Holstein Friesian mid-lactation Milk Polar Metabolite Composition in relation to Dietary Fat Composition and Diacylglycerol O-acyltransferase 1 Genotype

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Abstract

Background

The metabolite composition of cow milk is dependent on a large variety of animal associated factors including diet, genotype and gut microbiome composition. The objective of this study was to investigate changes in cow milk polar metabolite composition resulting from dietary and DGAT1 (Diacylglycerol O-acyltransferase 1) genotype perturbations.

Methods and Results

Cows were fed a standard diet and a diet supplemented with (poly)unsaturated fatty acids (experimental diet) for ten weeks. Metabolite profiles were determined using ¹H NMR (1-Hydrogen Nuclear magnetic resonance) technology. The results showed that the diet affected the polar metabolite composition of milk via the metabolism of the cow and via the metabolism of the gut and rumen microbiota. The experimental diet reduced the metabolic rate, especially the energy metabolism and the amino-sugar and amino acid metabolism, of the cows.

Conclusion

Our results suggests the DGAT1 genotype affects both the diet related polar metabolite metabolism of the cow as well as that of the rumen microbiota. Milk metabolite levels in animals with more DGAT1 A-alleles were higher than milk metabolite levels in animals with more K-alleles.

Keywords: Polar Metabolites; Unsaturated fatty acid diet composition; Diacylglycerol O-acyltransferase 1 genotype; Rumen Microbiota; 1-Hydrogen Nuclear magnetic resonance metabolomics

Introduction

The composition of milk is highly variable, even between lactation stages in a single animal [1, 2]. The composition of milk can be influenced by genetic factors of the cow and by the composition of the fodder [3, 4]. The metabolite composition of milk reflects the metabolism of the animal and that of the rumen microbiota [5-7].

The Diacylglycerol O-acyltransferase 1 (DGAT1) gene is known to affect milk yield and its fat composition [8]. The encoded enzyme catalyzes the terminal step of triglyceride synthesis. This reaction is important for triacylglycerol synthesis in the mammary gland, and for the absorption of dietary fats in the intestine [9]. In cattle DGAT1 showed two genotypes differing by one nucleotide substitution resulting in a non-conservative amino acid substitution. The DGAT1 gene has two known genotypes differing for the amino acid 232 and showing two alleles: A (Alanine) and K (Lysine). Mach *et al.* [10] showed that the interaction between the DGAT1 genotype and the cow's diet regulated the expression level of 30 genes affecting several cellular physiologic and developmental mechanisms and the immune system.

Diet is also known as an important factor in determining milk composition [4-5, 7]. Dietary components regulate both the cow's metabolism [11-13] and the rumen microbiome metabolism [5-6]. The underlying biological mechanisms probably is regulation of the metabolism via regulation of gene expression [10, 13-15].

Metabolomics is a high throughput methodology to study the metabolite composition of tissues or body fluids. The metabolome consists of both polar and nonpolar molecules of varying molecular weight such as lipids and fatty acids, amino acids and short peptides, but also a mixture of other reaction intermediate products from the metabolism. With ^1H NMR (1-Hydrogen Nuclear Magnetic Resonance) technology one

can detect, identify, and quantify molecules in complex matrices [16-17]. Sundekilde *et al.* [18, 19] previously used ^1H NMR to evaluate milk metabolite levels in relation to breed and milk properties.

Previously, we reported on the effects of a ten weeks unsaturated fatty acids addition to the diet of mid-lactation dairy cattle [10, 13, 20-22]. These studies showed that adding unsaturated fatty acids to the diet resulted in massive changes in the transcriptome of the mammary gland affecting many cellular metabolic processes and changing the non-polar milk composition. Furthermore, these studies showed interaction between the diet and the DGAT1 genotype, and the importance of the ruminal metabolism. The impact of these transcriptome changes on the polar metabolite profile remained poorly understood. The objective of this study was to study polar metabolite changes in milk related to unsaturated fatty acids feed composition and DGAT1 genotype during mid-lactation in the same animals.

Materials and Methods

Animals and experimental conditions

Holstein-Friesian cows in mid lactation were fed diets composed of a standard composition, or with plant oil supplementation (N=7 for each diet; For the DGAT1 genotypes: AA (N=2), AK (N=2), KK (N=3); For all experimental details see Mach *et al.*, 2011A, Jacobs *et al.*, 2011). In this study we focused on the cows receiving a mixture of rapeseed, soybean and linseed oil together (Table 1, [23]), excluding the animals receiving only one of these oils in an effort to maximize the measurable effects of the dietary differences. All other experimental conditions are as described in [13] because

the same samples were used. Milk samples were taken at the end of each feeding period and stored at -25°C until use.

Diacylglycerol O-acyltransferase 1 (DGAT1) genotyping

Genotyping of DGAT1 gene polymorphism was performed using a TaqMan allelic discrimination method in an Applied Biosystems 7,500 quantitative real-time (qRT) PCR System (Applied Biosystems, Bleiswijk, the Netherlands), as described by [24]. The genotype at the DGAT1 locus was designated homozygous Lys (KK), heterozygous Lys/Ala (KA), or homozygous Ala (AA).

Nuclear magnetic resonance Metabolomics

Milk samples (300µl) were taken out of the freezer and debris was removed by centrifugation for 10 minutes at 14.000xg at 4 °C. The interphase between pellet and fat layer was collected. To isolate only polar metabolites the non-polar metabolites were removed by chloroform treatment (milk : chloroform: 2:5 vol : vol) The water phases were collected after centrifugation for 10 min, 5000xg at room temperature. Initial ¹H NMR data showed too broad peaks indicating that additional purification was needed using ultrafiltration with Pall 3K Omega filters (OD003C34) (Pall corporation, Port Washington, NY, USA).

The ¹H NMR spectra were acquired on a 600Bruker 600MHz Avance III ¹H NMR spectrometer equipped with a CryoPlatform cryogenic cooling system, a BCU-05 cooling unit, and an ATM automatic tuning and matching unit (Bruker Biospin, Rheinstetten, Germany). The samples were measured in 3-mm ¹H NMR tubes (Bruker matching system). Measurements were performed at a temperature of 300 K. Baseline corrections

and zero alignment were performed manually for all spectra. The spectra were analyzed for background correction using machine-specific manufacturer software. The metabolites were identified using previous knowledge on the ^1H NMR spectra of cow milk [25-26].

Statistical analyses and Bioinformatics

The spectra were divided into integrals of 0.04 ppm and the mean signal strength was used as an indicator. Using a T-test approach we studied differences in the signal strength related to (1) feed composition irrespective of DGAT1 genotype generalizing the effect of feed over all animals using an unpaired T-test approach; (2) Because preliminary analysis of the data showed that differences between feeds differed considerably across animals, we studied feed composition per DGAT1 genotype using the paired nature of the experimental design (the same cows received both feeds) using a paired T-test approach, and (3) differences per DGAT1 genotype within one feed using an unpaired T-test approach. A higher stringency ANOVA analysis was performed using the function AOV of the R-package STATS. Two models were used: (1) Test for feed composition effects: $y = \mu + \text{feed composition} + e$ (where y = phenotype; μ = overall mean; e = residual) (Single-factor ANOVA); (2) Test for feed composition and DGAT1 genotype: $y = \mu + \text{feed composition} + \text{DGAT1 genotype} + e$ (Two-factor, no interaction ANOVA). For the DGAT1 genotype analysis the animals were grouped together per genotype. For all statistics: $P < 0.05$ was considered significant.

Biological functional analysis was done for all significant identified metabolites using available databases on the internet, e.g. KEGG (Kyoto Encyclopedia of Genes

and Genomes; <http://www.genome.ad.jp/kegg/> [27]) and Gene Ontology (<http://geneontology.org/>, 28).

Results

Identified polar metabolites in the milk of mid-lactation cows

The ¹H NMR spectra showed 83 identifiable peaks (Table 2), of which the identity of six peaks remained unknown. Since several metabolites were characterized / represented by more than one peak, in total 57 different metabolites were identified. Several of these metabolites are related to each other – e.g. phosphorylated and non-phosphorylated forms. After correction for multiple forms of a metabolite, 49 different polar metabolites were detected in the milk samples. Two of them were identified only as “a sugar”, without further specification. The integrals harboring the unidentified peaks were included in the analyses to evaluate the effects of diet and DGAT1 genotype. Interestingly, the databases indicated that several of the metabolites were derived from bacterial metabolism, e.g. Xantine, N-acetyl-glucosamine, and butyrate.

Associations between polar metabolite concentrations in the milk and diet

Table 3 shows the associations between identified metabolites and diet. The Supplementary Table S1 which is related to Table 3, shows all associations including those of the unidentified integrals of the ¹H NMR spectra. More specifically, Table 3 shows the association between the measured milk polar metabolite levels and the feed composition of the two diets with different fat composition. If more than one *P*-value is expressed in Table 3, the associations stretches over more than one integral and the integrals were taken together. After the first analysis, we included a high-stringent

analysis to confirm the associations. As expected, the number of associations in this high-stringency analysis was lower than in the first analysis, but integrals that related to the same metabolites were found in both analyses. Remarkably, many of the observed associations differed between the animals indicating an animal-specific component in the associations.

Effects of Diacylglycerol O-acyltransferase 1 genotype

Using the same ^1H NMR measurements the effects of the DGAT1 gene genotypes on the polar metabolite levels were studied. Table 4 and Supplementary Table S2 which is related to Table 4 show the associations between the two DGAT1 genotypes and the content levels of polar metabolites. More specifically, Table 4 shows the association between the measured milk polar metabolite levels differently expression among the three DGAT1 genotypes. Differences between the genotypes for polar metabolite levels in the milk were observed for both diets, but more for the experimental diet than the standard diet. The association between polar metabolites and DGAT1 genotype did not reveal any animal effects. Please note that the high stringent analysis indicated associations for a number of the associations found also with the low stringent analysis, but this high stringent analysis was unable to specify the genotypes.

Effect of Diacylglycerol O-acyltransferase 1 genotype on dietary-induced milk polar metabolite levels

In a next step, we studied the effect of the DGAT1 genotype on dietary-induced milk polar metabolite concentrations in order to investigate the interaction between the diet and the DGAT1 genotype effects on the content levels of polar metabolites. Table 5 and

Supplementary Table S3 which is related to Table 5 show that the DGAT1 A-allele displays more interactions with the diet than the K-allele. More specifically, Table 5 shows the association between the measured milk polar metabolite levels differently expression among the three DGAT1 genotypes specifically related to the interaction between the feed composition and the DGAT1 genotypes. Furthermore, the interaction between the diet and the DGAT1 genotype differed considerably between animals. The number of high-stringent analysis associations was low in this analysis, which suggests that the importance of the interaction between the diet and the DGAT1 genotype is relatively low.

Discussion

Dietary fats and milk polar metabolites

Previously it was demonstrated that dietary fatty acids affect the composition of non-polar metabolites in cow milk [5-7]. In this study we have identified a number of polar metabolites in cow milk that are related to dietary fat consumption. Together, this indicates that dietary fatty acids influence the entire cow's metabolism in such a way that it also affects a wide range of milk traits. This is in agreement with suggestions from several earlier studies [12], and [13, 20, 22] using these same samples.

Dietary and genetic influence on milk polar metabolite concentration

Feeding extra unsaturated fatty acids (UFA) to the cows was associated with changes in the concentration of several polar metabolites in the milk which were not directly related to the UFA. Thus, it may be concluded that the changed milk composition is related to changed metabolism – either of the cow, or of the gut or rumen bacteria of the cow.

Indeed, the results show examples of both. It has been reported that fatty acids may induce or inhibit the expression of many different gene (sets) and thereby change the metabolism of animals [10-11, 13-14].

General metabolism. In general, the concentration of most polar metabolites was lower in the milk of the animals with the UFA-supplemented diet as compared to the standard diet. Thus, UFA supplementation reduced the metabolism for polar metabolites of the animal. The UFA molecules are involved in many diverse metabolic pathways including energy metabolism. It is known that high energy intake affects animal metabolism rates [7]. However, importantly, lactose (milk sugar) showed increased levels in the milk of UFA supplemented cows. This suggests that the metabolic effects are specific and not general.

Energy metabolism and Nitrogen metabolism / urea cycle. The levels of ten polar metabolites with association to the dietary composition related to energy metabolism: succinate, formate, aconitate, lactate, galactose-1-phosphate, creatinine, carnitine, oxoglutarate, BHBA (beta-hydroxybutyrate), and butyrate. The concentrations of these metabolites were lower in the diet supplemented with the UFA suggesting that the effect of adding UFA to the diet is to reduce the rate of the cow's energy metabolism. This has been observed before [11, 15, 29]. It should be noted that butyrate is produced by the gut microbiota serving as an energy source for the colon cells metabolism rather than being produced by the cow's own metabolism. Effects of UFA in feed on ruminal metabolism has been reported before [5].

Hippurate and formate levels in milk were variable between dietary groups and animals, but no clear direction could be found. Aspartate showed reduced levels in the milk of UFA supplemented cows. We conclude that UFA supplementation did affect nitrogen metabolism in the cows, but probably the cow's genotype may specifically relate to the specific direction of the effect.

Animal-specific effects – the Diacylglycerol O-acyltransferase 1 genotype of cows

It is intriguing to note that the animals differed considerably for the majority of the associations. This indicates that the reaction of the animals to the diet was very different, so animal specific components such as genotype may be determining factors for dietary responses. The associations between metabolite concentrations and DGAT1 genotype did not show this animal-specific effect. Therefore, we suggest that the genetic component differentiating between animals may be the DGAT1 genotype, a new feature of this genotype. Although the number of cows per DGAT1 genotype is low, different types of analyses consistently indicate the same metabolites with different profiles per DGAT1 genotype. Furthermore, these differences are also consistent with previously reported general DGAT1 genotype effects. Therefore, these results can be trusted. DGAT1 genotype was already known to be related to milk yield and milk fat percentage and composition [8]. These DGAT1 genotype effects may also relate to the cow's metabolism or gut and rumen microbiome metabolism rate, and may therefore relate to this new feature of the DGAT1 genotype that we describe here. This feature may perfectly fit into the biological function of DGAT1, which regulates the final step before uptake of fat in the intestine [9]. Thus, the two genotypes of the DGAT1 gene may differ in the amount of uptake of fatty acids in the intestine, thereby differentially regulate the

animal's metabolism including energy metabolism leading to different milk yield and composition. Furthermore, Sorensen *et al.* [30] showed DGAT1 genotype effects on DGAT1 activity. This is a further argument that the DGAT1 genotype may also affect milk composition via regulation of the genome activity via regulation of the intestinal fatty acid uptake.

With the exception of Hippurate, valine, and Uridine-conjugate the levels of the metabolites in the milk were higher in animals with more DGAT1 A-alleles than in animals with more K-alleles (i.e. comparing the metabolite levels in the milk of animals with the AA-genotype with the milk metabolite levels of animals with the AK and KK genotypes, and comparing the metabolite levels in the milk of animals with the AK-genotype with the milk metabolite levels of animals with the KK genotype). This suggests that the DGAT1 genotype associates with the rate of metabolic processes. Metabolites showing association between metabolite levels and DGAT1 genotype were involved in energy metabolism and nitrogen metabolism including amino acid and pyrimidine metabolism pathways. On the other hand, proline shows the opposite content pattern in relation to the DGAT1 genotype, so we cannot conclude that amino acid metabolism is higher regulated to the K-allele than the A-allele. High stringency analysis confirmed several of the associations. Allele-specific effects on amino acid metabolism were not previously observed. General genotype-specific effects on amino acid plasma levels, which could be related to amino acid metabolism, have been reported before [31]. Such plasma level variations could affect amino acid composition in the milk, differentiating animals, which we detected in our metabolomics analyses.

Interactions between the effects of diet and the effects of DGAT1 genotype were prominent. Above we noted that most metabolites associated with diet showed lower

levels of the metabolites in the milk of animals fed the UFA-supplemented diet as compared to the control diet. However, here it is interesting to note that the opposite content pattern was mainly observed in animals with the AK-DGAT1-genotype. Grisart *et al.* [8] reported the lack of dominance effects for the DGAT1 alleles. Our results indicating the opposite content pattern in the heterozygous animals may relate to this, since both homozygous genotype animals showed the opposite content pattern. Alternatively, this result could indicate again a direct influence of the DGAT1 genotype on cow or rumen bacteria metabolism.

Indeed, the KEGG and other databases used identified several metabolites as compounds related to (unspecified) bacterial metabolism, e.g. Xantine (microbial metabolism in diverse environments) and N-acetyl-glucosamine (bacterial cell wall component), and butyrate (known for gut microbiome metabolism – it is an energy source for the colon epithelial cells). Since these metabolites are exclusively synthesized by bacterial metabolism and cannot be synthesized by a mammalian metabolism, finding these metabolites in the cow's milk may indicate a direct connection between the cows DGAT1 genotype and the rumen microbiota metabolic rate. An interaction between animal's genotype and the rumen bacterial population has been observed before [32]. Dietary composition effects on the gut bacterial composition has also been shown before [32-34]. So, this result may relate to both of these effects and may further show the importance of the interaction between the cow's genotype and the rumen bacterial population metabolic activity. In conclusion, we showed that both diet and DGAT1 genotype affect polar metabolite concentration in cow milk. Specifically, we showed effects on energy metabolism and nitrogen metabolism in the cow, and bacterial metabolism in the gut bacteria. Our data suggest that both cow metabolism and rumen

microbiota metabolism are important for the milk polar metabolite concentrations, and that a direct interaction between the cow's DGAT1-genotype and the activity of the rumen bacterial metabolism is important for the cow's milk polar metabolite composition. The interaction between diet and DGAT1-genotype also affect the cow's milk polar metabolite composition.

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Table 1. Diet compositions. Control feed is a standard fodder. Enriched feed is standard fodder supplemented with rapeseed, soybean and linseed oil (Data taken from [13, 23]). Mid-lactation cows were fed the control feed and the enriched feed for 10 weeks each.

Item	Control feed	Enriched feed ^a
Ingredients in the mixed rations (g/kg)		
Corn silage	520	520
Grass silage	120	120
Concentrate ^a	360	360
Ingredients in the concentrates (g/kg)		
Triticale	—	333.6
Rapeseed meal, formaldehyde treated	—	195
Soybean meal, formaldehyde treated	—	102
Dried brewers grain	—	79
Rapeseed oil	—	25
Soybean oil	—	25
Linseed oil	—	25
Rapeseed meal	108.1	68
Citrus pulp	370	60
Soybean meal	—	47
Palm kernel expeller	227.3	—
Sugar beet molasses	101	—
Coconut expeller	64.8	—
Wheat gluten feed	45	—
Wheat middlings	31	—

Soybean hulls	29.3	–
Protapec ^b	13.7	–
Calcium carbonate	2	16.3
Sodium chloride	2.4	10.5
Magnesium oxide (90%)	3.4	4.8
Urea	–	4.5
Monocalcium phosphate	–	2.3
Vitamin–mineral premix	2	2

a: Including the concentrate supplied through the automatic feeding station

b: Concentrated potato fruit juice mixed with soybean hulls.

Table 2. 1-Hydrogen Nuclear magnetic resonance detected polar metabolites in Holstein Friesian milk identified by their peak position in the 1-Hydrogen Nuclear magnetic resonance spectra.

Position spectra	Metabolite
8.457	Formate
8.142	Xanthine (putative)
7.877	Uridine
7.877	Uridine conjugate (putative) ; xanthine
7.840	Hippurate
7.644	Hippurate
7.557	Hippurate
6.526	Fumarate
6.525	Aconitate
6.198	Orotate
6.073	Maleate (putative)
5.944	Uridine conjugate
5.918	Uridine conjugate
5.909	Uridine conjugate
5.522	Galactose-1-phosphate
5.510	Galactose-1-phosphate
5.439	Sugar-phosphate
5.425	Sugar-phosphate
5.400	Sugar-phosphate
5.388	Sugar-phosphate
5.240	Lactose

4.592	Xylose (putative)
4.592	Galactose
4.517	Ascorbate (putative)
4.458	Lactose
4.271	Unknown
4.234	Uridine conjugate
4.223	UDP ^a
4.143	Glucolactone (putative)
4.125	Phosphoserine (putative)
4.115	Lactate
3.611	Phosphoglycerol (putative)
3.495	Galactose
3.475	Glucose
3.433	Acetoacetate
3.380	Unknown
3.286	Myo-Inositol (putative)
3.267	Betaine (putative)
3.266	Betaine
3.229	Lactose (putative)
3.229	Glycerophosphocholine
3.221	Choline ; Phosphocholine ; Glycerophosphocholine
3.221	Acetylcarnitine
3.203	Choline ; Phosphocholine ; Glycerophosphocholine
3.195	Acetylcarnitine ; isovalerylcarnitine (putative)
3.156	Unknown

3.133	Malonate (putative)
3.112	Unknown
3.056	Creatinine
3.040	Creatine-phosphate
3.010	Lysine
2.818	Aspartate
2.725	Unknown
2.698	Citrate
2.626	Citrate
2.556	Citrate
2.464	Carnitine
2.451	Oxoglutarate
2.445	Carnitine
2.424	Succinate
2.386	Pyruvate (putative)
2.377	Oxaloacetate
2.361	Proline (putative)
2.343	Proline (putative)
2.236	Glutamate
2.147	Acetylcarnitine; butyrate (putative)
2.070	N-acetylcarbohydrate gamma
2.068	N-acetylglucose (putative) ; galactosamine
2.059	N-acetylcarbohydrate beta
2.044	N-acetylcarbohydrate alpha
2.044	N-acetylmannosamine (putative) ; neuraminate

1.929	sialolactose ; lactose (total)
1.928	Acetate
1.563	Butyrate (putative)
1.483	Alanine
1.333	Lactate
1.204	BHBA ^b
1.044	Valine
0.933	Ethanol
0.933	Pantothenate (putative)
0.898	Butyrate
0.877	Butyrate
0.166	Unknown

a: Uridine diphosphate; b: Beta-hydroxybutyrate.

Table 3. Association between mid-lactation cow milk polar metabolite levels detected and quantified by 1-Hydrogen Nuclear magnetic resonance spectroscopy and diet with different fat composition. Mid-lactation cows were fed a standard fodder or a standard fodder supplemented with rapeseed, soybean, and linseed oil for 10 weeks each.

Metabolite	Spectra position range	P-value Feed (unpaired) ^a	P-value Feed (paired) ^b	P-value Stringent analysis ^c	Relative Residual Standard Deviation ^d
Formate	8.54-8.38		0.013-0.035		
Xanthine (putative)	8.14-8.1		0.024-0.041		
Hippurate	7.74-7.54	0.034-0.043	0.005-0.041		
Aconitate	6.62-6.34	0.012-0.031		0.012-0.034	0.08-0.84
Galactose-1-phosphate	5.02-5.58	0.04-0.046	0.049		
Lactose (total)	4.38-5.22	0.012	0.015-0.046	0.023	0.00-0.66
Lactate	4.1-4.02	0.009-0.33	0.011-0.037	0.016	0.53-0.65
Galactose	3.7-3.62		0.043-0.046		
Creatinine	3.06	0.008	0.02-0.05	0.16	0.38
Aspartate	2.98-2.78		0.001-0.023		

Carnitine ; Oxoglutarate	2.46-2.38		0.008-0.05		
; Succinate					
BHBA ^e	1.26-1.02	0.005-0.037	0.0002-0.02	0.009-0.045	0.46-0.84
Butyrate	0.94	0.033		0.028	0.23

a: Analyzed the dietary groups; b: Analyzed the metabolite levels between the two diets within each animal individually; c: High stringent statistical analysis using model 1; d: The control diet was set to 1.00; e: Beta-hydroxybutyrate

Table 4. Association between mid-lactation cow milk polar metabolite levels detected and quantified by 1-Hydrogen Nuclear magnetic resonance spectroscopy and diet with different fat composition. Mid-lactation cows were fed a standard fodder or a standard fodder supplemented with rapeseed, soybean, and linseed oil for 10 weeks each. The analysis compared the milk metabolite levels between Diacylglycerol O-acyltransferase 1 genotypes and diets.

Metabolite	Spectra position range	Genotype ^a	P-value (unpaired) ^b	Association trait ^c	P-value (paired) ^d	Stringent analysis ^e	P- value ^e	Relative Residual Standard Deviation AK ^f	Relative Residual Standard Deviation KK ^f
Hippurate	7.66	AK-AA (E)	0.016						
Uridine conjugate	4.22	KK-AK (E) ; AK-AA (E)	0.041 ; 0.025						
Lactate	4.1-4.06	KK-AA (E)	0.043-0.045			DGAT1	0.018- 0.023	150.14-197.38	55.41-127.43
Proline (putative)	2.34	KK-AA (C)	0.032-0.018						
Valine	1.06			AA-diet	0.018	DGAT1	0.015	259.88	156.37

a: DGAT1 genotypes with significant different milk polar metabolite levels, E: experimental diet (i.e. supplemented with oils), C: Control diet (i.e. standard fodder); b: Analyzed the dietary groups; c: Association between DGAT1 genotype and diet; d: Analyzed the metabolite levels between the two diets within each animal individually; e: High stringent statistical analysis using model 2; f: The value of the AA genotype was set to 1.00.

Table 5. Association between mid-lactation cow milk polar metabolite levels detected and quantified by 1-Hydrogen Nuclear magnetic resonance spectroscopy and diet with different fat composition. Mid-lactation cows were fed a standard fodder or a standard fodder supplemented with rapeseed, soybean, and linseed oil for 10 weeks each. The analysis compared the interaction between milk metabolite levels and diet-Diacylglycerol O-acyltransferase 1 genotype.

Metabolite	Spectra position range	Diet – DGAT1 genotype association	P-value (unpaired) ^a	Diet (paired) ^b	P-value (paired) ^b	Stringent analysis DGAT1 diet ^{c, d}	P-value ^c	Relative Residual Standard Deviation ^e
Formate	8.62- 8.42			AA ; AK	0.02- 0.45 ; 0.026			
Xanthine (putative)	8.14- 7.98			KK ; AA	0.049 ; 0.03- 0.042			
Xanthine ; Uridine conjugate (putative)	7.9	AA	0.016- 0.023	AA	0.05			

Hippurate	7.82-7.5	AA	0.002- 0.039	AA	0.002- 0.035 ; 0.004- 0.012			
Aconitate	6.62- 6.58					DGAT1	0.018-0.047	0.18-0.81
Galactose-1- phosphate ; Sugar Phosphate	5.02- 4.98			AA	0.012- 0.013			
Galactose (putative) ; Lactose (putative)	4.5-4.46			AK	0.0004- 0.015			
Uridine conjugate	4.26- 4.18	AA	0.015- 0.017	AA	0.002- 0.005			
Lactate	4.1-4.02	AA	0.032			DGAT1	0.003-0.044	0.64-0.81
Betaine	3.26			AA	0.05			

Choline ;	3.18			AA ; KK	0.048 ;
Phospho- choline					0.05
Creatine	3.02-			AK	0.02-
phosphate	2.94				0.05
(putative) ;					
Lysine					
Aspartate	2.82-	AA	0.024-	AA ; AK	0.044 ;
	2.28		0.049		0.037-
					0.041
Carnitine ;	2.46-	AA	0.03-0.04	AA	0.038-
Oxoglutarate	2.42				0.039
N-acetyl	2.1-1.98	AK-AA (E) /	0.042 /	AK	0.044
galactosamine		AA	0.014-		
(putative) ; N-			0.044		
acetyl					
glucosamine ;					
Neuraminate					

Lactose	1.94-1.9		AA	0.006-			
(total) ;				0.039			
sialolactose							
Butyrate	1.7-1.54;	AA	0.001-0.41	AK ; AA	0.033 ;		
(putative)	1.7-1.06				0.023-		
					0.047		
BHBA ^f	1.26-		AA	0.013-	DGAT1	0.002-0.045	0.33-0.61
	1.02			0.046			
Valine	1.06		AA	0.018	DGAT1	0.015	0.42
(putative)							
Butyrate	0.94-		AA ; AK ;	0.006-			
	0.82		KK	0.015 ;			
				0.028-			
				0.044 ;			
				0.024-			
				0.045			

a: Analyzed the dietary groups; b: Analyzed the metabolite levels between the two diets within each animal individually; c: High stringent statistical analysis using model 2; d: DGAT1: Diacylglycerol O-acyltransferase 1 e: The control diet was set to 1.00; f: Betahydroxybutyrate

Supplementary Table 1

Supplementary Table related to Table 3. Association between mid-lactation cow milk polar metabolite levels detected and quantified by 1-Hydrogen Nuclear magnetic resonance spectroscopy and diet with different fat composition. Mid-lactation cows were fed a standard fodder or a standard fodder supplemented with rapeseed, soybean, and linseed oil for 10 weeks each. This supplementary Table also shows the associations with unidentified integrals of the 1-Hydrogen Nuclear magnetic resonance spectra.

Metabolite ^a	Spectra position range	P-value Feed (unpaired) ^b	P-value Feed (paired) ^c	P-value Stringent analysis ^d	Relative Residual Standard Deviation ^e	
Formate	9.1-8.96	0.049	0.015-0.045	0.036	0.65	
	8.86-8.62		0.020-0.047			
	8.54-8.38		0.013-0.035			
	8.26		0.029			
Xanthine (putative)	8.14-8.1	0.034-0.043	0.024-0.041	0.036	0.65	
Hippurate	8.02		0.005-0.41			
Hippurate	7.7-7.62					0.002-0.006
	7.74-7.54					
	7.42-7.38					
	7.42-6.34					
	7.3	0.02-0.041		0.036	0.65	
	7.34-7.22		0.036-0.043			
	7.02-6.98					0.016-0.046
	6.9-6.7					
	6.82-6.74		0.03-0.046	0.03-0.046	0.69-0.82	
Aconitate	6.62-6.58		0.012-0.034			0.012-0.034
Aconitate	6.62-6.34	0.012-0.031				
	6.34				0.044	0.18
Galactose-1-phosphate	5.62-5.58	0.04-0.046				
Galactose-1-phosphate	5.02		0.049			
Galactose ; Lactose (total)	4.5-4.38		0.015-0.046			

	4.42-4.38	0.033-0.046			
	4.18	0.018	0.02	0.026	0.41
Lactate	4.1-4.02	0.009-0.33	0.011-0.037	0.016	0.53-0.65
	3.94-3.86	0.006-0.014	0.009-0.22	0.013-0.025	0.65-0.92
Galactose	3.7-3.62		0.043-0.046		
Creatinine	3.06		0.05		
Creatinine	2.98-2.94	0.014-0.023		0.027-0.043	0.83-0.87
	2.94			0.042	0.83
Aspartate	2.98-2.78		0.001-0.023		
Carnitine ; Oxoglutarate	2.46-2.38		0.008-0.017		
Carnitine ; Oxoglutarate ; Succinate	2.3-2.02		0.001-0.038		
	2.26-2.22	0.045-0.046			
	2.26-2.02		0.001-0.015		
	1.9		0.004		
	1.82-1.42		0.003-0.021		
BHBA ^f	1.3-1.02	0.005-0.037	0.0002-0.02	0.009-0.045	0.46-0.81
BHBA ^f	1.26-1.14			0.01	0.61-0.81
Ethanol	0.94	0.033			
	0.02		0.006		

a: Open cells indicate unknown metabolites; b: Analyzed the dietary groups; c: Analyzed the metabolite levels between the two diets within each animal individually; d: High stringent statistical analysis; e: The control diet was set to 1.00; f: Beta-hydroxybutyrate.

Supplementary Table 2

Supplementary Table related to Table 4. Association between mid-lactation cow milk polar metabolite levels detected and quantified by 1-Hydrogen Nuclear magnetic resonance spectroscopy and diet with different fat composition. Mid-lactation cows were fed a standard fodder or a standard fodder supplemented with rapeseed, soybean, and linseed oil for 10 weeks each. The analysis compared the milk metabolite levels between Diacylglycerol O-acyltransferase genotypes and diets. This supplementary Table also shows the associations with unidentified integrals of the 1-Hydrogen Nuclear magnetic resonance spectra.

Metabolite ^a	Spectra position range	Genotype ^b	P-value (unpaired) ^c	Animal-diet relation ^d	P-value (paired) ^e	Stringent analysis ^f	P-value ^f	Relative Residual Standard Deviation AK ^g	Relative Residual Standard Deviation KK ^g
Hippurate	7.66	AK-AA (E)	0.016						
	7.5-7.46	KK-AA (E)	0.008-0.031						
	7.46	KK-AK (E)	0.031						
	7.5	AK-AA (E)	0.002						
Uridine conjugate (putative)	4.98	AK-AA (E)	0.006						
Uridine conjugate	4.22	KK-AK (E)	0.041						
Uridine conjugate	4.22	AK-AA (E)	0.025						
	4.18					DGAT1	0.037	313.32	109.33
Lactate	4.1-4.06	KK-AA (E)	0.043-0.045						
Lactate	4.1-4.06					DGAT1	0.018-0.023	150.14-197.38	55.41-127.43
	3.46	KK-AK (E)	0.049						
Proline (putative)	2.34	KK-AA (C)	0.018						
	1.14					DGAT1	0.036	167.89	346.96
Valine (putative)	1.06			AA-diet	0.018	DGAT1	0.015	259.88	156.37

a: Open cells indicate unknown metabolites; b: DGAT1 genotypes with significant different milk polar metabolite levels, E: experimental diet (i.e. supplemented with oils), C: Control diet (i.e. standard fodder); c: Analyzed the dietary groups; d: Association between DGAT1 genotype and diet; e: Analyzed the metabolite levels between the two diets within each animal individually; f: High stringent statistical analysis; g: The value of the AA genotype was set to 1.00.

Supplementary Table 3

Supplementary Table related to Table 5. Association between mid-lactation cow milk polar metabolite levels detected and quantified by 1-Hydrogen Nuclear magnetic resonance spectroscopy and diet with different fat composition. Mid-lactation cows were fed a standard fodder or a standard fodder supplemented with rapeseed, soybean, and linseed oil for 10 weeks each. The analysis compared the relation between milk metabolite levels and diet- Diacylglycerol O-acyltransferase genotype. This supplementary Table also shows the associations with unidentified integrals of the 1-Hydrogen Nuclear magnetic resonance spectra

Metabolite ^a	Spectra position range	Diet – DGAT1 genotype association	P-value (unpaired) ^b	DGAT1 genotype animal-specificity diet	P-value (paired) ^c	Stringent analysis DGAT1 – diet ^d	P-value ^d	Relative Residual Standard Deviation ^e
Formate Formate	9.06			AA	0.043			
	8.62-8.5	AA	0.029-0.046					
	8.62-8.42			AA	0.02-0.45			
	8.34	AA	0.03	AA	0.04			
Xanthine (putative)	8.26-8.22			AA	0.018-0.038			
	8.14-7.98			AA	0.03-0.042			
Xanthine ; Uridine conjugate (putative)	8.06	AA	0.049					
	7.9	AA	0.0160.023	AA	0.05			
Hippurate	7.9-7.5			AA	0.002-0.035			
Hippurate	7.82-7.5	AA	0.002-0.039					
Hippurate	7.74			AA	0.004			
	7.42-7.3	AA	0.005-0.032	AA	0.005-0.027			
	7.22-6.62	AA	0.003-0.042	AA	0.023-0.035			
	6.82					DGAT1	0.046	0.62
	6.74					DGAT1	0.047	0.82
	6.74-6.62			AK	0.002-0.018			
Aconitate	6.62-6.58					DGAT1	0.018-0.047	0.18-0.81

Galactose-1-phosphate ; Sugar-phosphate beta	5.02-4.98			AA	0.012-0.013			
Galactose (putative) ; Lactose (putative)	4.5-4.46			AK	0.0004- 0.015			
	4.42			KK	0.024			
	4.42-4.34	KK	0.002-0.19					
	4.38					DGAT1	0.044	0.53
	4.34-4.3			KK	0.017-0.047			
Uridine conjugate	4.26-4.22			AA	0.002-0.005			
Uridine conjugate (putative)	4.22-4.18	AA	0.015-0.017					
	4.18					DGAT1	0.009	0.70
Lactate	4.1	AA	0.032					
Lactate	4.1-4.02					DGAT1	0.003-0.044	0.64-0.81
	3.94-3.86					DGAT1	0.009-0.014	0.74-1.03
	3.78-3.62			AA	0.0004- 0.034			
	3.74			AK	0.045			
	3.54			AA	0.00009			
Betaine	3.26			AA	0.05			
Phosphocholine ; Glycerophosphocholine	3.18			KK	0.05			
	3.1			AA	0.015			
	3.1			AK	0.028			
Creatine phosphate (putative) ; Lysine	3.02-2.94			AK	0.02-0.05			
	2.98	AK	0.036					
	2.94	AA	0.041			DGAT1	0.049	0.86
Aspartate	2.82	AA	0.049	AA	0.044			
Aspartate	2.82			AK	0.037			
	2.28	AA	0.024	AK	0.041			

Carnitine ; Oxoglutarate ; Succinate	2.46-2.42	AA	0.03-0.04	AA	0.038-0.039			0.77-0.88
Succinate	2.26-2.22	AA	0.027-0.034					
N-acetyl galactosamine (putative) ; N-acetyl glucosamine ; Neuraminate	2.1-1.98	AA	0.014-0.044					
Lactose (total) ; sialolactose	1.94-1.9			AA	0.031-0.039			
	1.82-1.78	AA	0.024-0.043					
	1.82-1.42			AA	0.007-0.031			
Butyrate (putative)	1.7-1.54; 1.7-1.06	AA	0.001-0.41	AA	0.023-0.047			
BHBA ^f	1.34-1.14			AA	0.013-0.046			
BHBA ^f	1.26-1.02					DGAT1	0.002-0.045	0.33-0.61
Valine (putative)	1.06			AA	0.018	DGAT1	0.015	0.52
Ethanol ; Butyrate	0.94-0.86			AA	0.006-0.015			
	0.7			AK	0.032			
	0.3--0.06			AA	0.003-0.038			
	-0.02	AA	0.049					

a: Open cells indicate unknown metabolites; b: Analyzed the dietary groups; c: Analyzed the metabolite levels between the two diets within each animal individually; d: High stringent statistical analysis; e: The control diet was set to 1.00; f: Betahydroxybutyrate