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Content and Distribution of Phytanic Acid Diastereomers in Organic Milk As Affected by Feed Composition

Brita N. Che,[†] Troels Kristensen,[‡] Caroline Nebel,[†] Trine K. Dalsgaard,[†] Lars I. Hellgren,[§] Jette F. Young,[†] and Mette K. Larsen*[,†]

[†]Department of Food Science and [‡] Department of Agroecology, Aarhus University, AU Foulum, Blichers Allé 20, DK-8830 Tjele, Denmark

[§]Department of System Biology, Center for Biological Sequence Analysis, Technical University of Denmark, Kemitorvet, Building 208, DK-2800 Kgs. Lyngby, Denmark

ABSTRACT: Phytanic acid (PA) is a bioactive compound found in milk that is derived from the phytol chain of chlorophyll, and the content of PA in milk fat depends on the availability of phytol from feed. In this study, the content of PA diastereomers was analyzed in milk sampled from five organic herds twice during the grazing season (May and September). The total content of PA was higher in September compared to May, but was not affected by breed (Danish Holstein or Danish Jersey). Total PA could not be directly related to intake of green feed items. The distribution between diastereomers was closely related to the amount of grazed clovers, where a higher intake resulted in a higher share of the *RRR* isomer.

KEYWORDS: *organic milk, phytanic acid diastereomers, pasture, clovers, silage*

INTRODUCTION

Recent findings point to an inverse correlation between increased dairy consumption and a cluster of parameters that promote the prevalence of cardiovascular diseases, the metabolic syndrome,[1,2] even though dairy products are a rich source of saturated fatty acids.[3] This apparent conflict has directed the focus toward the search for components in milk that are responsible for counteracting the adverse effects of the metabolically detrimental saturated fatty acids. One such component is 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid (PA)), a saturated branched-chain fatty acid (see Figure 1).

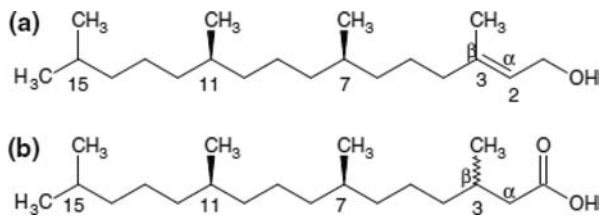

Figure 1. Structures of phytol (a) and phytanic acid (b) with the methyl group of carbon 3 of phytanic acid existing in either an *S* or *R* configuration (numbers denote carbon atom position from the carboxyl terminal). Adapted from Schröder et al.[15].

In the human food chain, PA is primarily found in dairy products, ruminant meat, and some marine fats.[4] PA is formed from phytol, which is cleaved from chlorophyll and subsequently oxidized by the rumenal microbiota and certain marine organisms.[5,6] The formation of PA involves an oxidation of the alcohol moiety, as well as a biohydrogenation of the double bond in phytol, but the order of these reactions is not clear.[6]

As PA is derived from chlorophyll, the content in milk fat is dependent on the feed composition, and increasing the amounts of green feed increases the content of PA. Due to the higher use of grass-based products, PA content is higher in organic, compared to conventional, milk and a threshold value of the PA content in milk fat of at least 200 mg/100 g has been suggested as a marker of organic milk products.[7]

PA and its primary metabolite, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), are natural agonists of the peroxisome proliferator activated receptor-α (PPARα) and the retinoid X receptor (RXR).[8] As RXR and PPARα are known to control fatty acid and glucose metabolism,[9] PA has been suggested to have health-improving properties and a protective effect on the metabolic syndrome.[8,10] Heim et al.[11] have shown that PA can increase glucose uptake in rat primary hepatocytes. Recently, we observed that PA can increase glucose uptake in primary porcine myotubes (unpublished data), although there was no concomitant increase in glycogen synthesis.

PA has three chiral centers positioned on carbons 3, 7, and 11(Figure 1). Carbons 7 and 11 are *R*-configured as in phytol, whereas carbon 3 is either *R*- or *S*-configured as a result of the biohydrogenation of oxidized phytol. As such, PA occurs naturally as two diastereomers; *SRR* PA and *RRR* PA.[5] The distribution profile of the PA diastereomers in lipid fractions in serum varies, with s higher *SRR*/*RRR* ratio in phospholipids than in other lipid fractions.[12] Moreover, the biological activities of the diastereomers of PA in humans differ. At higher PA concentrations, the oxidation of *SRR* PA is preferred by up to 50%, compared to the *RRR* form,[13] whereas at lower PA concentrations, both forms exhibit similar oxidation rates.[11,13] The initial step in PA oxidation is peroxisomal α-oxidation to pristanic acid, which is further degraded by β-

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oxidation.[14] The β-oxidation is stereospecific and requires α-methylacyl-CoA racemase (AMCAR) to convert 2*R*-pristanic acid to its 2*S* isomer.[14] These differences in the oxidation and distribution profiles of PA diastereomers hint toward potential variation in their physiological functions and warrant an elucidation of their origin in ruminants.

The distribution of the diastereomers of PA varies in food, with the *SRR* form predominant in marine animals and some terrestrial mammals having more of the *RRR* form.[5,15] The distribution between the diastereomers varies between bovine milks of different origin, where organic farming gives higher shares of the *RRR* isomer.[15,16] On the basis of these results it has been suggested that the threshold value of 200 mg/100 g PA in organic milk fat[7] should be replaced by a combination of total PA content and the ratio between the diastereomers for authentication of organic milk.[16]

Organic dairy management varies between countries due to tradition as well as climatic differences.[17] In Danish organic dairy farming, there is a compulsory use of grazing when climatic conditions allow, which is in accordance with the definition in the EC regulations;[18] however, the extent of grazing varies considerably between farms. Other main feed items include grass and maize silage and concentrates.[17] This feed composition differs significantly from the ratios of pure hay or pure grass silage investigated by Schröder et al.,[16] and PA concentrations in milk fat may be lower as the content of grass-based feed is lower.

The present study was carried out as a survey of five Danish commercial organic herds in the grazing season, and the purpose was to test whether the suggested target value of 200 mg/100 g PA in milk fat[7] could be met, to investigate how the content of the diastereomers of PA varied in bulk milk, and to test the hypothesis that these differences could be related to specific feeding patterns.

MATERIALS AND METHODS

The experiment was conducted at five Danish commercial organic dairy herds (100 ± 17 lactating cows per herd). Registration of feed consumption and milk production was made during two periods of 14 days within the grazing season; the first period started in spring about 2 weeks after start of grazing, and the second period started in late summer at the end of August. Milk was sampled from the bulk tank on two consecutive days at the end of each period. Milk was sampled in the morning and consisted of milk from the morning milking and the preceding afternoon milking.

Herds and Feeding. Three herds (DH1, DH2, and DH3) were Danish Holstein cows (DH), whetrsd the two other herds (DJ1 and DJ2) were Danish Jersey (DJ). All farms practiced calving all year round, and all lactating cows participated in the experiment, as the herd was the experimental unit. Feeding of supplemental feed as roughage (grass silage, maize silage, hay, or straw) or a mix of roughage and concentrate wsd applied indoors at the feeding lane with one feeding space to each cow, whereas concentrate (commercial mix and cereals) in all herds was applied in the milking parlor, divided in equal amounts and given twice daily.

Pastures. At all farms, pastures dominated by perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) were part of a cropping rotation with arable crops. Rotations were typically based on 3-year leys established by reseeding cereals with grass clover mixtures.

Registration and Calculation of Feed Intake. Milk yield was recorded once in each period for each cow, and the contents of fat, protein, and urea were analyzed (Milkoscan 4000, Foss Electric A/S, Hillerød, Denmark). Milk yield as energy-corrected milk (ECM) was calculated according to the method of Sjaunja et al.,[19] and average contents of fat, protein, and urea at herd level were calculated on the basis of the milk recordings from each lactating cow.

Assessment of the pasture was made three times during each period. Samples were collected in 25 places at each farm by hand-plucking the pasture at the height at which cows were observed to graze. The botanical composition of the dry matter (DM) was determined after drying at 60 °C to constant weight. The relative amounts of the individual species were recorded, and the relative amount of clovers was calculated as the sum of white clover and red clover.

Three times during each period, the mass intake of supplemental feed over a 1 day period was determined at herd level. Feed samples of concentrates and roughage were taken once during each period. Each sample of roughage and supplemental feed was analyzed by near-infrared reflectance spectroscopy. Net energy value of lactation (NE) was estimated on the basis of the Hvelplund et al.[20] method.

The energy requirements for maintenance, grazing activity, lactation, and live weight change were calculated as described by Macoon et al.[21] on the basis of animal performance data at group level, as the intake of the indoor feed was registered at group level. Pasture DM intake (DMI) was estimated as the difference between the requirement and NE in supplemental feed at group level divided by the NE concentration of the hand-plucked samples.

The DMI of the different species in the pasture was estimated proportionally to the botanical analysis of the hand-plucked samples assuming no selectivity. DMI of individual feed items was expressed as average relative distribution (kg DM/kg total DMI) at herd level for each period.

Fatty Acid Extraction from Milk. Milk fat was extracted as described previously with some modifications.[22] Milk (10 mL) was centrifuged at 1700*g* at 4 °C for 20 min and the cream phase transferred to a new tube. The cream was centrifuged for 10 min at 13000*g* at 20 °C and afterward placed on a 60 °C heater for 10 min. The heated cream was then centrifuged for 10 min at 13000*g* at 40 °C, and fat was sampled from the liquid fat layer. A volume of 15 mg of fat was weighed out and mixed with 1 mL of heptane containing 0.4 mg/mL of C12:1 *cis* 11 triglyceride (99% purity; Nu-Chek-Prep Inc., Elysian, MN, USA) as an internal standard (IS). For methylation, 10 μL of a 2.2 M sodium methylate was added. The mixture was vortexed for 1 min and allowed to stand for 10 min at room temperature. The methylated mixture was centrifuged for 5 min at 13000*g* at 4 °C and the heptane phase collected for GC-MS analysis.

GC-MS Analysis of Methyl Esters of PA Diastereomers. Methylated milk fat extracts were analyzed in detail for PA isomers on a GC-MS (GC 6890N from Agilent Technologies (Waldbronn, Germany)) equipped with a Restek 2560 column (100 m × 0.25 mm × 0.20 μm, distributed by VWR and Bie and Berntsen, Herlev, Denmark). A 1 μL aliquot of the extracts was injected in split mode (20:1) into an inlet with a temperature of 250 °C. The programmed column temperature was isothermal at 100 °C for 5 min followed by an increase to 140 °C at a rate of 3 °C/min, a hold time of 5 min, followed by a raise to 160 °C at a rate of 5 °C/min, and a hold time for 20 min. Subsequently, the temperature was raised to 220 °C at a rate of 12 °C/min and held there for 8 min. Finally, an increase to 240 °C was performed at a rate of 25 °C/min and held there for 10 min before the next injection. Mass spectral analysis was performed on a quadrupole MSD 5975 (Agilent Technologies) using an external standard curve in SIM mode, using the ion m/z 101 as target and the ions1 m/z 57, 74, and 17 as qualifier ions for the quantification of the PA isomers (*SRR* and *RRR*). For the IS (C12:1) the ion m/z 96 was used as target ion, and m/z 110, 123, and 138 were used as qualifier ions. The analysis was performed with a quadrupole temperature of 150 °C and a fragmentation voltage of 70 eV. The ion source temperature was 230 °C, and the interface was 280 °C. The flow rate for helium carrier gas was set at 1.0 mL/min.

The content of PA in the milk samples was calculated on the basis of a standard curve made from synthetic PA methyl esters (≥95% purity; Sigma-Aldrich A/S, Brøndby, Denmark), and variation in response between different samples was corrected using the IS.

Calculations and Statistical Treatment. The total amount of PA in milk fat was calculated as the sum of the two isomers, and the share of *RRR* isomer in total PA was calculated as a measure of the distribution of isomers. Data were treated statistically by use of SAS,

version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). The first model investigated the main effects of breed and period, whereas the second model was a one-way ANOVA of the effect of combinations of herd and period. Pearson correlation coefficients between PA results and share of individual feed items were calculated to determine relationships between feed components and PA.

RESULTS

Identification of PA Diastereomers in Milk Fat. GC-MS analysis showed two PA peaks of milk samples and three peaks of the synthetic standard (see Figure 2). A constant ratio

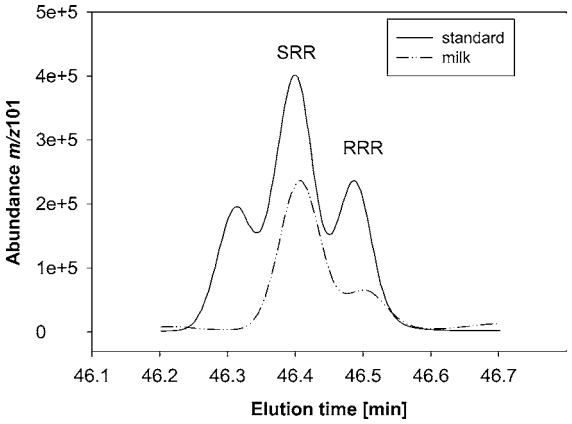

Figure 2. GC-MS-SIM chromatograms (m/z 101) of methyl esters of a sythetic phytanic acid standard and a milk sample.

between the target (m/z101) and the qualifier ions (m/z 57, 74, 171) over the whole peak was used for peak authentication, and the first peak of the milk samples was assigned as the *SRR* isomer of PA; the second peak was assigned as the *RRR* isomer of PA according to the literature.[5,15]

Effect of Breed and Period. The concentration of total PA in milk fat was generally higher in September compared to May (1.21 and 0.96 mg/g fat, respectively, $p < 0.001$), and this difference could be ascribed to results for September of one DH herd and one DJ herd (DH3 and DJ1) (see Figure 3). No overall breed difference was detected. There were no significant

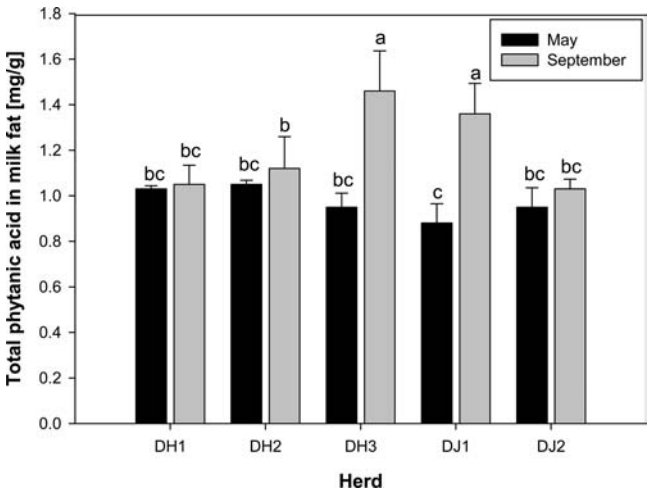

Figure 3. Total content of phytanic acid in milk fat of samples from five herds in May and September. Error bars denote standard deviations, and different letters above columns indicate significant differences ($p < 0.05$).

effects of period or breed on the share of *RRR* isomer in total PA, despite large variations (0.24–0.49) between different herds at different periods as shown in Figure 4.

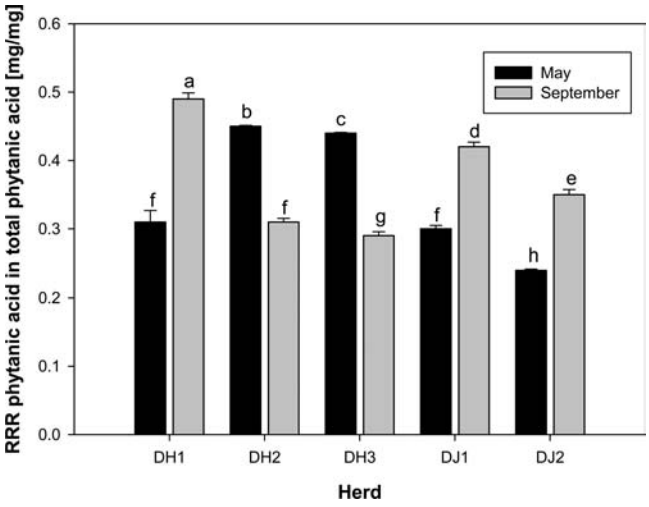

Figure 4. Share of *RRR* phytanic acid in total phytanic acid in milk fat of samples from five herds in May and September. Error bars denote standard deviations, and different letters above columns indicate significant differences ($p < 0.05$).

Effect of Feed Composition. The milk yield and feed consumption data are presented in Table 1. Concentrations of fat and protein were higher and milk yields lower for DJ, compared to DH, as normally observed for these breeds. There was a large variation in feed composition between herds; the share of pasture ranged from 0.21 to 0.76, the share of maize silage ranged from 0 to 0.48, and the share of grass silage including hay and straw ranged from 0 to 0.31, whereas the share of concentrate ranged from 0.10 to 0.34, corresponding to a total share of roughage from 0.66 to 0.90. Due to weather conditions, DMI from grazing was lower in September, which was accompanied by a higher DMI of silage, as well as concentrate. Clovers constituted a larger part of pastures in September.

Pearson correlation coefficients of total PA, as well as share of *RRR* isomer in total PA, on individual feed groups are given in Table 2. Total PA was negatively correlated to intake of pasture and positively correlated to intake of concentrate, whereas the share of *RRR* isomer was positively correlated to the grazed amount of clovers. This latter correlation coefficient was most significant, and the share of *RRR* isomer in milk fat from different herds at different periods related to the grazed amount of clovers (see Figure 5).

DISCUSSION

The concentrations of total PA in milk fat were only about half of the suggested threshold value of 200 mg PA per 100 g milk fat for organic milk.[7] This could be due to differences in organic feeding regimens, for example, between countries. Other investigations have shown very diverse values of the content of PA in milk fat depending on feeding. Thus, for example, feeding red clover silage instead of grass silage has increased the PA content in milk fat from 30 to 60–70 mg/100 g.[23]

In a study by Schröder et al.,[16] in which cows were switched from a conventional total mixed ration to a ration consisting entirely of hay and afterward back to the mixed ration or to a

Table 1. Milk Production, Milk Content of Fat and Protein, and Feed Consumption and Composition of Five Herds (DH1−DH3, DJ1, DJ2) in May and September

	May					September				
	DH1	DH2	DH3	DJ1	DJ2	DH1	DH2	DH3	DJ1	DJ2
ECM, kg $cow^{-1}day^{-1}$	29.4	29.8	27.1	22.3	26.5	30.9	26.7	25.6	21.6	23.1
fat, g $kg\ milk^{-1}$	39.0	38.4	38.5	58.0	55.5	42.1	35.1	40.2	56.8	51.7
protein, g $kg\ milk^{-1}$	32.7	32.1	33.0	41.0	40.0	34.7	32.6	36.1	41.1	38.1
feed dry matter intake (DMI), kg day^{-1}	20.0	19.2	19.6	15.7	17.7	20.5	20.2	19.8	15.8	16.8
feed composition, kg kg DMI^{-1}										
total pasture[a]	0.47	0.76	0.52	0.66	0.33	0.42	0.21	0.28	0.43	0.29
grazed grass	0.33	0.50	0.29	0.46	0.20	0.17	0.09	0.13	0.25	0.17
grazed clovers	0.11	0.20	0.18	0.14	0.01	0.23	0.11	0.13	0.16	0.09
grazed other species	0.03	0.06	0.05	0.06	0.12	0.02	0.01	0.02	0.02	0.03
maize silage	0.23	0.06	0.13	0.00	0.15	0.17	0.48	0.20	0.00	0.11
grass silage, hay, and straw	0.04	0.07	0.06	0.14	0.30	0.17	0.00	0.19	0.23	0.31
total concentrates	0.25	0.10	0.30	0.19	0.22	0.24	0.31	0.32	0.34	0.29

[a]Total pasture is a sum of grazed grass, grazed clovers and grazed other species.

Table 2. Pearson Correlation Coefficients and Corresponding *p* Values for the Relationships between Total Relative Amount of Dry Matter Intake of Individual Feed Groups and Content of Phytanic Acid in Milk Fat and Share of *RRR* Isomer of Phytanic Acid, Respectively

	total phytanic acid in milk fat		share of *RRR* isomer in total phytanic acid	
	correlation coefficient	*p* value	correlation coefficient	*p* value
total pasture	−0.41	0.019	0.33	0.060
grazed grass	−0.42	0.014	0.11	0.526
grazed clovers	0.07	0.697	0.85	<0.001
grazed other species	−0.52	0.002	−0.32	0.070
maize silage	0.08	0.650	−0.28	0.110
grass silage, hay and straw	0.15	0.401	−0.02	0.898
total concentrates	0.58	<0.001	−0.11	0.529

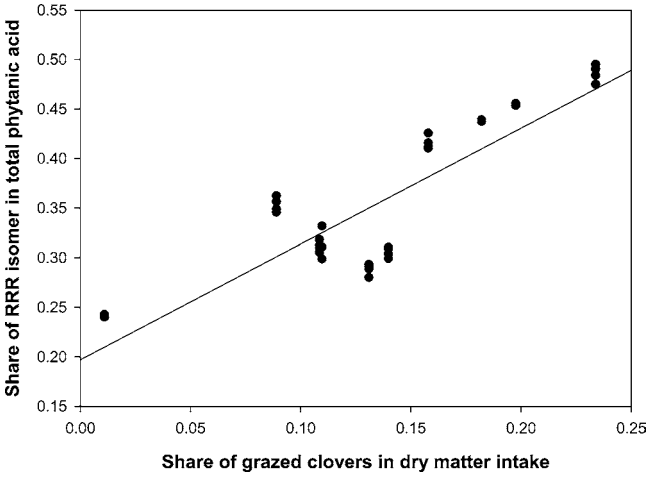

Figure 5. Relationships between share of grazed clovers in dry matter intake and share of *RRR* phytanic acid in total phytanic acid in milk fat.

ration of only grass silage, average PA content in milk fat was reported as 98−116 mg/100 g after feeding the mixed ration, 153 mg/100 g at hay feeding, and 259 mg/100 g at grass silage feeding. These results demonstrate a positive relationship between the consumption of, and type of, green fodder and the PA content in milk fat. Such a relationship has also been documented by Schröder et al.,[24] where 50 or 87.5% of green fodder resulted in PA contents in milk fat of 146 and 314 mg/100 g, respectively. Leiber et al.[25] have shown a 3-fold increase in PA content in milk fat (from 150 to 450 mg/100 g) when cows were shifted from a mixed ration based on hay, grass silage, maize silage, and concentrate, to a ration based entirely on fresh grass (either barn fed or pasture). Likewise, Baars et al.[26] has shown twice as high PA content in milk fat in summer compared to winter and a 50% higher PA content in biodynamic milk fat compared to conventional.

In the present study grazing was negatively correlated, whereas concentrate feeding was positively correlated, to the content of PA in milk fat, which to some extent was due to a high negative correlation between grazing and concentrates ($r = -0.75$). The positive effect of concentrate could be attributed to the use of grass pellets in concentrate mixtures, which is common in Danish organic farming.[27] During the drying of grass pellets, increased hydrolysis of chlorophyll may occur and lead to higher phytol concentrations, similar to what happens during drying of herbs.[28] However, during heat treatment some of the naturally occurring *trans*-phytol may be converted to *cis*-phytol, similar to what has been observed in refined plant oils.[29] Subsequently, the microbial formation of PA may be affected by the *cis* or *trans* configuration of phytol. Other studies have shown positive effects of grazing in comparison to barn feeding[25,26] or organic in comparison to conventional.[15,24,26] However, increased intensity of grazing under organic management has shown no effect on PA content in milk fat.[26] Thus, it is likely that a positive effect of grazing as such on the PA content in milk fat exists, but this effect is less dependent on the level of grazing. The overall effects of feeding on PA synthesis should, as such, not be considered for the single feed items, but should integrate the effects of all feed items in interaction with the ruminal microbiota. These complex interactions need to be further investigated. Therefore, changes in the feed composition from one season to another could interfere with the ruminal microbiota, with altering of the fatty acid profile as a possible consequence.

The distribution between the diastereomers of PA varies with dairy management, and the share of *SRR* isomer has been reported as 39−71% in organic cheeses and 51−84% in conventional cheeses.[15] In milk from one organically and one

conventionally fed cow, the share of *SRR* isomer has been reported as 47 and 85%, respectively.[15] In the experiment in which feed rations have been shifted between a mixed ration, pure hay, and pure grass silage (see above),[16] the share of *SRR* isomer is reported as 50% during hay feeding and 70−80% during feeding the mixed ration, as well as during grass silage feeding. These results demonstrate that feed composition regulates the distribution of isomers, and hay favors the formation of the *RRR* isomer. Our present results showed a high positive correlation between the intake of grazed clovers and the share of *RRR* isomer. This relationship between clovers and isomer distribution could be an explanation for the higher share of *RRR* isomer in organic products, as the use of clovers and other legumes is increased in organic agriculture to ensure nitrogen fertilization.[30] Milk collected during the summer has also been shown to have a higher share of *RRR*, owing to the increased intake of green products in this period, when compared to the winter season.[26]

The mechanisms involved in the formation of either *SRR* or *RRR* isomers have, to our knowledge, not been previously reported. The *R* or *S* configuration of carbon 3 (see Figure 1) is formed during the hydrogenation of the double bond of phytol, and possibly different microbial strains form either predominantly *SRR* or *RRR* isomers. The biohydrogenation of most unsaturated fatty acids takes place at carbon 9 or higher, and different microorganisms are involved in the hydrogenation step depending on the position and number of double bonds.[31] Similarly, the biohydrogenation of phytol may take place before or after the oxidation of the alcohol moiety,[6] which may involve different groups of microorganisms. The biohydrogenation of unsaturated fatty acids from feed has been reported to be reduced by secondary plant metabolites from legumes.[25] Likewise, these secondary plant metabolites in clovers may favor microbial strains forming predominantly *RRR* isomer or suppress strains forming predominantly *SRR* isomer.

Very little is known on whether the intake of PA with different isomeric composition has any physiogical relevance. There is no difference in the agonist activity on PPARα between the isomeric forms of PA.[11] However, pristanic acid, produced by α-oxidation of PA, is a considerably more potent PPARα agonist than PA[32] and hence a shift toward more pristanic acid would be expected to enhance PPARα activation. It has been shown that the *SRR* form of PA is more rapidly oxidized in mitochondrial preparations at high PA concentrations.[13] However, because peroxisomal α-oxidation is required for efficient β-oxidation of PA, the in vivo relevance of this is uncertain. But, as the *RRR* form requires isomerization by AMCAR prior to β-oxidation,[14] it is reasonable to assume that the *SRR* form of pristanic acid is more rapidly oxidized during β-oxidation than the *RRR* form, thereby decreasing the PA/pristanic acid ratio. Thus, the intake of PA with a greater portion of *SRR* might be desirable as its oxidation would be faster and less dependent on AMCAR; on the other hand, a greater portion of *RRR* might lead to higher concentrations of pristanic acid and, hence, more efficient activation of PPARα. Which of these situations is most advantageous for the consumer cannot be concluded at present. In either case, feeding strategies can be manipulated, for example, by changing the content of legumes in the feed, to achieve an altered content of *RRR* share of PA in dairy products.

In conclusion, this study shows that total PA content in milk fat cannot be directly related to the intake of green fodder items, and other elements in the feed composition may also affect PA synthesis. The distribution of isomers is highly dependent on the amount of clovers in feed, and the results suggest that it should be possible to increase the share of *RRR* isomer of PA by increasing the content of clovers in feed. However, our results do not show whether this effect relates only to grazed clovers or if similar effects could be obtained with preserved clovers. Thus, the concentrations of PA diastereomers in milk are variable and the influencing factors not fully understood. The physiological consequences of this variation have also not been investigated.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mette.larsen@agrsci.dk. Phone: +45 87 15 80 62. Fax: +45 87 15 48 91.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AMCAR, α-methylacyl-CoA racemase; DH, Danish Holstein; DJ, Danish Jersey; DMI, dry matter intake; ECM, energy corrected milk; GC-MS, gas chromatography−mass spectroscopy; IS, internal standard; NE, net energy value of lactation; PA, phytanic acid; PPARα, peroxisome proliferator activated receptor-α; RXR, retinoid X receptor; SIM, selected ion monitoring

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