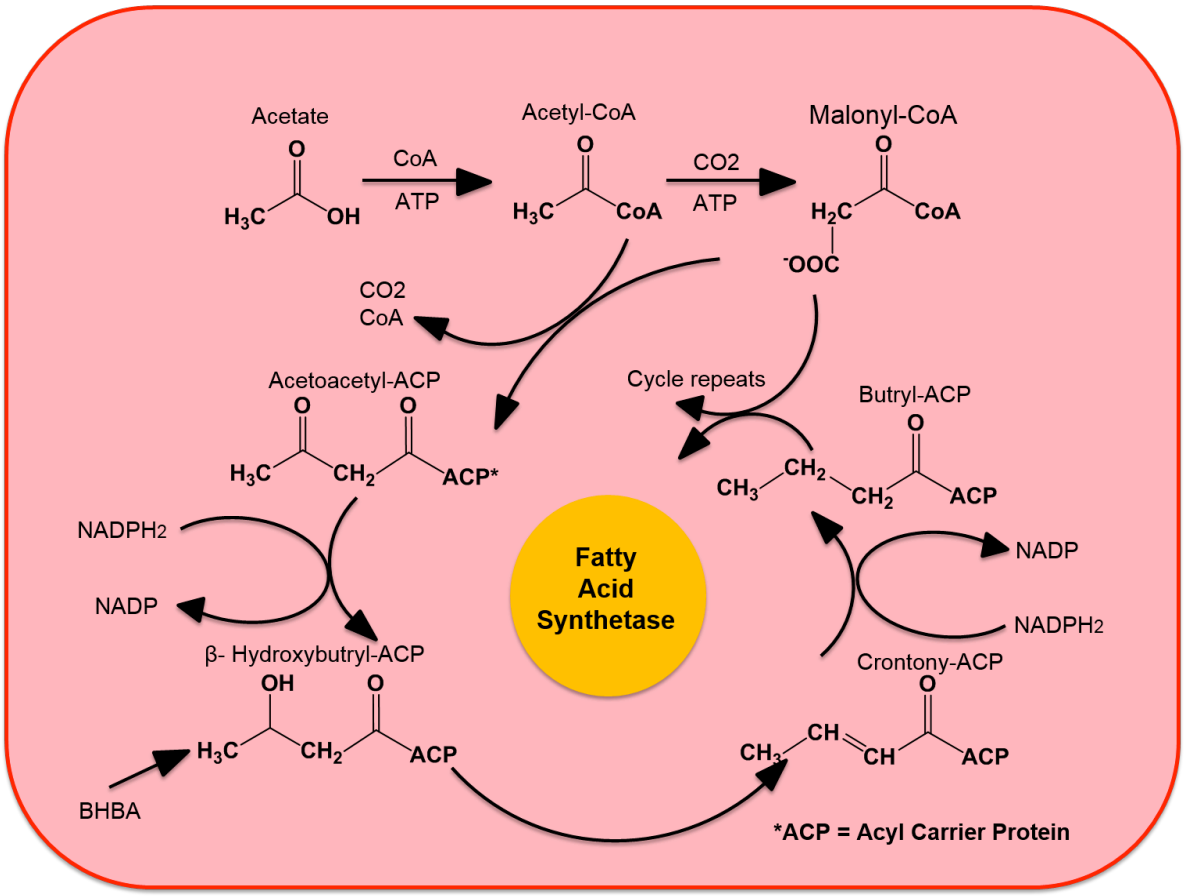


Figure 1.3 Milk fat globules structure. Milk fat globule membrane structure: large amount of TAGs and a phospholipid trilayer with embedding proteins.

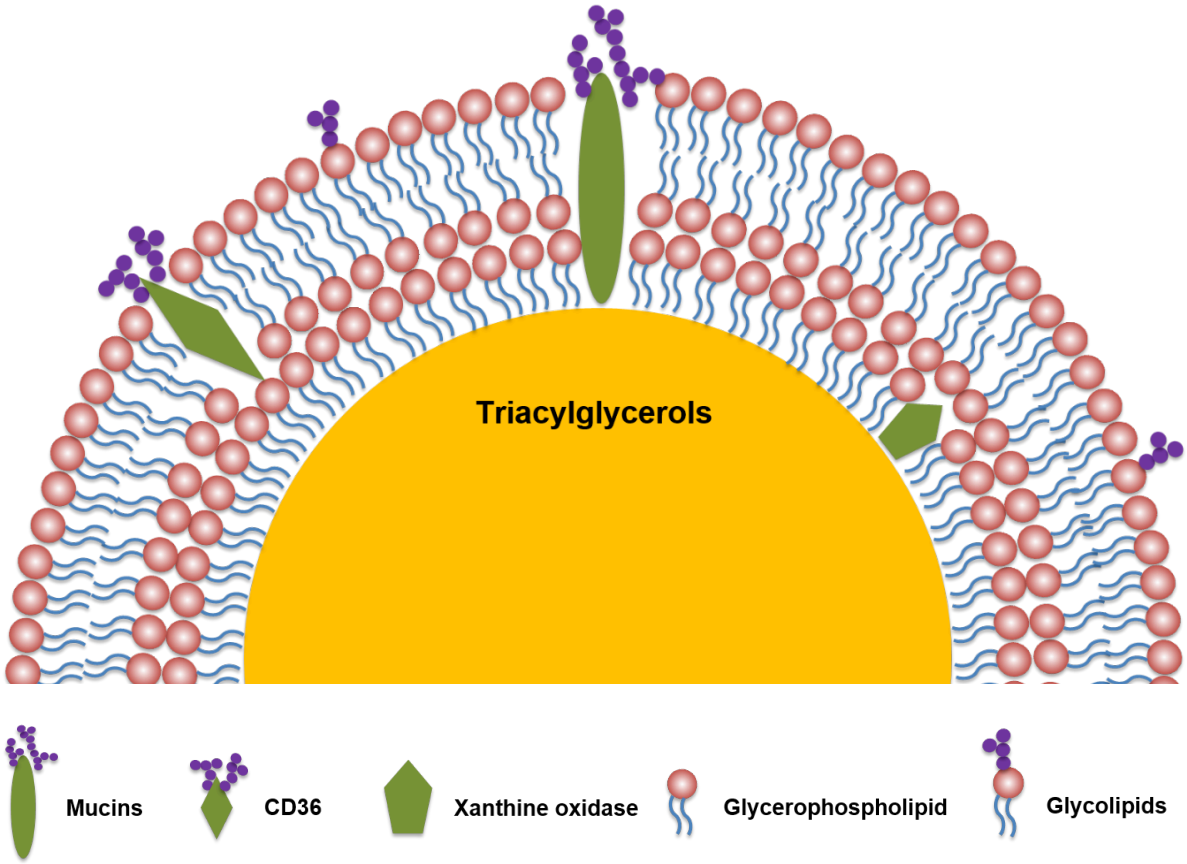


Figure 1.4 Average annual milk yield in the United States (US) from 1950 to 2015 (in kilograms per cow). The figure is plotted based on the data from the USDA-NASS (https://www.nass.usda.gov).

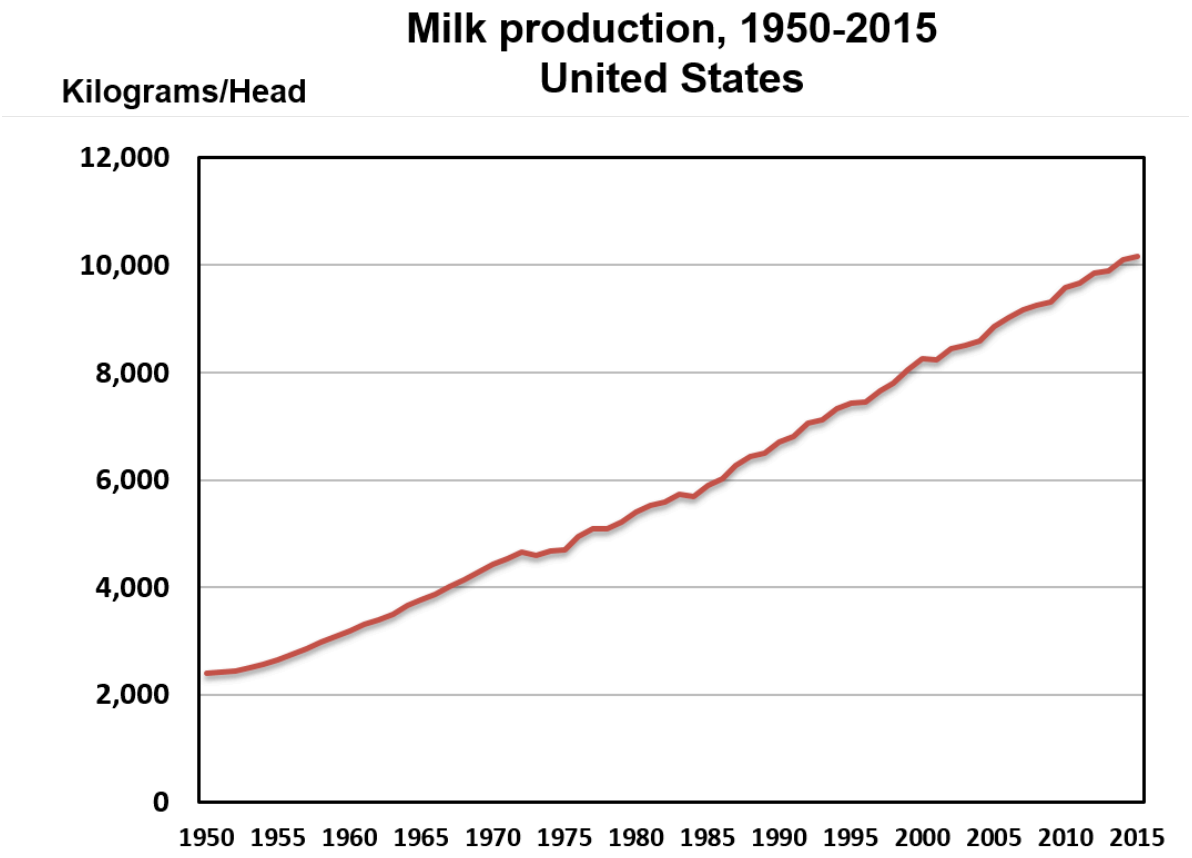
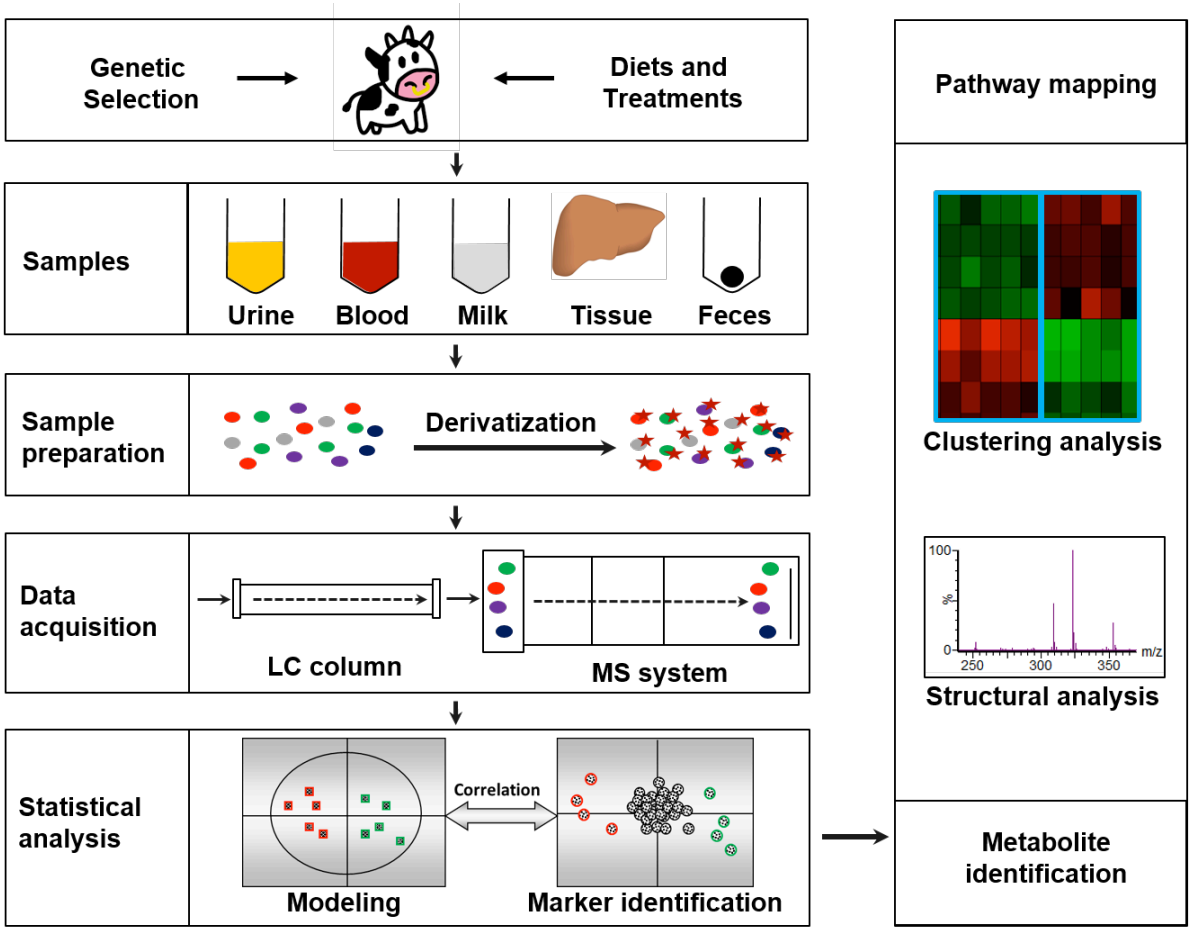


Figure 1.5 General workflow of a LC-MS based metabolomics study. Four main steps of LC-MS-based metabolomic analysis are sample preparation, data acquisition, statistical analysis, metabolite identification and pathway mapping.



Chapter 2

Lipidomic analysis revealed increased contribution of performed fatty acids to milk fat synthesis as a metabolic consequence of genetic selection in early lactation of multiparous Holstein cows

[2]**KEYWORDS:** triacylglycerol, fatty acids, lipidomics, milk

ABBREVIATIONS: ACN, acetonitrile; **ALA**, alpha linolenic acid; **BHBA**, β-hydroxybutyrate; **CH**, contemporary Holstein; **DHA**, docosahexaenoic acid; **DPDS**, 2,2'-dipyridyl disulfide; **EPA**, eicosapentaenoic acid; **FAs**, fatty acids; **GLA**, gamma linolenic acid; **HCA**, hierarchical clustering analysis; **HMDB**, Human Metabolome Database; **HQ**, 2-hydrazinoquinoline; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **LA**, linoleic acid; **LC-MS**, liquid chromatography-mass spectrometer; **OA**, organic acid; **OPLS-DA**, orthogonal partial least squares-discriminant analysis; **PCA**, principal components analysis; **PTA**, predicted transmitting ability; **QTOFMS**, quadrupole time-of-flight mass spectrometer; **RT**, retention time; **SD**, standard deviation; **SIC**, single ion counts; **TAGs**, triacylglycerols; **TIC**, total ion counts; **TPP**, triphenylphosphine; **UH**, unselected Holstein; **UPLC**, ultra-performance liquid chromatography; **VLDL**, very low density lipoproteins.

2.1 INTRODUCTION

Most US dairy cattle in the United States have been genetically selected for higher milk yield in the last past half century, resulting in the increase of over 5,500 kg of milk annually for a US Holstein cow [76]. However, this dramatic increase in milk yield is associated with other undesirable consequences, including the decreased reproductive efficiency and the increased incidence of mastitis and other metabolic discorders [77]. The University of Minnesota initiated a genetic selection project of dairy cattle in 1964 [83]. In this project, female descendants of the original foundation cows were mated each year to the bulls with highest predicted transmitting ability (PTA) for milk. After nearly 40 years of artificial selection, a line of high-merit contemporary Holstein (CH) was established and is still under continuous selection for higher milk production. At the same time, the original line of low-merit unselected Holstein (UH) was maintained as the control. The difference between these two lines on annual milk yield is over 4,500 kg [83]. Besides the increase in milk yield, CH cows have larger body size and better udder conformation than UH cows [83].

In cow milk, lipids are the most abundant and energy-intensive nutrients in cow milk. Milk lipids are dominated by triacylglycerols (TAGs). Fatty acids used in milk TAG synthesis are mainly from two sources. Fatty acids containing less than 16 carbons are mainly originated from de novo synthesis in mammary epithelial cells while fatty acids containing more than 16 carbons are performed fatty acids

from serum lipids. In addition, both sources contributed more equally to the level of palmitic acid in milk [36]. De novo synthesis of fatty acids occurs in the cytoplasma of mammary epithelial cells, utilizing acetate and β-hydroxybutyrate (BHBA) from the rumen and the liver as the carbon sources. Performed fatty acids from blood lipids are mainly derived from chylomicrons and very low density lipoproteins (VLDL) synthesized in the intestine and the liver, respectively [38].

Cows at the early phase of lactation are under dramatic physiological changes caused by calving, hormonal signaling, and energy imbalance [137]. Significant adjustments in the metabolic system are required to accommodate the need for milk production and the challenge of reduced feed intake, which place the cows in negative energy balance. Considering that different milk yield is a consequence of different metabolic processes, it is likely that the compositional differences also exist between the milks from UH and CH cows. Therefore, analyzing the compositions of milk might provide useful insights on the metabolism. In this study, comprehensive metabolomic analysis covering lipids and organic acids were conducted to determine the correlations between milk yield phenotypes and specific milk metabolites alter the compositions of FAs in TAGs and TAGs in cow milk fat during early lactation.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

LC-MS-grade water and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Houston, TX); 2-hydrazinoquinoline (HQ) and triphenylphosphine (TPP) from Alfa Aesar (Ward Hill, MA); 2,2'-dipyridyl disulfide (DPDS) from MP Biomedicals (Santa Ana, CA); dansyl chloride (DC) and n-butanol from Sigma-Aldrich (St. Louis, MO); fatty acid standards from Nu-Chek-Prep (Elysian, MN); palmitic acid-1,2-$^{13}C_2$ from CDN Isotopes (Pointe-Claire, QC). The other metabolite standards used for structural confirmation were from Sigma-Aldrich, Thermo Fisher Scientific, Alfa Aesar, Ark Pharm (Libertyville, IL), Frontier Scientific (Logan, UT), and Steraloids (Newport, RI), respectively.

2.2.2 Animals and sample collection

Multiparous UH and CH cows (n = 12/genotype) were fed the same diet ad libitum and housed together prepartum for at least 5 weeks. Cows were milked at approximately 12-h intervals. Milk aliquots from every Tuesday night milking during the first 9 weeks of lactation were collected and stored at -80°C prior to further metabolite analysis. Additional aliquots were preserved with dichromate and submitted to Minnesota DHIA (Zumbrota, MN) for determination of milk protein, lactose, and fat contents were analyzed by infrared spectroscopy.

2.2.3 LC-MS analysis of milk lipids

To solubilize the neutral lipids in whole milk, 5 μL of milk was mixed with 245 μL of n-butanol. After vortexing, the mixture was centrifuged at 18,000×g for 10 min to remove proteins and precipitates. The supernatant was transferred to a HPLC vial for LC-MS analysis. For LC-MS analysis, 5 μL of aliquot of the supernatant was injected in to an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) and components separated by a BEH C18 column (Waters) using the a mobile phase gradient, A: H_2O:ACN=60:40 (v/v) containing 0.1% formic acid (v/v) and 10 mmol/L ammonium acetate and B: IPA:H_2O=90:10 (v/v) containing 0.1% formic acid (v/v) and 10mmol/L ammonium acetate. LC eluant was introduced into a Xevo-G2-S quadrupole time-of-flight mass spectrometer (QTOFMS, Waters) for accurate mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization were set at 3 kV and 30 V for positive-mode detection, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as the cone gas (50 L/h) and the desolvation gas (600 L/h) and argon as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution with mass-to-charge ratio (*m/z*) of 50–1000 and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M + H]^+$ = *m/z* 556.2771 and ($[M - H]^-$ = *m/z* 554.2615) in real time. Mass chromatograms and mass spectral

data were acquired and processed by MassLynx^{TM} software (Waters) in centroided format. Additional structural information was obtained tandem MS (MSMS) fragmentation with collision energies ranging from 15 to 40 eV.

2.2.4 LC-MS analysis of milk fatty acids

Milk lipids were hydrolyzed to release free fatty acid using a modified alkaline hydrolysis method [138]. Briefly, 5 μL of milk sample was mixed with 200 μL of methanol containing 200 μM deuterated palmitic acid as the internal standard, and then hydrolyzed by adding 35 μL of 40% potassium hydroxide (w/v). The mixture was incubated at 60 °C for 30 min and then neutralized by 60 μL of 2.5M HCl and 200 μL of phosphate buffer (75mM, pH=7). After a 10-min centrifugation at 18,000×g, the supernatant containing free fatty acids was collected and further derivatized for LC-MS analysis. To derivatize free fatty acids, 2 μL of sample or fatty acid standard was mixed with 100 μL of freshly prepared master reaction mix containing 1mmol/L DPDS, 1mmol/L TPP, and 1mmol/L HQ in ACN solution. The mixture was incubated at 60 °C for 30 min and then chilled on ice. The reaction was terminated by mixing with 100 μL of H_2O. After a 10-min centrifugation at 18,000×g, the supernatant was transferred into a HPLC vial. For LC-MS analysis, 5 μL of HQ-derivatized sample was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA)

and separated by a BEH C18 column (Waters) using a gradient ranging from water to 95% aqueous ACN containing 0.1% formic acid over a 10-min run.

2.2.5 LC-MS analysis of milk organic acids

Milk samples were derivatized by HQ, without hydrolysis, for the LC-MS analysis of organic acids [138]. The derivatization procedure and the MS parameters are the same as the ones used for the LC-MS analysis of free fatty acids, except the mobile phase, which contains A: H_2O containing 0.05% acetic acid (v/v) and 2 mM ammonium acetate and B: H_2O:ACN=5:95 (v/v) containing 0.05% acetic acid (v/v) and 2 mM ammonium acetate.

2.2.6 Marker identification through multivariate data analysis

Chromatographic and spectral data from LC-MS analysis of milk samples were deconvoluted by MarkerLynx^{TM} software (Waters). A multivariate data matrix containing information on sample identity, ion identity [retention time (RT) and *m/z*], and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single ion counts (SIC) versus the total ion counts (TIC) in the whole chromatogram. The processed data matrix was further exported

into SIMCA-P+^{TM} software (Umetrics, Kinnelon, NJ), transformed by *Pareto* scaling and then analyzed by principal components analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). Major latent variables in the data matrix were described in a scores scatter plot of defined multivariate model. Potential metabolite markers of genetic selection for high milk yield were identified by analyzing ions contributing to the separation of sample groups in an S-loadings plot of OPLS-DA model. After *Z* score transformation, the concentrations or relative abundances of identified metabolite markers in examined samples were presented in the heat maps generated by the R program (http://www.R-project.org), and correlations among these metabolite markers were defined by hierarchical clustering analysis (HCA).

2.2.7 Marker characterization and quantification

The chemical identities of metabolite markers were determined by accurate mass measurement, elemental composition analysis, database search, MSMS fragmentation, and comparisons with authentic standards if available. Database searches were performed using Human Metabolome Database (HMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Lipid Maps databases. Individual metabolite concentrations were determined by calculating the ratio

between the peak area of metabolite and the peak area of internal standard and fitting with a standard curve using QuanLynx^{TM} software (Waters).

2.2.8 Statistics

Effects of genetic selection, lactation week and their interaction on milk yield and lipidomic profiles were analyzed by repeated measures using PRO MIXED procedure of SAS with lactation week as the repeated effect. Effects of genetic selection at specific time point of lactation was analyzed by two-tailed Student t-tests for unpaired data. Experimental values are expressed as mean ± standard deviation (SD), with $P < 0.05$ considered statistically significant.

2.3 RESULTS

2.3.1 Milk yield and its macronutrient contents

Dairy cows undergo significant physiological challenges in the early phase of lactation due to decreased dry matter intake and the need to use body reserves as substrates for milk production. To determine the effects of genotype for low and high milk yield during this period, the yields and macronutrient contents of CH and UH milk were monitored in the first 9 weeks of lactation. Both CH and UH cows had relatively stable milk production in these 9 weeks (Figure 2.1A). As

expected, CH cows produced much more milk than UH cows (20.87 vs. 12.53 kg/12h, $P<0.01$). Among macronutrient contents in milk, CH milk had comparable levels of lactose (4.75% vs. 4.69%, $P=0.39$) as UH milk (Figure 2.1B), but CH milk contained less protein (2.98% vs. 3.13%, $P<0.05$) and more fat (4.33% vs 3.55%, $P<0.01$) than UH milk (Figure 2.1C-D). The significantly different fat content of UH and CH milk indicates genetic selection has altered the lipid metabolism in Holstein cows during early lactation.

2.3.2 Lipidomic analysis of intact lipids in UH and CH milk

The composition of UH and CH milk lipids were compared by the LC-MS-based lipidomic analysis. The detection of neutral lipids, especially TAGs, was facilitated by using ammonium acetate and isopropanol in the mobile phase for forming ammonium adducts and stronger washing, respectively. Examination of 2D plots of LC-MS data indicated that these milk samples were dominated by hundreds of different TAG species with great variance in abundance (Figure 2.2A). As shown by the MSMS spectrum of TAG (4:0, 16:0, 18:1), the fatty acid composition of individual TAG can be resolved by analyzing the product ions from MSMS fragmentation (Figure 2.2B). The influences of genotypes on the compositions of milk lipids were defined by a PCA model, in which the kinetic profiles of UH and CH milk lipids are distinguishable from each other (Figure

2.3A). One prominent feature revealed by the distribution of CH and UH samples in the models is that the differences between UH and CH in the first three weeks of lactation were much greater than the differences at the end of 9-week sample collection (Figure 2.3A). In addition, milk from CH cows had more dramatic changes in milk composition than milk from UH cows in the first 9 weeks of lactation (Figure 2.3A). The top 40 markers contributing to the separation of milk from CH and UH cows were identified in the loadings S-plot of an OPLS-DA model (Figure 2.3B), and their chemical identities were defined by MSMS fragmentation and database search (Table 2.1). Among them, 27 TAGs were present at greater concentration in milk from CH cows and 13 TAGs were greater in milk from UH cows (Figure 2.3C). More importantly, all TAG markers that were present at greater concentrations in CH milk contained at least two fatty acids with aliphatic tails of no less than 16 carbons, while all TAG markers in UH milk contained at least two fatty acids with aliphatic tails of no more than 16 carbons (Figure 2.3C). In the 9^{th} week of lactation, the amounts of the majority of these markers became more comparable in their levels, which is consistent with the results from the PCA model (Figure 2.3A and C). Overall, there were time-dependent changes in lipid profiles in milk from both UH and CH milk during the early lactation, but the composition of UH milk was relatively more stable than that of CH milk.

2.3.3 Analysis of fatty acids in UH and CH milk

To confirm the different distributions of fatty acids in milk TAGs from UH and CH cows, fatty acid compositions of CH and UH milk were determined by the LC-MS analysis of hydrolyzed milk samples. Fatty acids in hydrolyzed milk were derivatized by 2-hydrazinoquinoline (HQ) to form carbohydrazines to improve chromatographic separation and spectroscopic detection of fatty acids, as demonstrated by the MSMS spectrum of myristic acid (Figure 2.4A). Using this method, more than 20 fatty acids with aliphatic chains ranging from 4 to 24 carbons, together with other metabolites, can be effectively detected and quantified (Figure 2.4B and Table 2.2). The PCA model revealed time-dependent changes of fatty acid composition in both UH and CH milk (Figure 2.5A). It appeared that during the first two weeks of lactation, dramatic changes occurred in the FA content of CH milk, but not in UH milk (Figure 2.5A). Analyzing the metabolites contributing to the differences between CH and UH in the loadings S-plot of OPLS-DA model revealed the positive correlations between CH milk and the fatty acids with aliphatic tails of no less than 16 carbons (Figure 2.5B).

The total fatty acid concentration in CH milk was consistently greater than that in UH milk during the first 6 weeks of lactation (Figure 2.6A), which is consistent with difference in fat concentrations in these milk samples (Figure 2.1D). Greater concentrations of fatty acids containing no less 16 carbons in CH milk were observed in CH milk during 9 weeks of lactation (Figure 2.6H-L), while the

concentrations of fatty acids containing 4-14 carbons were largely comparable between CH and UH milk (Figure 2.6B-G). In contrast, the percent contribution of these de novo FAs were or tended to be greater in milk from UH cows (Figure 2.6B-G, Table 2.3) while percent contribution of the preformed FAs were or tended to be greater in milk from CH cows (Figure 2.6H-L, Table 2.3). Calculated ratios between the fatty acids containing more than 16 carbons and the fatty acids containing less than 16 carbons further indicated that UH milk had relatively stable fatty acid composition during the first 9 weeks of lactation while CH milk underwent more dramatic changes due to continuous decease in the percentage of fatty acids containing more than 16 carbons (Figure 2.6M, Table 2.4). Moreover, the concentrations of unsaturated fatty acids in CH milk were higher than that in UH milk (reduced SFA/UFA ratio), indicating a higher level of desaturase activity in CH cows (Figure 2.6N, Table 2.4).

2.3.4 Analysis of organic acids in UH and CH milk

Mammary epithelial cells utilized organic acids, especially acetic acid and 3-hydroxybutyric acid for de novo synthesis of fatty acids [38]. Because there were differences in amounts of de novo synthesized fatty acids in UH and CH milk, the organic acid profiles of these milk samples were analyzed by the LC-MS analysis of HQ-derivatized milk samples. The distribution of milk samples in the scores

plot of PCA model revealed clear differences between CH and UH milk in their organic acid profiles, which was represented by more dramatic changes in CH than UH, especially in the first two weeks of lactation (Figure 2.7A). The markers contributing to the differences between CH and UH were identified in the loading S-plot of an OPLS-DA model (Figure 2.7B). Among these organic acid markers, butyric acid, propionic acid, 3-hydroxybutyric acid and 2-ketoglutaric acid were positively associated with CH milk, while carnitine, acetylcarnitine, propionylcarnitine, butyrylcarnitine, valerylcarnitine, pyroglutamic acid and lactic acid were positively associated with UH milk (Figure 2.7B). Quantifying the concentrations of individual organic acids in milk indicated that the levels of carnitine and short-chain acylcarnitines in UH milk were persistently higher than that in CH milk, but the associations between genotypes and the organic acids, such as butyric acid, 3-hydroxybutyric acid, lactic acid, and pyroglutamate, were partially attributed to the presence of a few of outlier samples (Figure 2.8A-F).

To examine the associations among TAG, FA and organic acid markers, a HCA-based heatmap was constructed (Figure 2.9). Metabolites associated with de novo synthesized fatty acids and metabolites associated with preformed fatty acids differed greatly between CH and UH, indicating the significant effects of genetic selection on milk composition and the biosynthesis of milk fat-related metabolites.

2.4 DISCUSSION

Genetic selection for greater milk yield is expected to alter the metabolism of dairy cattle since higher levels of metabolite synthesis and secretion are required to achieve these increased yields. The increase of metabolite production in CH cows could be due to the increased capacity of whole metabolic system, but it could also be affected by specific components of metabolic system. Lipidomic comparisons of TAGs and fatty acids in UH and CH milk collected during the first 9 weeks of lactation indicate the use of performed fatty acids to synthesize milk TAG synthesis is a major contributor to the higher levels of TAGs in CH milk, especially in the early weeks of lactation (Figure 2.1D and 2.6H-L). Blood lipids that contribute to the performed fatty acids in milk lipids originate from lipogenesis in the liver and intestine, lipolysis in the adipose tissue, and fermentation in the rumen. Even though the metabolic profiles of serum and tissue samples were not examined in this study, the presence of higher levels of liver- and rumen-derived propionic acid, butyric acid, and 3-hydroxybutyric acid in CH milk implicated dramatic metabolic changes in CH cows.

The metabolic status of UH and CH cows, including milk composition and growth hormone level, have been examined in previous studies [53, 83]. Significant differences have been observed in growth hormone but not milk composition between these two lines of cows in these studies [53, 83]. The weekly sample collection and the selection of LC-MS-based metabolomics platform likely

contribute to the observation of significant metabolic differences in this study. For example, the kinetic profiling and metabolite analysis in this study indicated that, despite the differences in the earlier weeks of lactation, the TAG and fatty acid compositions of UH and CH milk became comparable at the end of 9-week sample collection. Since FA analysis in a previous study was conducted during week 1, 8, and 16 of lactation, the metabolic changes occurred during early weeks of lactation were not examined [53]. In addition, compared to traditional targeted fatty acid analysis using GC-based techniques, the approach used in this study provided a more comprehensive analysis of lipids, TAGs and FAs that generated novel information about temporal changes in lipidomics due to selection-induced alterations in metabolism in the Holstein in early lactation. Even though the molecular mechanisms of observed differences between UH and CH milk were not examined in this study, new phenotypic information on the sources of fatty acids in milk TAG synthesis provides a foundation for further investigation on genetic selection on lipid metabolism.

Milk is an important source of energy and nutrients for human and calves. Genetic selection-induced changes in fatty acids and other associated metabolites in milk alter the nutritional profile and values of cow milk. For example, the concentrations of de novo synthesized fatty acids were comparable between UH and CH milk (Figure 2.6B-G). However, the relative abundances of these de novo synthesized fatty acids in CH milk were lower than those in UH milk due to higher

levels of total fatty acids in CH milk (Figure 2.6B-G). Considering that medium-chain fatty acids, the main products of de novo fatty acid synthesis, have diverse bioactivities that differs from preformed long-chain fatty acids in milk, such as their effects on fatty acid oxidation and food intake [139, 140], it is possible that the consumption of UH and CH milk might lead to subtle metabolic differences in humans and calves. In addition, the differences in omega-3 fatty acids percentages of UH and CH milk might be another metabolic consequence of genetic selection. The relative abundances of EPA and DPA were higher in UH cows (Table 2.3) while α-linolenic acid (ALA), the precursor of EPA and DPA, were higher in CH milk [141]. The increase of ALA and the decrease of EPA and DPA in CH milk, imply that the conversion efficiency of ALA to EPA and DPA might be reduced by genetic selection which would alter the nutritional values of UH and CH milk.

2.5 CONCLUSION

Genetic selection for greater milk yield has significant effects on composition and biosynthesis of milk fat from Holstein cows in early lactation. The compositional change of fatty acids in milk fat was mainly due to an increased incorporation of preformed fatty acids in milk from CH cows.

Table 2.1 TAG markers identified by the LC-MS-based lipidomic analysis of UH and CH milk samples.

ID	$[M+NH4]^+$	$[M+H]^+$	Structure
T1	544.4554	527.4310	TAG(4:0 ,8:0 ,16:0)
T2	572.4897	555.4617	TAG(4:0 ,10:0,16:0)
T3	600.5204	583.4922	TAG(4:0 ,12:0,16:0)
T4	614.5369	597.5071	TAG(4:0 ,14:0,15:0)
T5	628.5532	611.5221	TAG(4:0 ,14:0,16:0)
T6	642.5656	625.5376	TAG(4:0 ,15:0,16:0)
T7	654.5687	637.5369	TAG(4:0 ,14:0,18:1)
T8	656.5828	639.5530	TAG(6:0 ,14:0,16:0)
T9	680.5827	663.5515	TAG(4:0 ,16:0,18:2)
T10	682.5979	665.5685	TAG(4:0 ,16:0,18:1)
T11	694.5989	677.5665	TAG(4:0 ,17:1,18:1)
T12	696.6154	679.5827	TAG(4:0 ,17:0,18:1)
T13	706.5997	689.5660	TAG(4:0 ,18:1,18:2)
T14	708.6165	691.5818	TAG(4:0 ,18:1,18:1)
T15	710.6267	693.5989	TAG(6:0 ,16:0,18:1)
T16	736.6479	719.6120	TAG(6:0 ,18:1,18:1)
T17	740.6809	723.6447	TAG(10:0,16:0,16:0)
T18	792.7117	775.6729	TAG(10:0,18:1,18:1)
T19	796.7385	779.7054	TAG(14:0,16:0,16:0)
T20	820.7418	803.7042	TAG(14:0,16:1,18:1)
T21	822.7551	805.7212	TAG(14:0,16:0,18:1)
T22	824.7753	807.7383	TAG(14:0,16:0,18:0)
T23	846.72	829.7185	TAG(14:0,18:1,18:2)
T24	848.7706	831.7360	TAG(16:0,16:1,18:1)
T25	850.7887	833.7522	TAG(16:0,16:0,18:1)
T26	852.8022	835.7683	TAG(16:0,16:0,18:0)
T27	862.7823	845.7492	TAG(16:0,17:1,18:1)
T28	864.8052	847.7666	TAG(16:0,17:0,18:1)
T29	872.7696	855.7325	TAG(16:0,18:2,18:2)
T30	874.7888	857.7483	TAG(16:0,18:1,18:2)
T31	876.8065	859.7651	TAG(16:0,18:1,18:1)
T32	878.8219	861.7806	TAG(16:0,18:0,18:1)
T33	888.8087	871.7639	TAG(17:1,18:1,18:1)
T34	890.8115	873.7785	TAG(17:0,18:1,18:1)
T35	892.8389	875.7952	TAG(17:0,18:0,18:1)
T36	898.7876	881.7465	TAG(18:1,18:2,18:2)
T37	900.8056	883.7619	TAG(18:1,18:1,18:2)
T38	902.8163	885.7760	TAG(18:1,18:1,18:1)
T39	904.8294	887.7950	TAG(18:0,18:1,18:1)
T40	906.8386	889.8110	TAG(18:0,18:0,18:1)

Table 2.2 Identities and masses of milk FAs and their HQ derivatives.

ID	Name	Neutral mass of FAs [M]	Exact mass of FA-HQ derivatives $[M+HQ]^+$
F1	C4:0	88.0524	230.1293
F2	C6:0	116.0837	258.1606
F3	C8:0	144.1150	286.1919
F4	C10:0	172.1463	314.2232
F5	C12:0	200.1776	342.2545
F6	C12:1	198.1620	340.2389
F7	C14:0	228.2089	370.2858
F8	C14:1	226.1933	368.2702
F9	C15:0	242.2246	384.3015
F10	C15:1	240.2089	382.2858
F11	C16:0	256.2402	398.3171
F12	C16:1	254.2246	396.3015
F13	C17:0	270.2559	412.3328
F14	C17:1	268.2402	410.3171
F15	C18:0	284.2715	426.3484
F16	C18:1	282.2559	424.3328
F17	C18:2	280.2402	422.3171
F18	C18:3	278.2246	420.3015
F19	C19:0	298.2872	440.3641
F20	C20:0	312.3028	454.3797
F21	C20:1	310.2872	452.3641
F22	C20:2	308.2715	450.3484
F23	C20:3	306.2559	448.3328
F24	C20:4	304.2402	446.3171
F25	C20:5	302.2246	444.3015
F26	C22:0	340.3341	482.4110
F27	C22:1	338.3185	480.3954
F28	C22:2	336.3028	478.3797
F29	C22:3	334.2872	476.3641
F30	C22:4	332.2715	474.3484
F31	C22:5	330.2559	472.3328
F32	C22:6	328.2402	470.3171
F33	C24:0	368.3654	510.4423
F34	C24:1	366.3498	508.4267

Table 2.3 Effect of genetic selection on fatty acid composition in the first 9 weeks of lactation. More than 22 FAs (C4:0, C6:0, C8:0, C10:0, C12:0, C12:1, C14:0, C14:1, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2 (Linoleic acid, LA), C18:2 (Conjugated linoleic acid, CLA), C18:3 (Alpha linolenic acid, ALA), C18:3 (Gamma linolenic acid, GLA), C20:5, C22:5, C22:6) were quantitated. Others refer to the sums of C20:0, C20:1, C20:4, C22:4 FAs. Data are the means of CH and UH (n=108) samples in the first 9 weeks lactation. Statistical significance was calculated by repeated measures ANOVA.

FA	CH (%) week1-9	UH (%) week1-9
C4:0	5.40	6.05*
C6:0	1.30	1.55
C8:0	0.83	1.03**
C10:0	1.86	2.37**
C12:0	1.99	2.63**
C12:1	0.07	0.09**
C14:0	8.48	9.93**
C14:1	0.71	0.79**
C15:0	1.10	1.43**
C16:0	26.52	27.00**
C16:1	2.52**	1.97
C17:0	0.53*	0.50
C17:1	0.55**	0.49
C18:0	9.94**	7.71
C18:1	33.94**	32.18
LA	2.48**	2.21
CLA	1.06	1.12
ALA	0.23**	0.22
GLA	5.40E-02	5.82E-02
C20:5	2.21E-02	3.50E-02**
C22:5	5.14E-02	1.02E-01**
C22:6	2.97E-03	1.03E-02
Others	0.46	0.49

(** P<0.01; *P<0.05.)

Table 2.4 Effect of genetic selection on fatty acid saturation and origin in the first 9 weeks of lactation. Data are means of all CH and UH weekly samples (n=108) collected during first 9 weeks of lactation. Statistical significance was calculated by repeated measures ANOVA.

	CH	UH	P value
FA orgin and saturation			
SFA	**58.0%**	**60.3%**	**<0.01**
UFA	**37.8%**	**35.7%**	**<0.01**
PUFA	**4.1%**	**4.0%**	**<0.01**
De novo FA	**21.7%**	**25.9%**	**<0.01**
16:0 + 16:1	**29.0%**	**29.0%**	**0.022**
Preformed FA	**48.7%**	**44.7%**	**<0.01**

Figure 2.1 Milk yield and macronutrient contents. A. Milk yield of CH and UH in the first 9 week of lactation; **B.** Percentage of total milk lactose content; **C.** Percentage of total lactose content; **D.** Percentage of total fat content. Data represent means from CH and UH (n=12) cows. Differences between CH and UH in each week 1-3, 4-6, 7-9, 1-9 were calculated by Student's t-test (** P<0.01; *P<0.05).

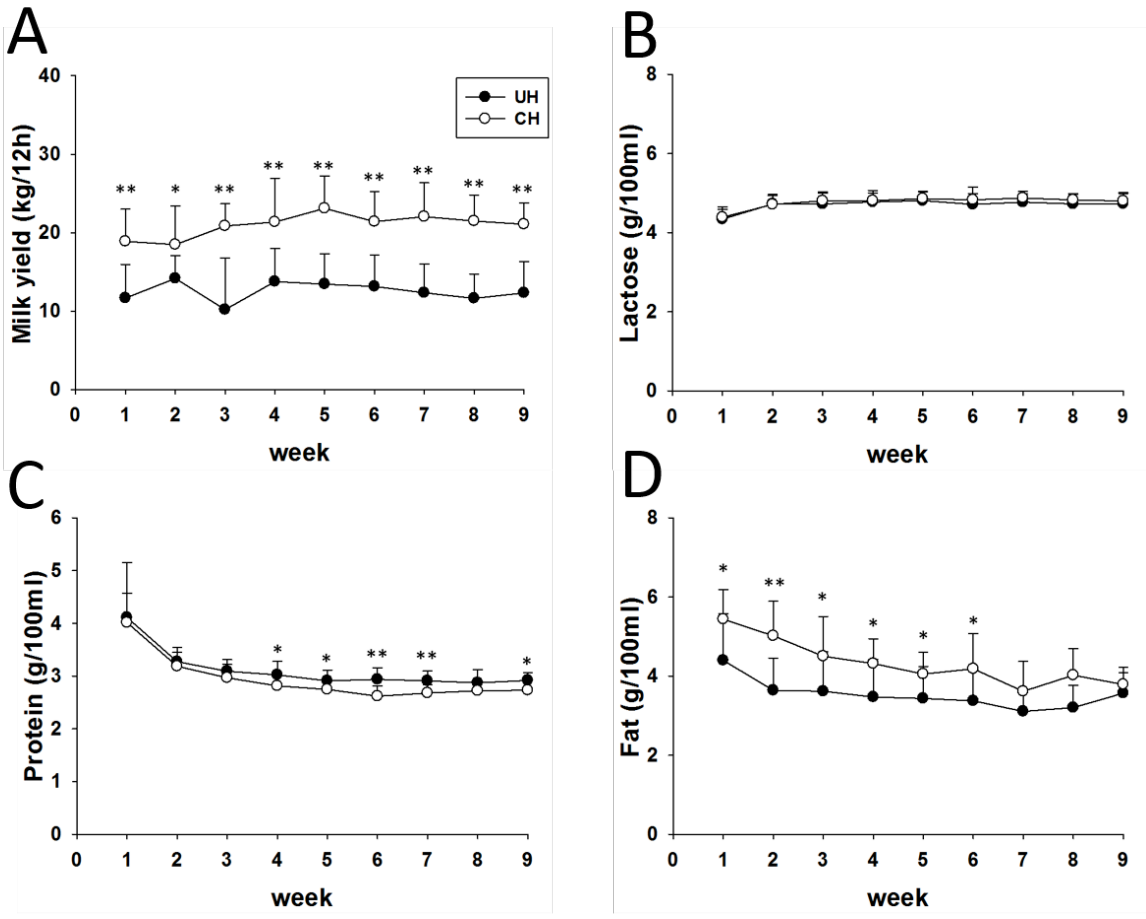
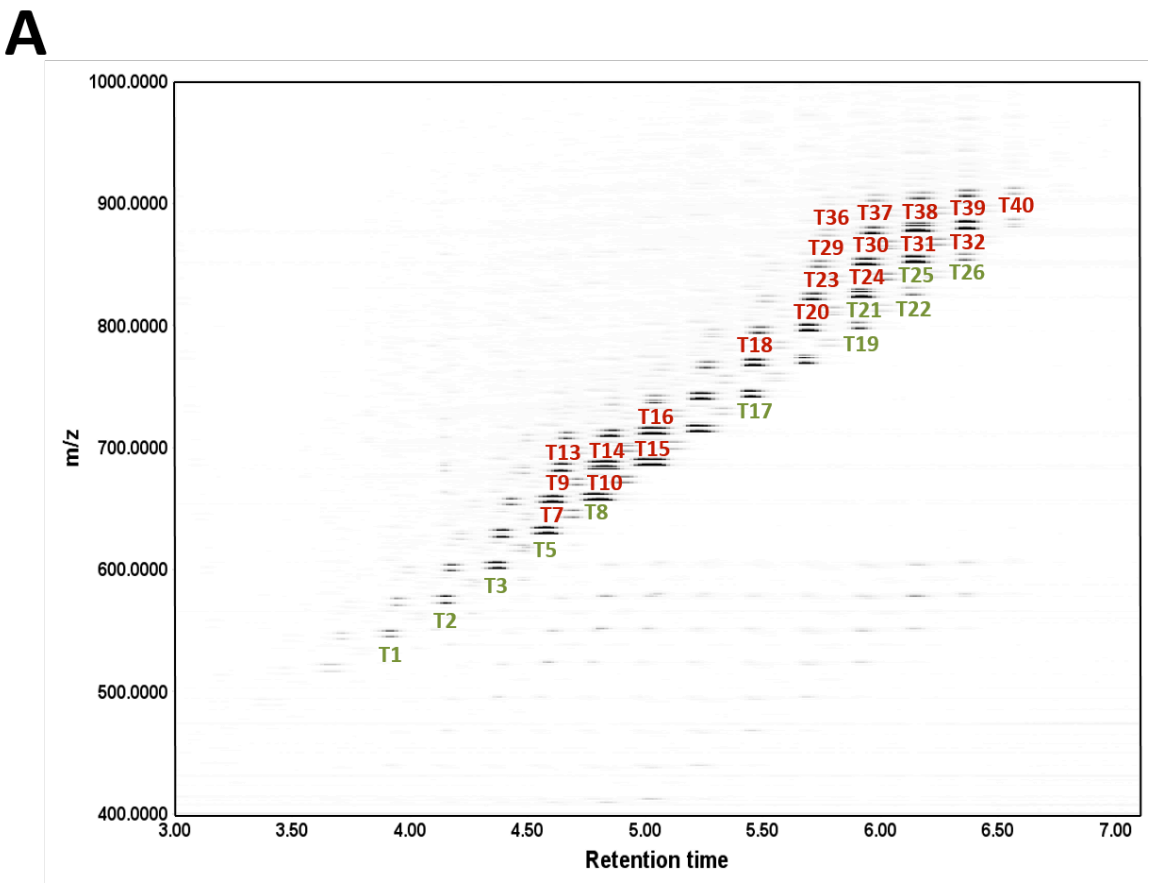


Figure 2.2 LC-MS based lipidomic analysis of milk TAGs. A. A representative 2D-plot of LC-MS data on milk lipids; **B.** MSMS fragmentation of TAG (4:0, 16:0, 18:0). Outstanding TAG markers high and low in CH are labeled in red and green, respectively.



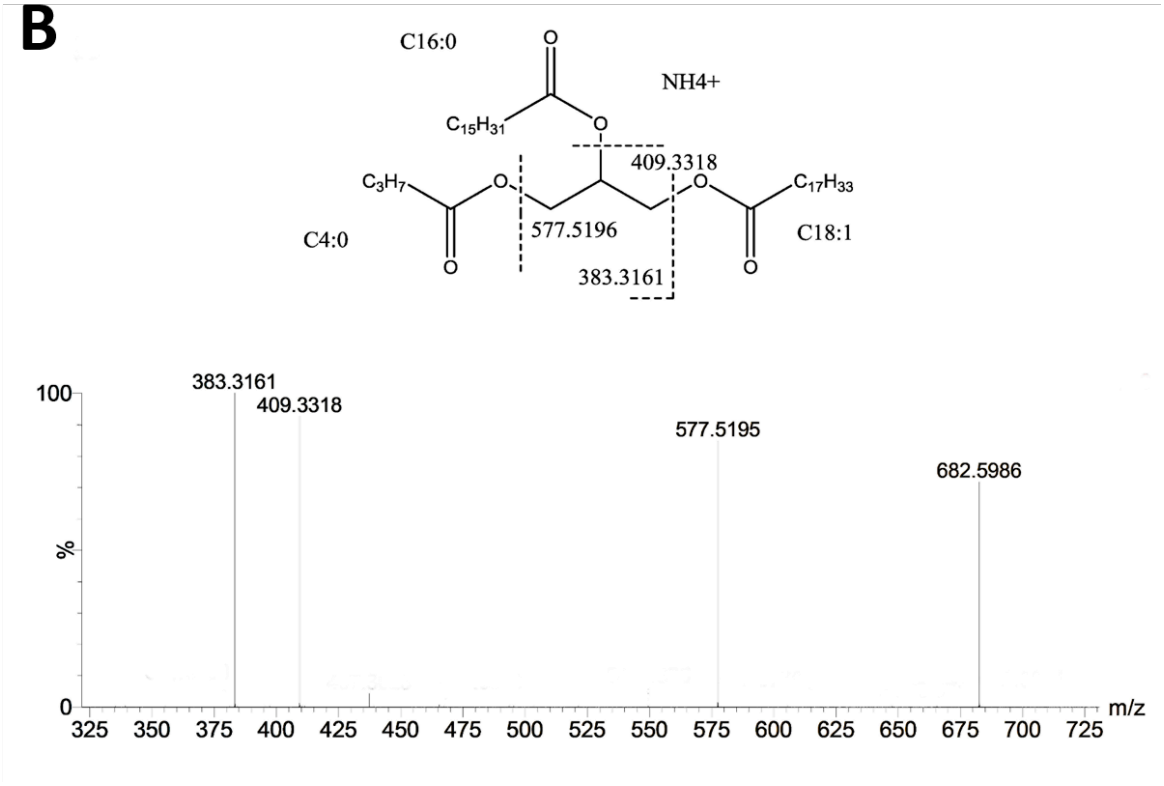
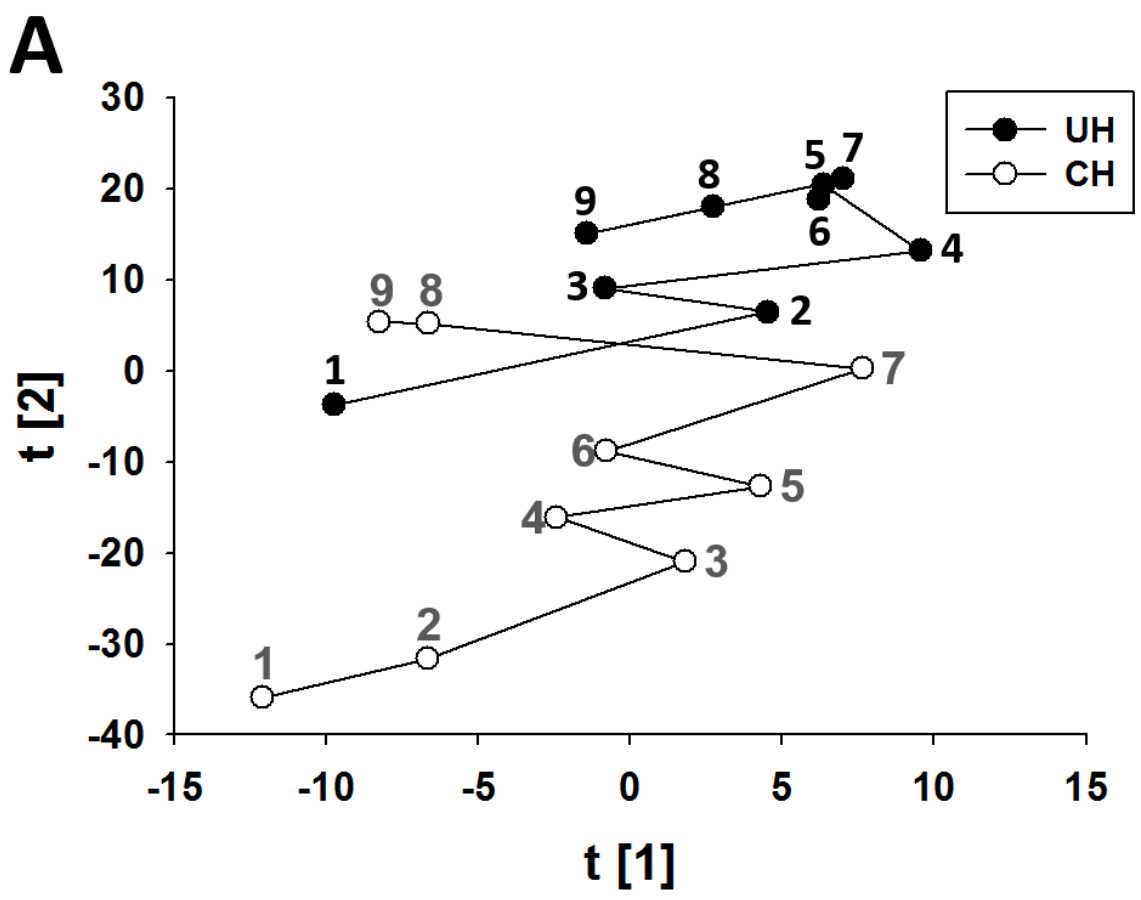
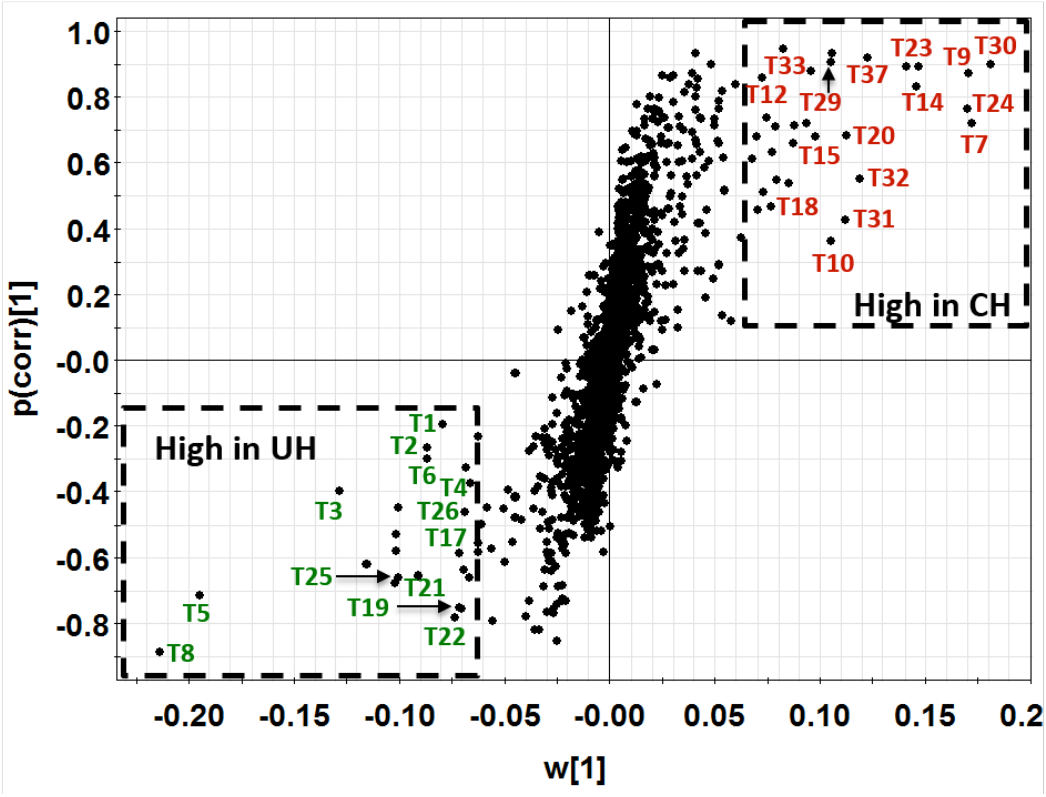

B
C16:0
NH4+
C15H31
409.3318
C3H7
C17H33
577.5196
C4:0
C18:1
383.3161
100
%
0
383.3161
409.3318
577.5195
682.5986
325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700 725
m/z

Figure 2.3 LC-MS based metabolomic analysis of milk neutral lipids. A. The score plot of a PCA model on CH and UH samples (n=12) in the first 9 weeks of lactation. The t[1] and t[2] values represent scores of each sample in the principal component 1 and 2, respectively. Each dot is the mean of 12 sample in the same group; **B.** The loading S-plot of lipid metabolites detected by the LC-MS analysis, 13 markers high in UH and 27 markers high in CH were selected for further structure elucidation; **C.** Heat map from HCA of 40 TAG markers.



B


p(corr)[1]
w[1]
1.0
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-0.20
-0.15
-0.10
-0.05
-0.00
0.05
0.10
0.15
0.2
High in CH
High in UH
T33
T23
T9
T30
T37
T12
T29
T14
T24
T20
T7
T15
T32
T18
T31
T10
T1
T2
T6
T4
T3
T26
T17
T25
T21
T19
T5
T22
T8

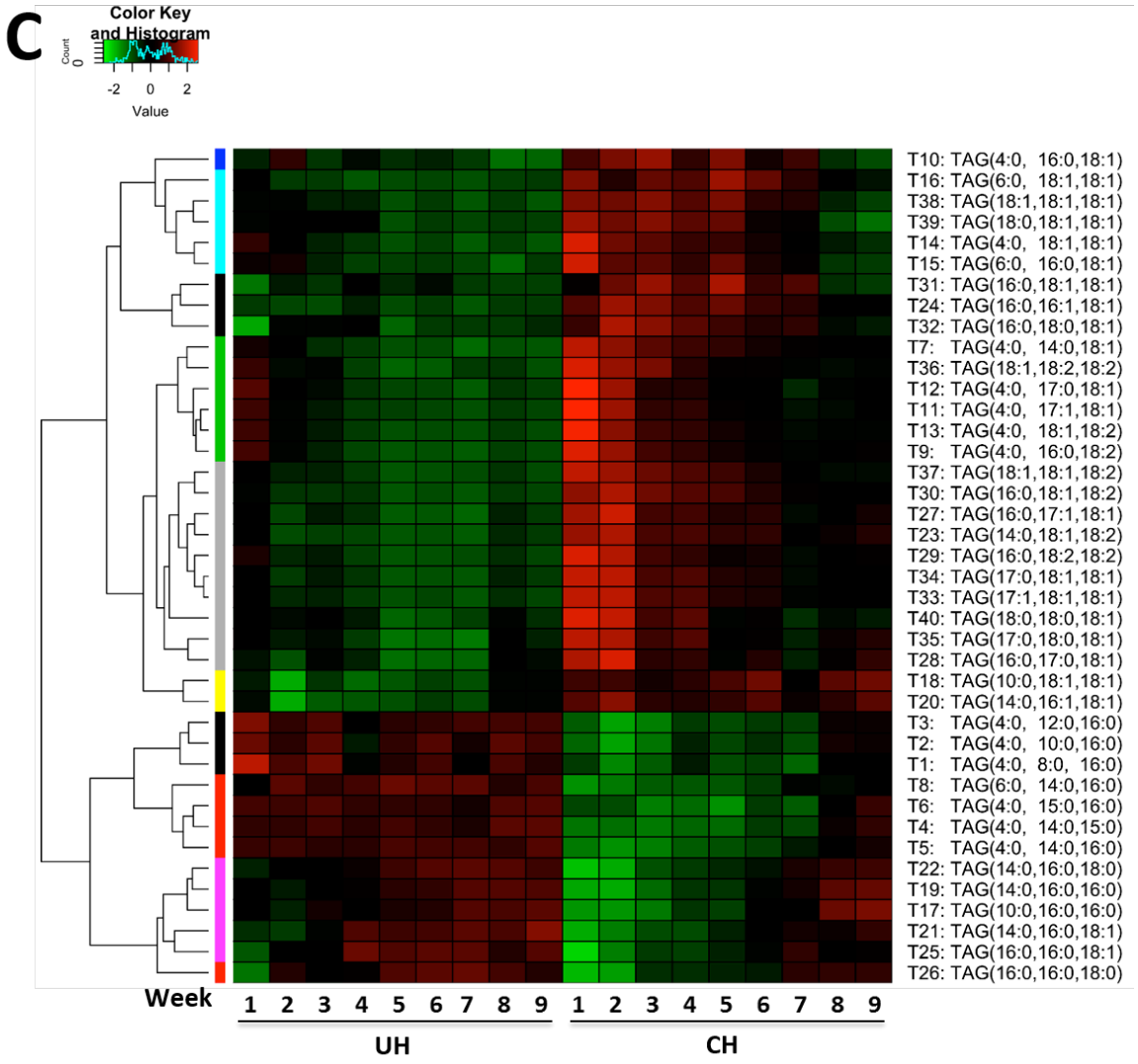
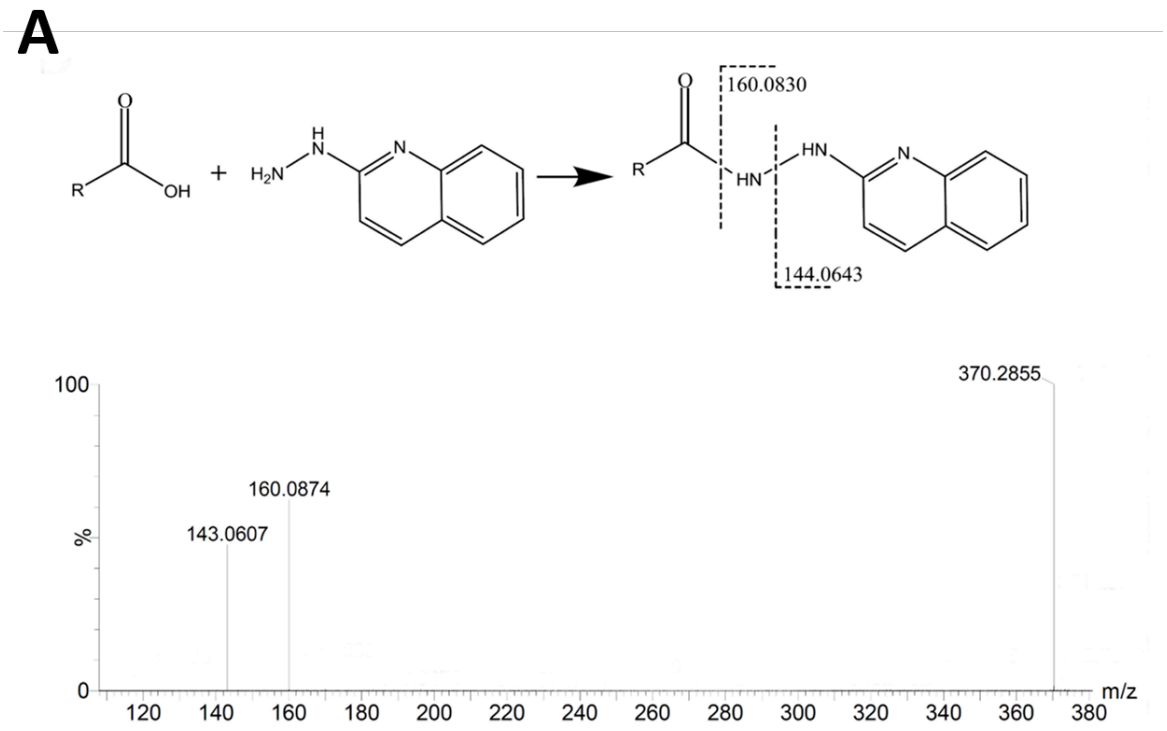

C
Color Key and Histogram
Count
0
-2
0
2
Value
T10: TAG(4:0, 16:0,18:1)
T16: TAG(6:0, 18:1,18:1)
T38: TAG(18:1,18:1,18:1)
T39: TAG(18:0,18:1,18:1)
T14: TAG(4:0, 18:1,18:1)
T15: TAG(6:0, 16:0,18:1)
T31: TAG(16:0,18:1,18:1)
T24: TAG(16:0,16:1,18:1)
T32: TAG(16:0,18:0,18:1)
T7: TAG(4:0, 14:0,18:1)
T36: TAG(18:1,18:2,18:2)
T12: TAG(4:0, 17:0,18:1)
T11: TAG(4:0, 17:1,18:1)
T13: TAG(4:0, 18:1,18:2)
T9: TAG(4:0, 16:0,18:2)
T37: TAG(18:1,18:1,18:2)
T30: TAG(16:0,18:1,18:2)
T27: TAG(16:0,17:1,18:1)
T23: TAG(14:0,18:1,18:2)
T29: TAG(16:0,18:2,18:2)
T34: TAG(17:0,18:1,18:1)
T33: TAG(17:1,18:1,18:1)
T40: TAG(18:0,18:0,18:1)
T35: TAG(17:0,18:0,18:1)
T28: TAG(16:0,17:0,18:1)
T18: TAG(10:0,18:1,18:1)
T20: TAG(14:0,16:1,18:1)
T3: TAG(4:0, 12:0,16:0)
T2: TAG(4:0, 10:0,16:0)
T1: TAG(4:0, 8:0, 16:0)
T8: TAG(6:0, 14:0,16:0)
T6: TAG(4:0, 15:0,16:0)
T4: TAG(4:0, 14:0,15:0)
T5: TAG(4:0, 14:0,16:0)
T22: TAG(14:0,16:0,18:0)
T19: TAG(14:0,16:0,16:0)
T17: TAG(10:0,16:0,16:0)
T21: TAG(14:0,16:0,18:1)
T25: TAG(16:0,16:0,18:1)
T26: TAG(16:0,16:0,18:0)
Week
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
UH
CH

Figure 2.4 LC-MS analysis of HQ derivatives of milk FAs. A. HQ derivatization reaction and fragmentogram of HQ derivative of myristic acid; **B.** A representative chromatograph of major milk FAs detected by HQ derivatization and LC-MS analysis.



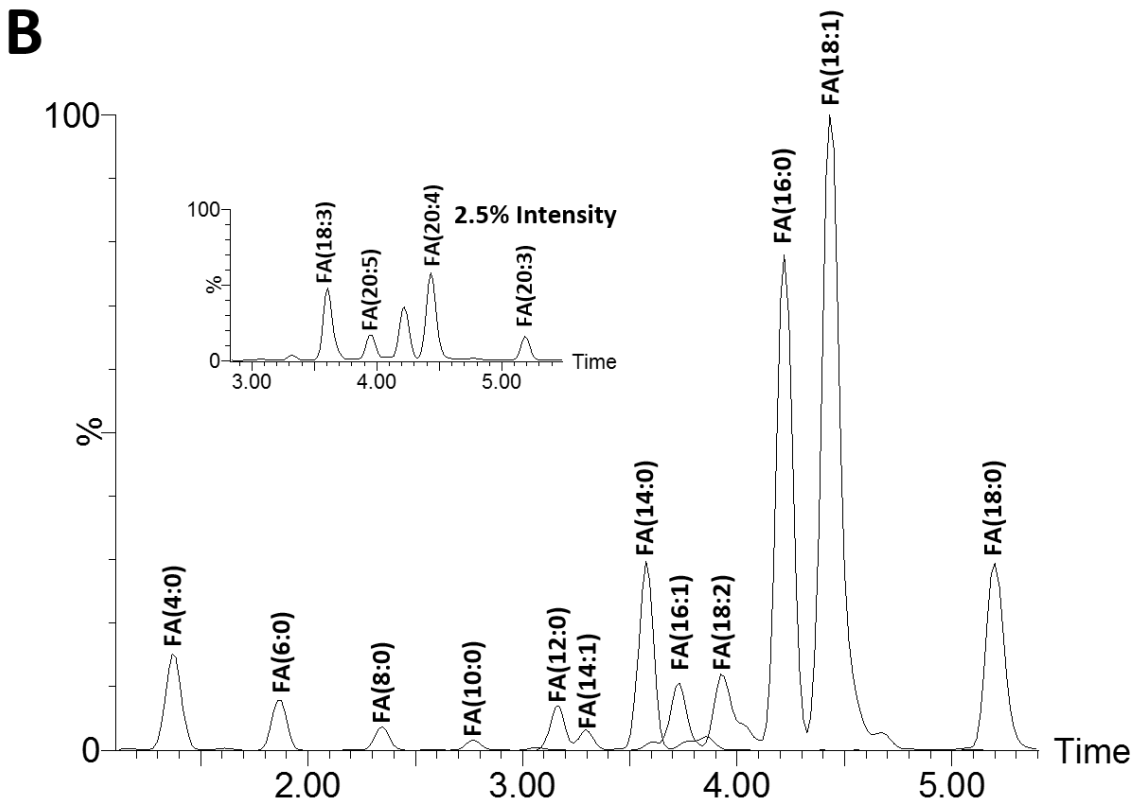
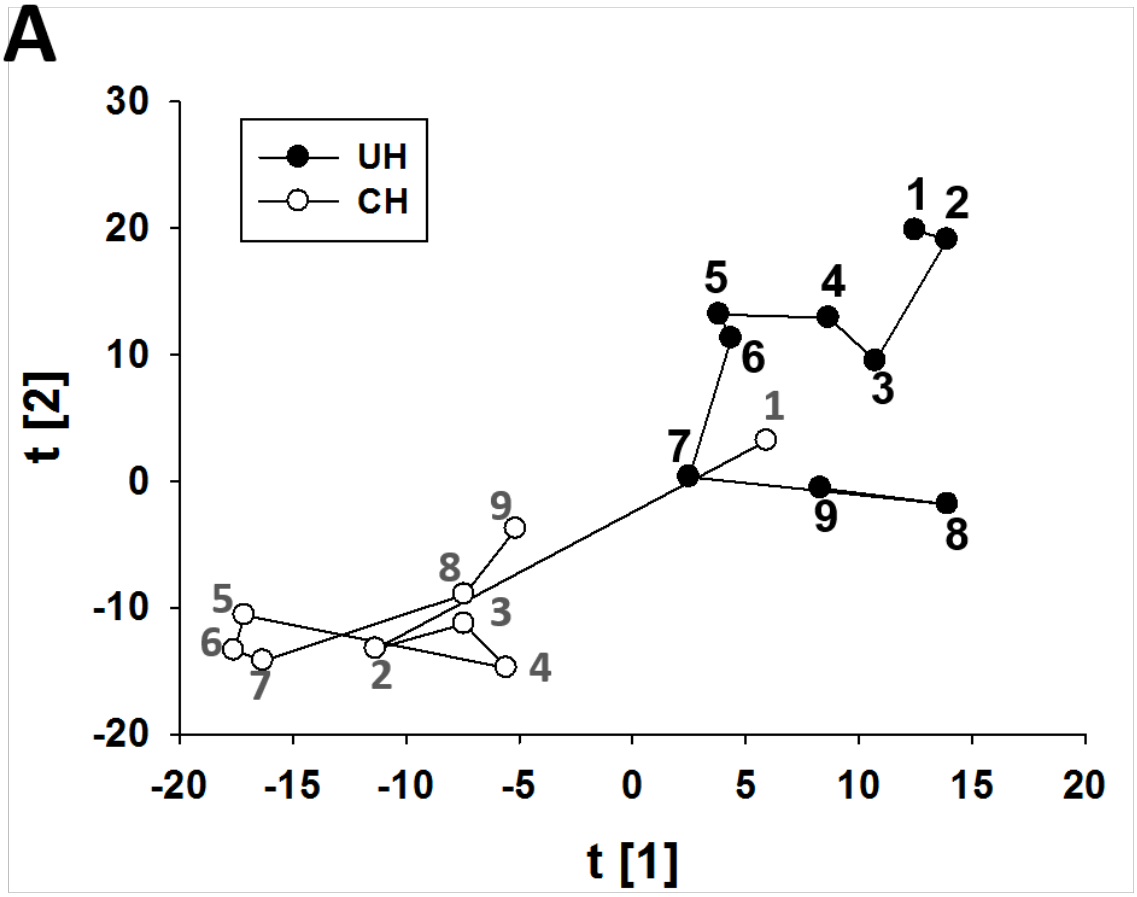

B
100
%
0
2.00
3.00
4.00
5.00
Time
FA(4:0)
FA(6:0)
FA(8:0)
FA(10:0)
FA(12:0)
FA(14:1)
FA(14:0)
FA(16:1)
FA(18:2)
FA(16:0)
FA(18:1)
FA(18:0)
2.5% Intensity
FA(18:3)
FA(20:5)
FA(20:4)
FA(20:3)

Figure 2.5 LC-MS based metabolomic analysis of milk fatty acids. A. The score plot of a PCA model on CH and UH samples (n=12) in the first 9 weeks of lactation. The t[1] and t[2] values represent scores of each sample in the principal component 1 and 2, respectively. Each dot is the mean of 12 sample in the same group; **B.** The loading S-plot of lipid metabolites detected by the LC-MS analysis.



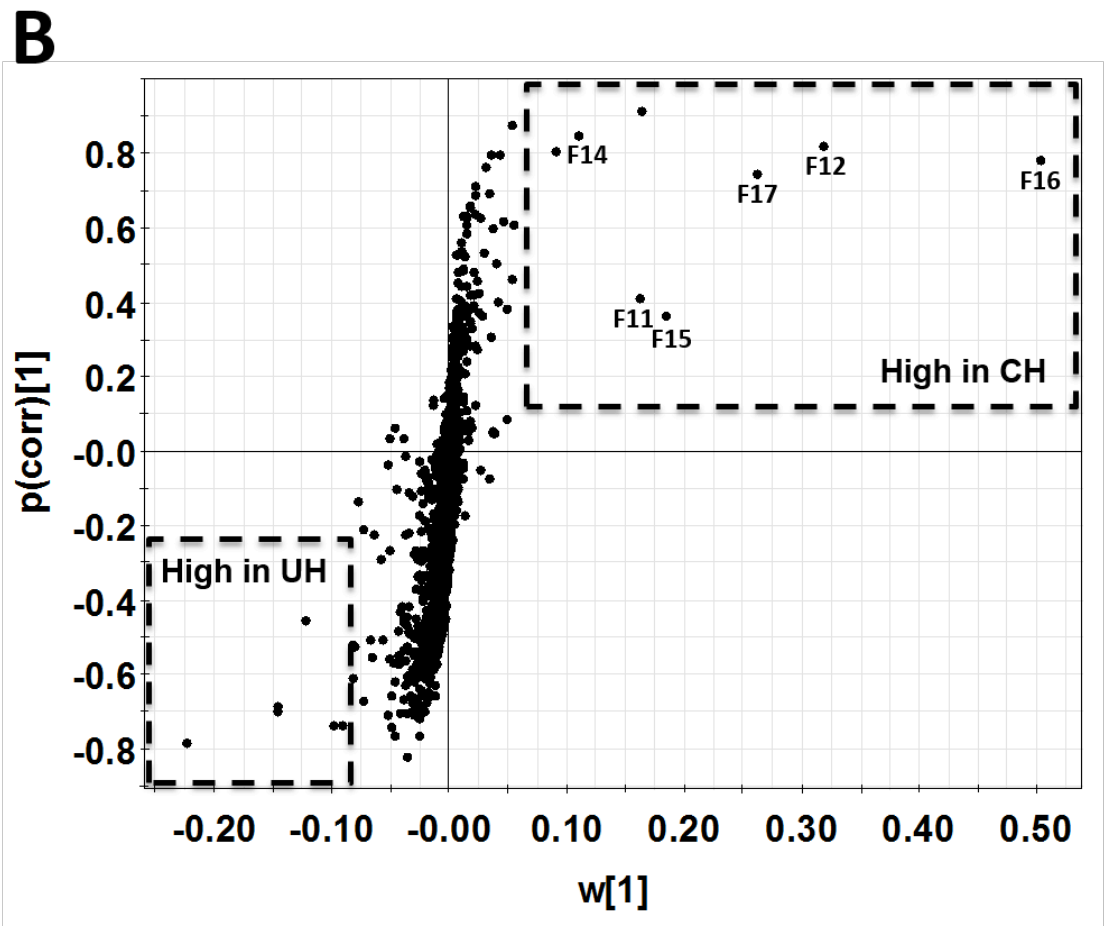
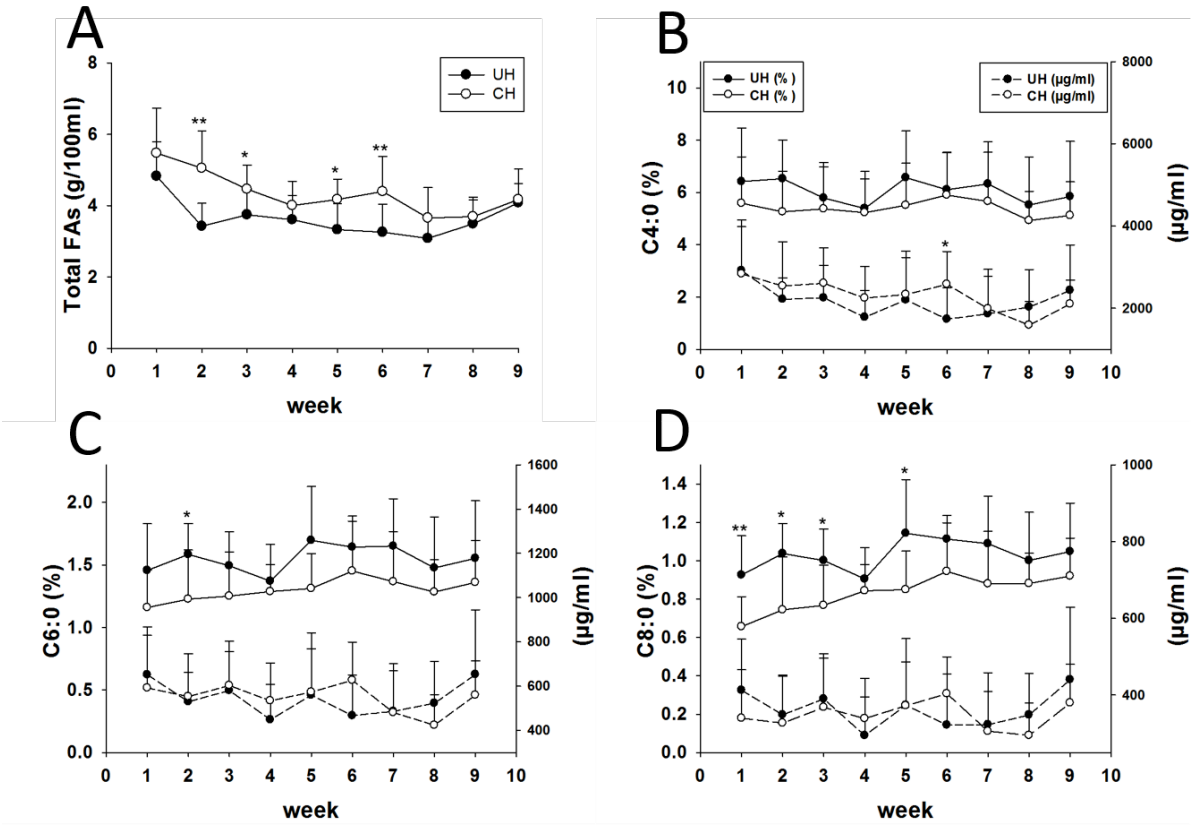
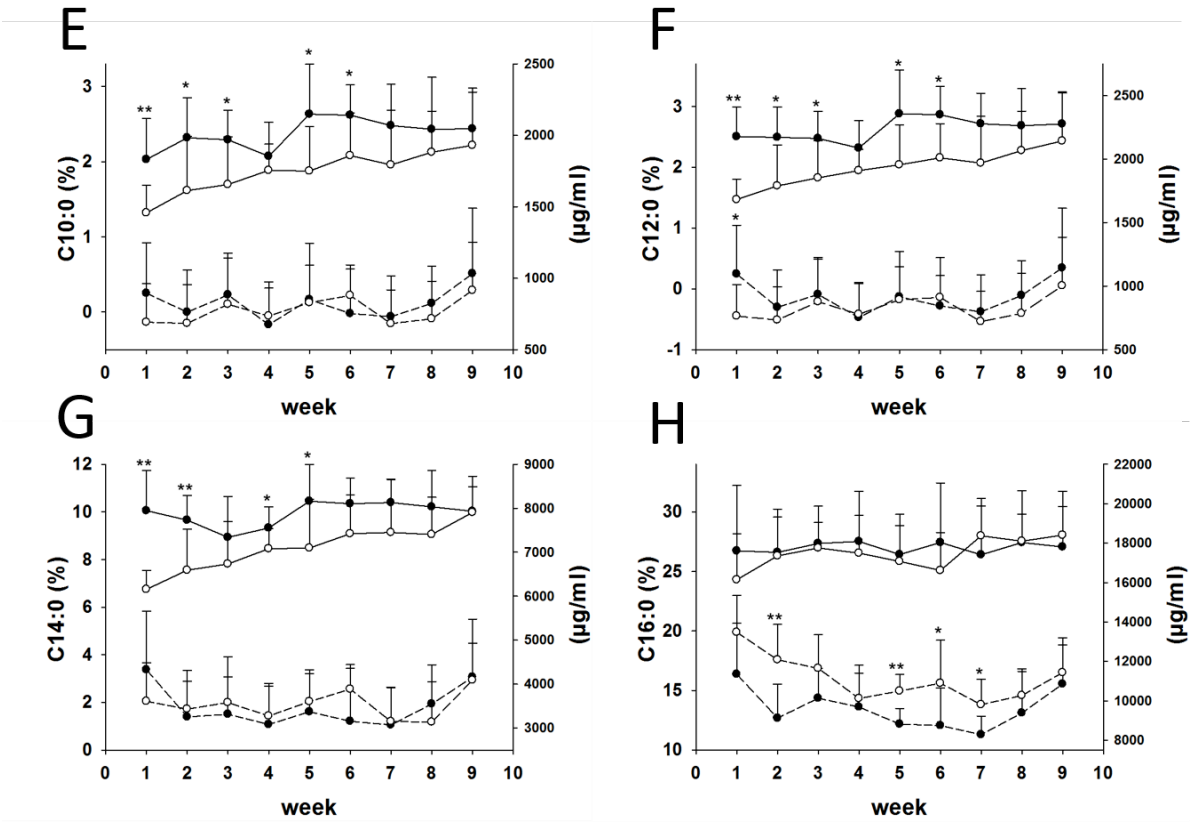

B
p(corr)[1]
w[1]
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-0.20
-0.10
-0.00
0.10
0.20
0.30
0.40
0.50
F14
F12
F17
F16
F11
F15
High in CH
High in UH

Figure 2.6 Effect of genetic selection and weeks of lactation on milk fatty acids. The concentration of individual milk fatty acid and percentage of individual FA to total FAs were measured. **A.** Total FAs; **B.** C4:0; **C.** C6:0; **D.** C8:0; **E.** C10:0; **F.** C12:0; **G.** C14:0; **H.** C16:0; **I.** C16:1; **J.** C18:0; **K.** C18:1; **L.** C18:2 (LA). **M.** The ratio of total preformed FAs versus total de novo synthesized fatty acids; **N.** The ratio of total saturate FAs versus total unsaturated FAs. Each data point is the mean of CH or UH sample at one time point (n=12). Difference between CH and UH at the same week of lactation was calculated by the Student's t-test (** P<0.01; *P<0.05).




E
C10:0 (%)
(µg/ml)
week
F
C12:0 (%)
(µg/ml)
week
G
C14:0 (%)
(µg/ml)
week
H
C16:0 (%)
(µg/ml)
week

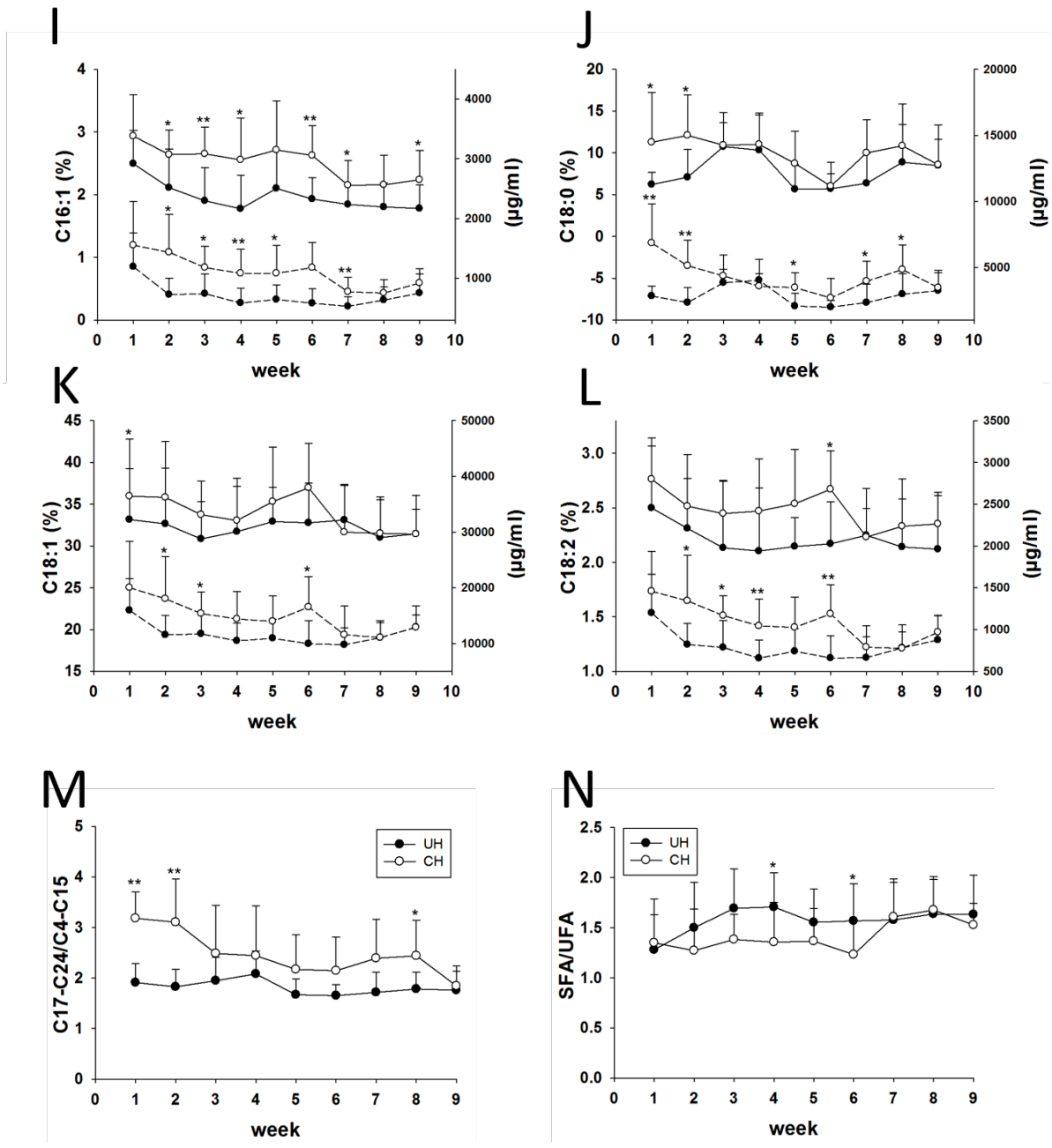
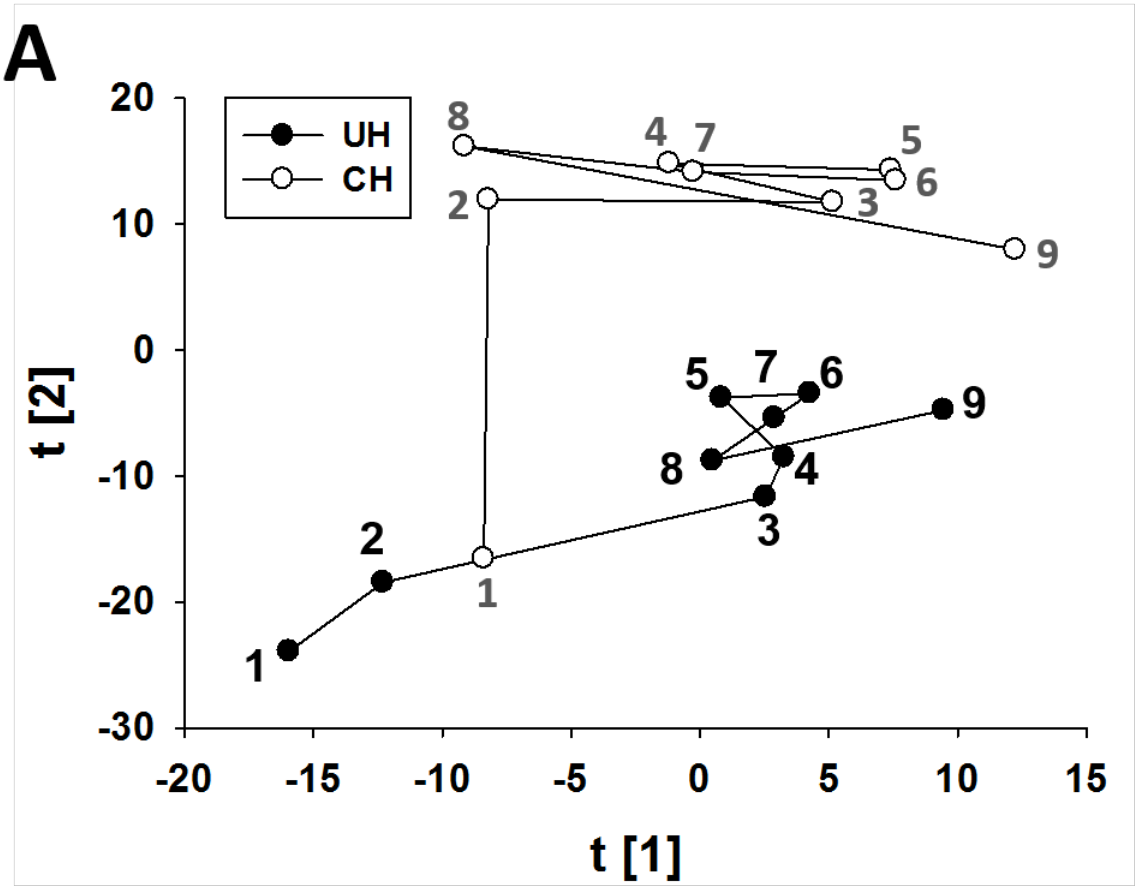

I
C16:1 (%)
(μg/ml)
week
J
C18:0 (%)
(μg/ml)
week
K
C18:1 (%)
(μg/ml)
week
L
C18:2 (%)
(μg/ml)
week
M
C17-C24/C4-C15
UH
CH
week
N
SFA/UFA
UH
CH
week

Figure 2.7 LC-MS based metabolomic analysis of milk organic acid. A. The score plot of a PCA model on CH and UH samples (n=12) in the first 9 weeks of lactation. The t[1] and t[2] values represent scores of each sample in the principal component 1 and 2, respectively. Each dot is the mean of 12 sample in the same group; **B.** The loading S-plot of organic acids detected by the LC-MS analysis.



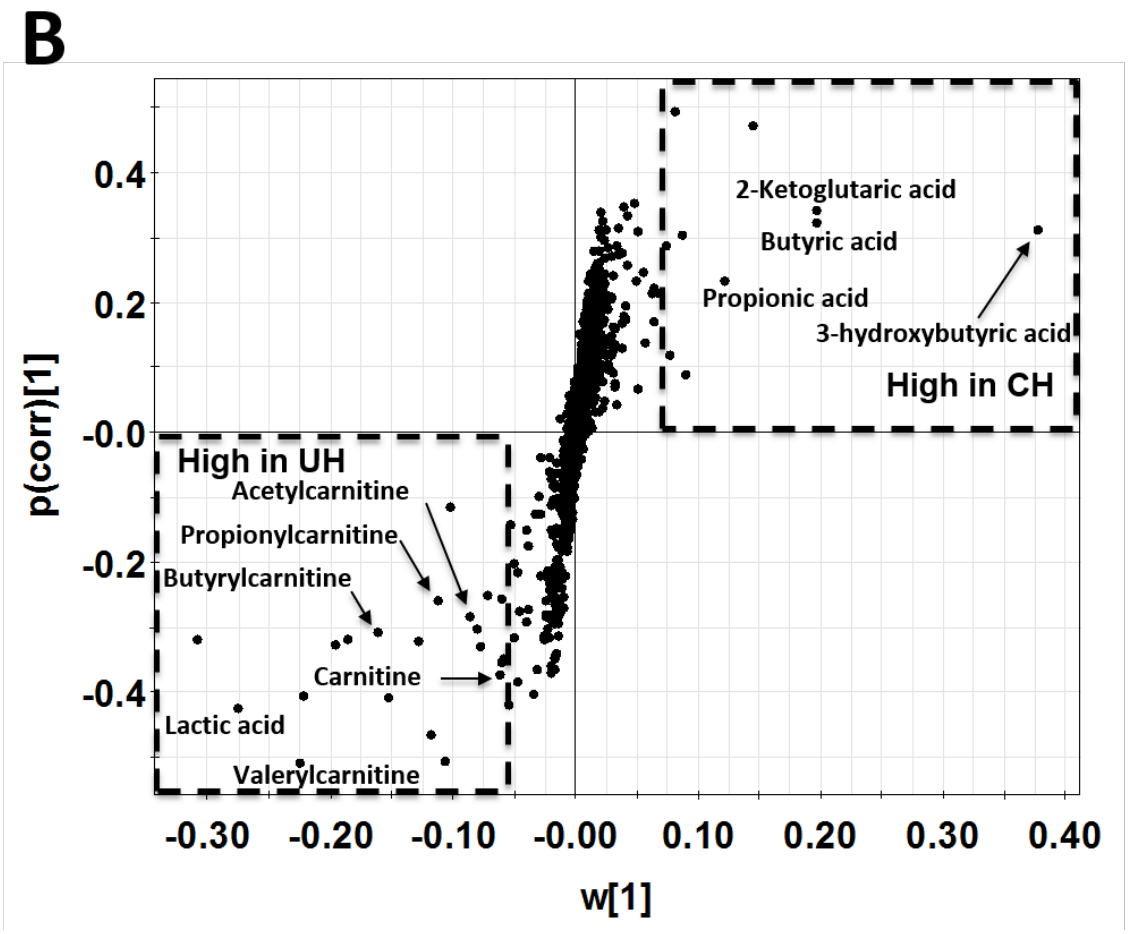
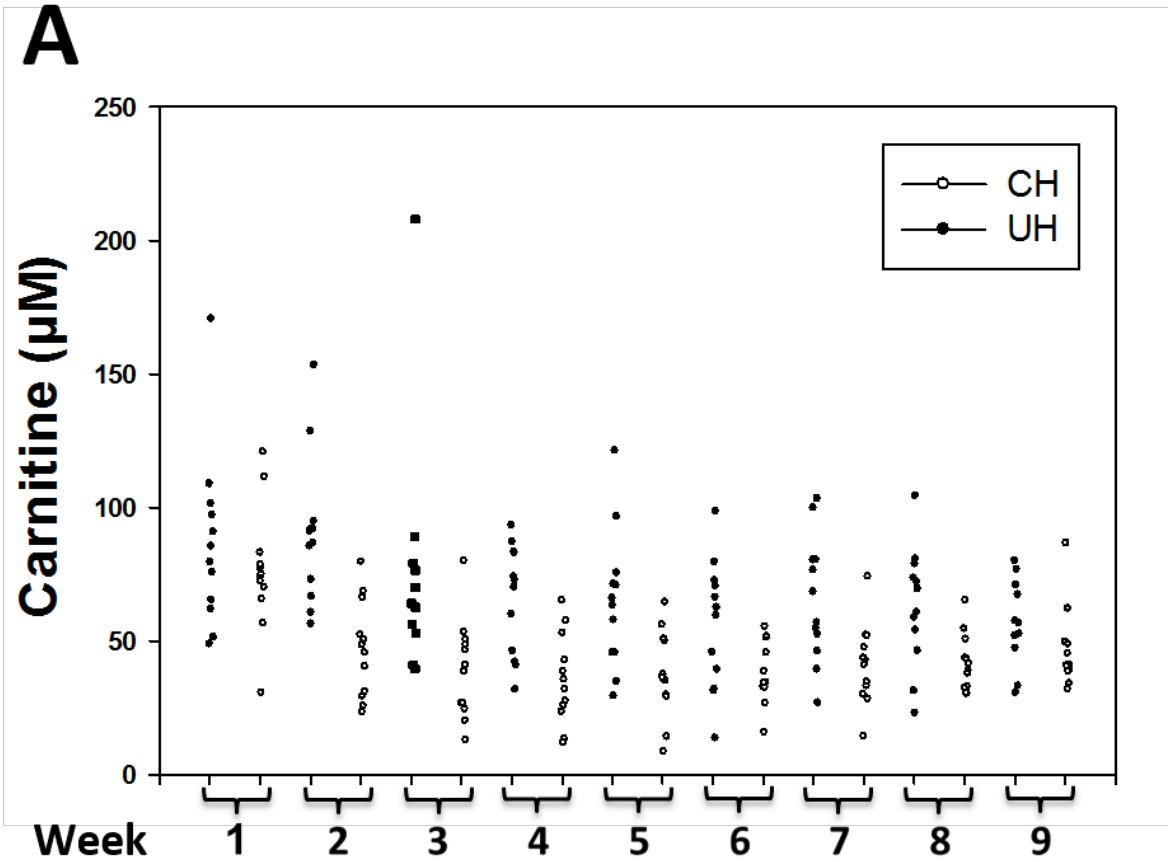
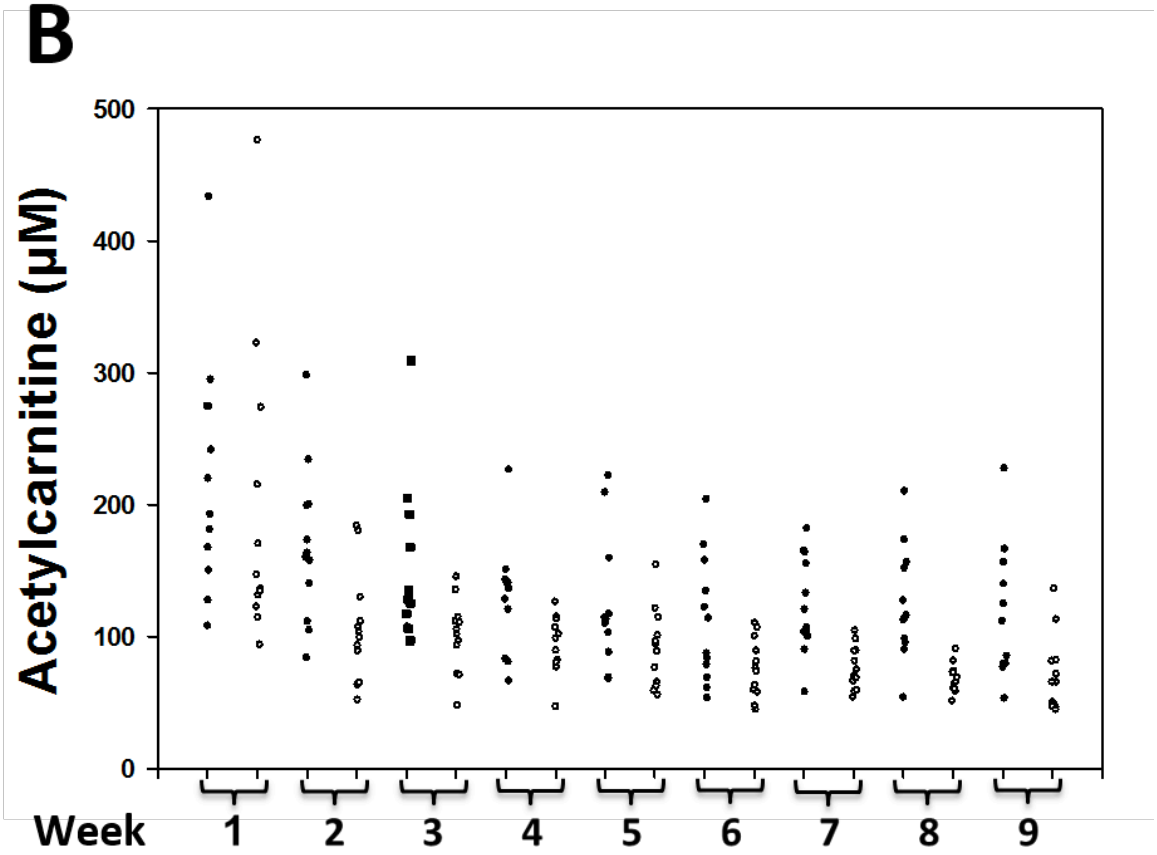
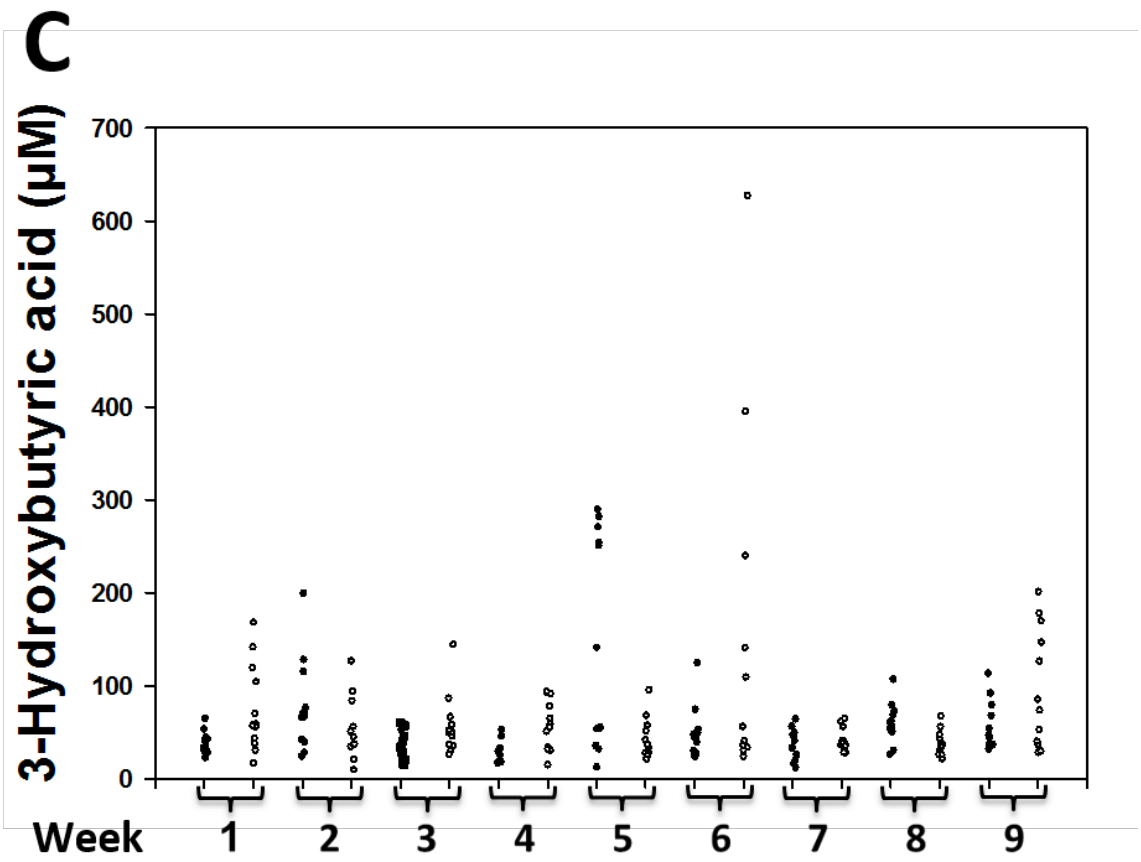
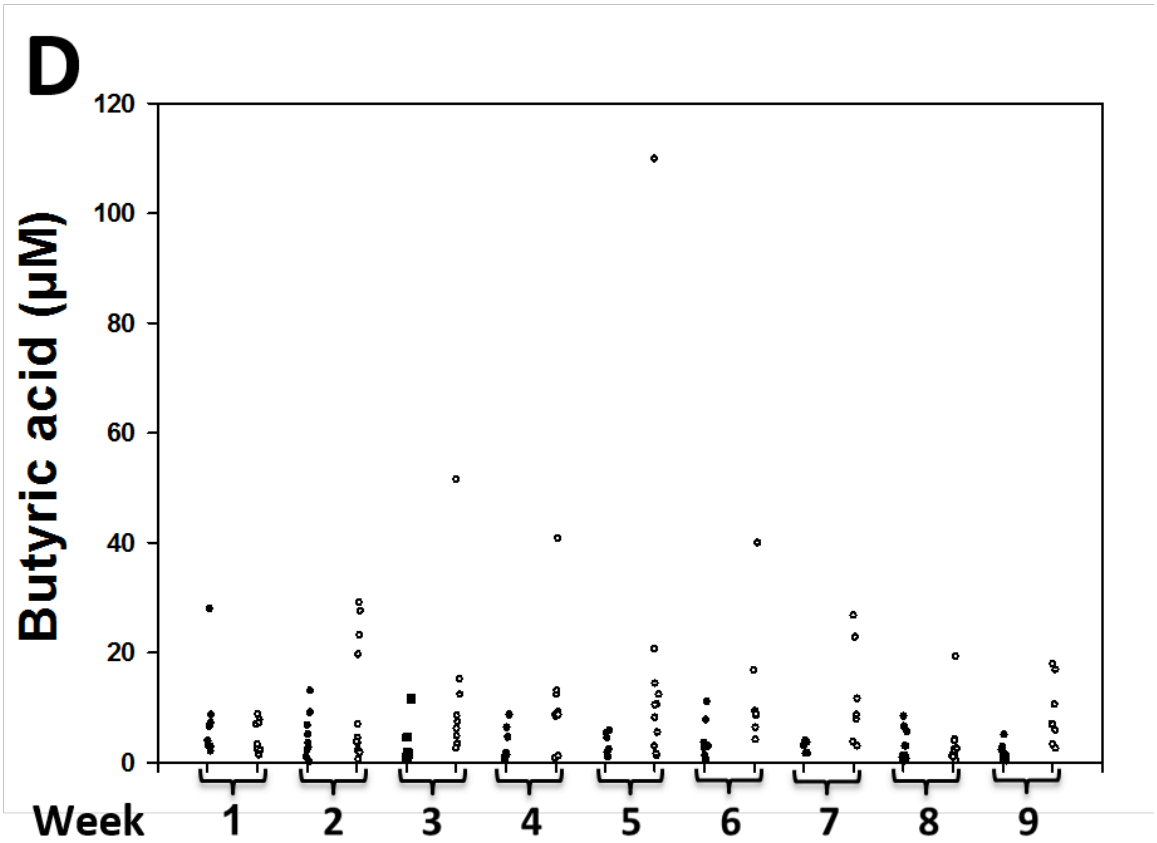

B
0.4
0.2
-0.0
-0.2
-0.4
p(corr)[1]
2-Ketoglutaric acid
Butyric acid
Propionic acid
3-hydroxybutyric acid
High in CH
High in UH
Acetylcarnitine
Propionylcarnitine
Butyrylcarnitine
Carnitine
Lactic acid
Valerylcarnitine
-0.30
-0.20
-0.10
-0.00
0.10
0.20
0.30
0.40
w[1]

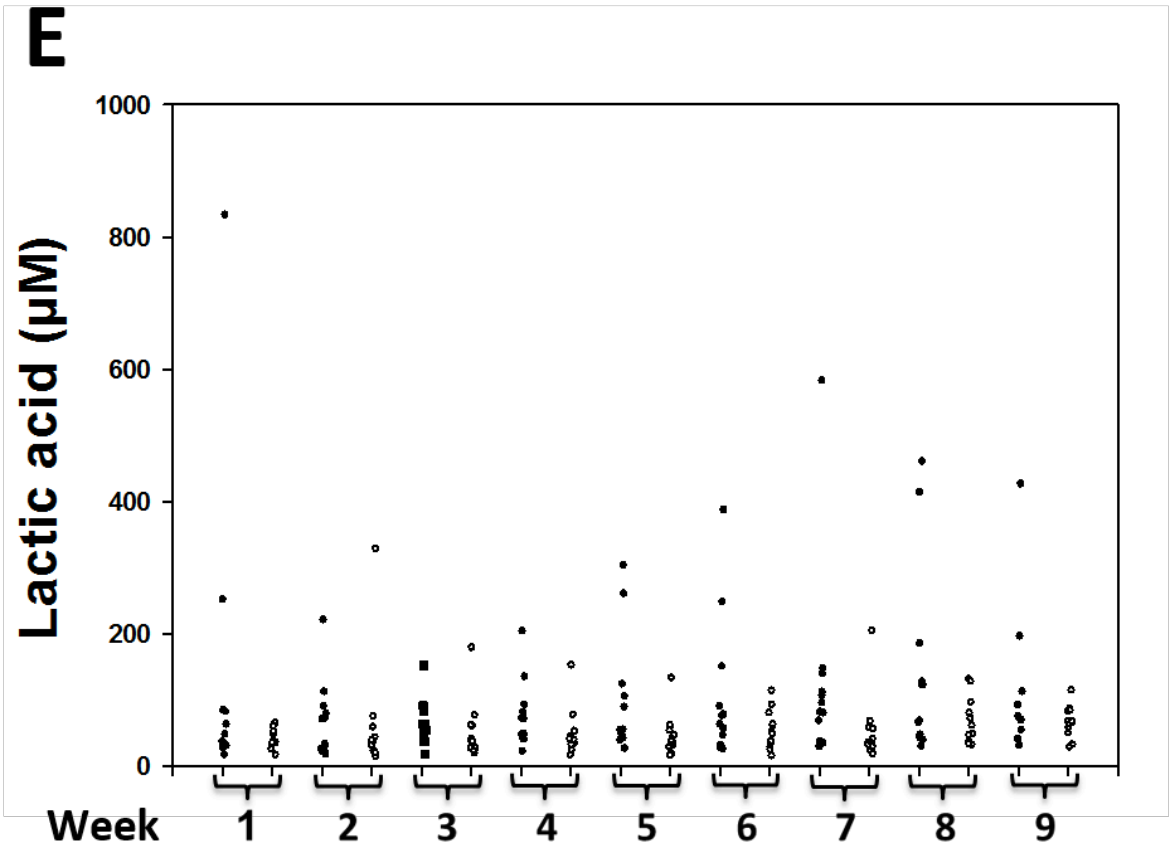
Figure 2.8 Concentrations of major organic acid markers. The concentrations of major organic acid markers in UH and CH milk were quantified and presented in respective scatter plots. **A.** Carnitine; **B.** Acetylcarnitine; **C.** Hydroxybutyric acid; **D.** Butyric acid; **E.** Lactic acid; **F.** Pyroglutamic acid.




B
Acetylcarnitine (μM)
500
400
300
200
100
0
Week
1
2
3
4
5
6
7
8
9


C
3-Hydroxybutyric acid (μM)
700
600
500
400
300
200
100
0
Week
1
2
3
4
5
6
7
8
9


D
Butyric acid (μM)
120
100
80
60
40
20
0
Week
1
2
3
4
5
6
7
8
9


E
Lactic acid (μM)
1000
800
600
400
200
0
Week
1
2
3
4
5
6
7
8
9

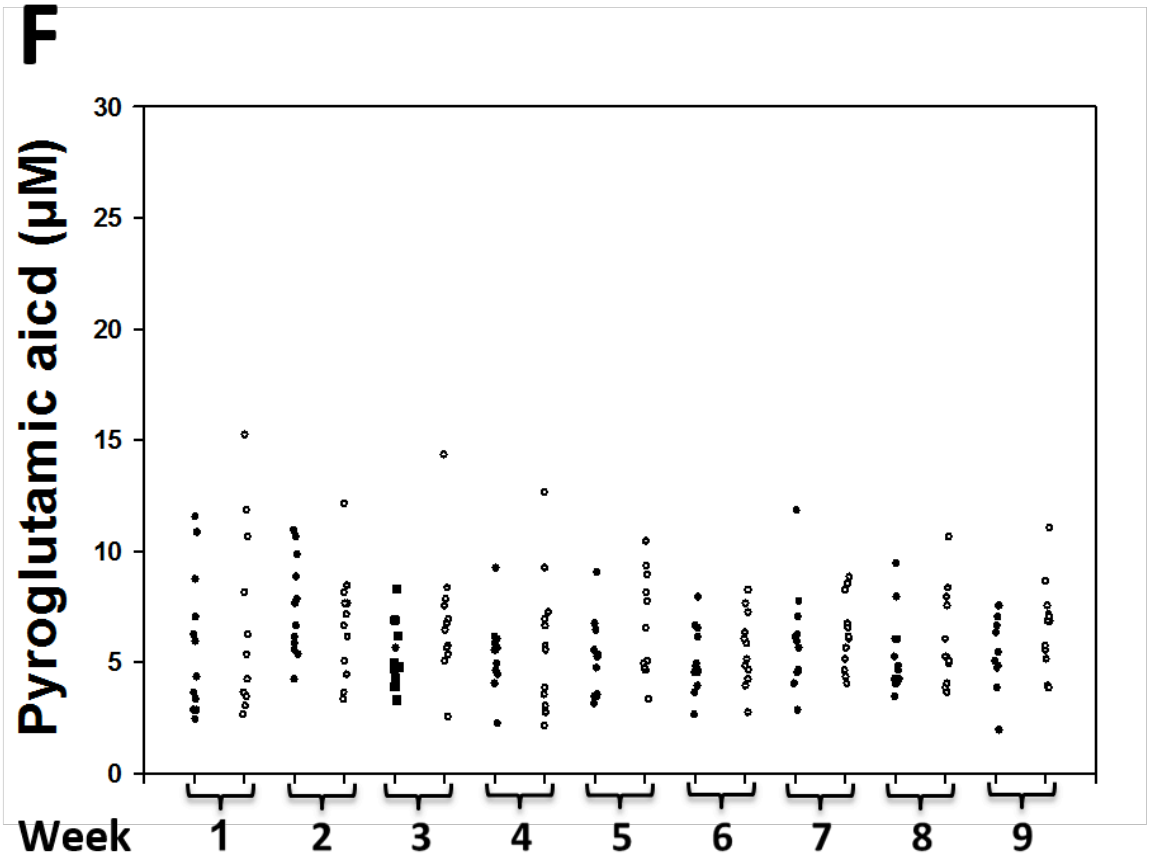
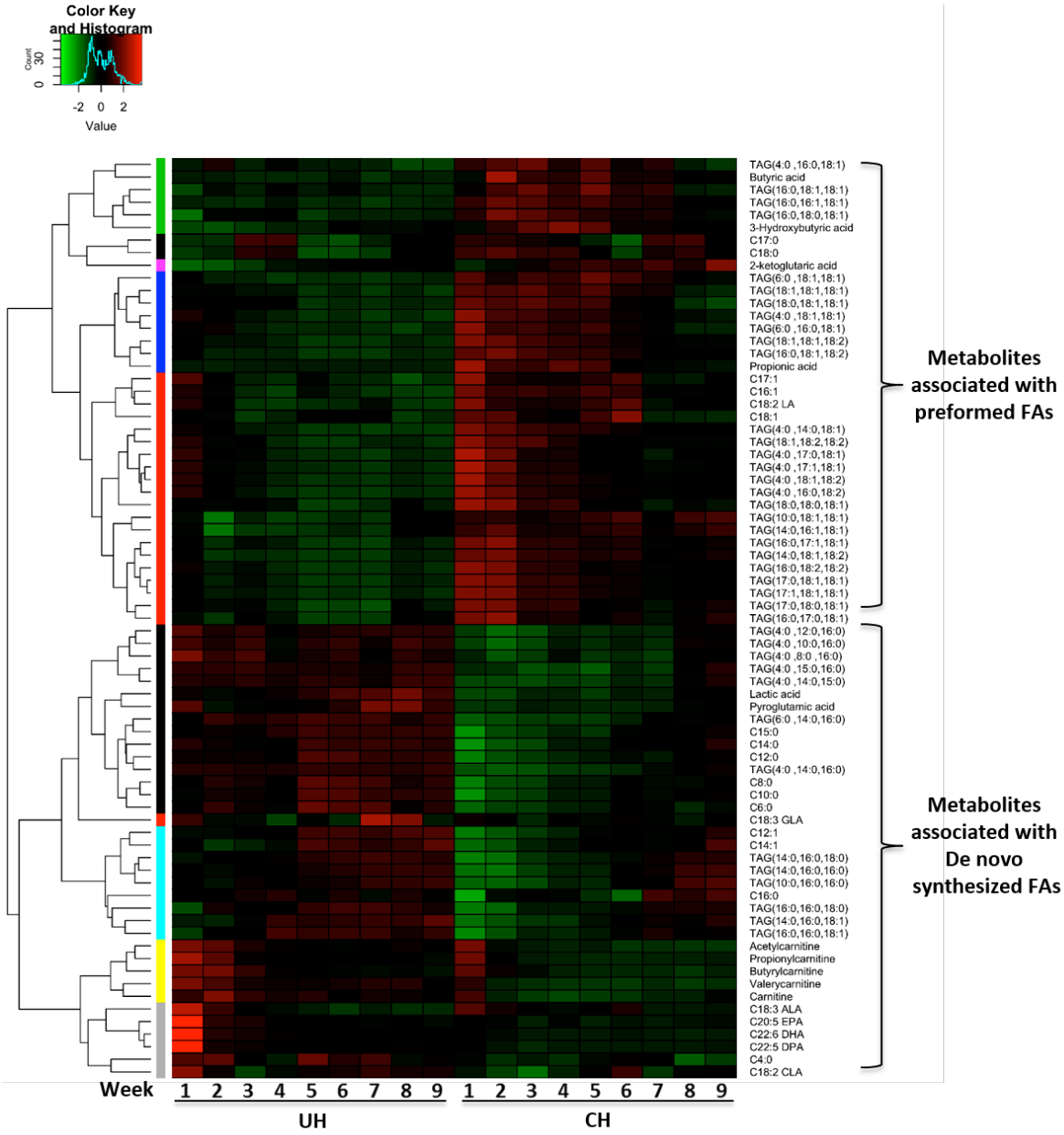

F
Pyroglutamic aicd (μM)
30
25
20
15
10
5
0
Week
1
2
3
4
5
6
7
8
9

Figure 2.9 HCA-based heatmap on TAG, FA and organic acid markers in UH and CH milk.



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