Multivariate Modelling of Milk Fatty Acid Profile to Discriminate the Forages in Dairy Cows' Ration

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Abstract

Although there are many studies on the importance of fatty acids (FA) in our diet and on the influence of cows' diet on FA metabolism, only a few investigate their predictive capacity to discriminate the type, amount and conservation method of the dietary forages. This research quantitatifies differences in FA concentrations and, using a supervised factorial discriminant analysis (FDA), assesses which could be biomarkers when replacing maize silage with other silages, grass/lucerne hays or fresh grass. The statistical modelling identified three main clusters in milk FA profiles associated with silages, hays and fresh grass as dominant roughages. The main implication of a dairy cow feeding system based on poliphytic forages from permanent meadows is to enhance milk's nutritional value thanks to an increase in beneficial FA belonging to omenga-3 polyunsaturated FA, conjugated linoleic acids and odd chain FA, compared to the use of maize silage from arable land. The study also identified a small but powerful and reliable pool of FA that can act as biomarkers to authenticate feeding systems: C16:1, C17:0, C18:0, C18:3n-3, C18:1 c-9, C18:1 t-11, C20:0.

Introduction

Dairy lipids have a number of different fat classes although they are dominated by triacylglycerols comprising of more than 400 different fatty acids (FA) of varying length and saturation. There is also a small fraction of other lipids like vitamins, phospholipids and glycerolipids [1]. So far, numerous studies highlight certain FA and vitamins are essential for humans and many contribute to the sensory characterization of the product [1–3]. Despite the high number of FA thought to have a negative impact on human health, therefore creating some concernes on dairy consumption, specific research suggests milk constituents can actually have positive health effects thanks to short chain (≤ C10) FA (SCFA), conjugated linoleic acids (CLA), omega-3 (n-3) polyunsaturated FA (PUFA) and odd- and branched-chain FA (OCFA and BCFA) [1,4,5]. SCFA were showed to have antiviral activities and to delay tumors' growth [6,7]. CLA and n-3 have numerous beneficial functions for human health as reported by Tunick & Van Hekken [2] and Patterson, *et al.* [8], and there is an increasing interest in milk OCFA and BCFA, which are mainly derived from rumen bacteria and reported to have anticarcinogenic effects on cancer cells [9].

The milk FA profile varies with a number of factors. For instance, FA profile depends on the breed, season, stage of lactation [10,11], although feed management is recognised as having the strongest impact [12–14]. Furthermore, many studies investigated the impact feeding strategies have on milk FA composition within intensive lowland dairy systems [10,15,16].

Talking about intensive lowland dairy systems, milk produced by cows fed on forage from diverse meadow compared with that obtained by feeding maize silage has been demonstrated to be rich in beneficial FA such as CLA and n-3, both in studies considering grazing animals [16] and animals receiving such forages as TMR [10]. Meanwhile, the role of forage type and conservation method, especially ensiling, on modifying rumen FA metabolism is still under debate [15,17–19]. Most reported studies to date have been

carried out under experimental conditions and it would be good to know if the findings can be replicated under the challenges and variety of diets on commercial farms. The literature lacks research carried out under field conditions, considering the potentially contrasting effects from a range of diet ingredients, whose actions may work in synergy on these metabolic pathways. O'Callaghan, *et al.* [16], report both grass hay and maize silage, if supplemented with high levels of concentrates, increase the concentrations of C18:2n-6 *cis*, C18:3n-6 *cis*, C22:0, C22:1n-9 and C18:2 *c*-10, *t*-12 in milk. However, increasing the dietary proportion of fresh or ensiled poliphytic (mixed or diverse) forages leads to significantly more n-3 and CLA as well as vaccenic acid (VA, C18:1 *t*-11), even if cows are kept indoors and fed a total mixed ration (TMR) [10,15,20]. Despite the wide knowledge on the role of forages influencing FA metobolism, there is limited information on the application of multifactorial models predicting FA in milk in relation to the forages' botanical origin, conservation method and dietary proportion [21,22]. The use of supervised multivariate models could allow achieving a comprehensive answer on the impact of forages on the metabolic pathways involved from rumen to mammary gland and responsible for the FA release in milk [23,24]. Furthermore, a chemometric approach based on a pattern recognition supervised modelling can determine the functional relationship between the analytes (i.e., FA) and the predictor classes (i.e., forage groups) highlighting a pool of useful features as biomarkers able to discriminate among the classes [25].

This research aims to estimate quantitative differences in dairy milk FA from replacing maize silage with silages from other cereal and legume crops, grass and lucerne hays or fresh grass. For this purpose, a supervised factorial discriminant analysis was carried out to verify if significant differences in FA can be used to fingerprint dairy production chains. Moreover, a linear regression model and clustering of the variability by a set of descriptive statistics were performed to predict milk FA profile in relation to forages.

Materials And Methods

Ethical statement.

The experimental trial did not influence the farm activities or management strategies, nor involved any invasive procedure or manipulation of the lacating dairy cows. Therefore, there was no implication on the animals' welfare status. Ethical review and approval from the local or national ethics committeee was not required because only diet, refusal and bulk milk samples were collected by a trained veterinarian with consent from the owners of the animals.

Experimental design.

The study involved 14 dairy farms in the middle of the Italian lowland area, Po Valley (North East of Italy, 45°19'49"N 9°47'56"E). The farms were selected to represent average herd size and milk yield characterizing of the Italian intensive dairy system. All were associated with the Regional Breeders' Association, ensuring herd performances were recorded monthly over the experimental period (Table 1). The daily milk production records were also used to calculate standardised 'Fat Protein Corrected Milk'

(FPCM) [26], considering fat as 4.0% and the true protein as 3.3% (true protein as crude protein × 0.93). Herd dry matter intake (DMI) was recorded at each sampling visit (5 recordings *per* farm) by calculating the difference between total amount of total mixed rations (TMR) distributed to the lactating cows and refusals after 24 hrs or before the subsequent distribution. The experimental protocol was designed to allocate each farm to one of five feeding groups (FG), according to the main roughage source (% of TMR on dry matter basis): i. high maize silage (HMS; maize silage ≥ 32%; 4 farms and 20 milk samples); ii. medium maize silage (MMS; maize silage = 12-26%; 4 farms and 18 milk samples); iii. mixed crop silages (MCS; other crop silages ≥ 37% and maize silage = 0%; 2 farms and 11 milk samples); iv. grass and lucerne hays (HAY; permanent meadow and lucerne hays ≥ 42%, maize silage = 0%, other crop silages < 9%; 2 farms and 12 milk samples); v. green grass (GRG; fresh grass > 20% and maize silage = 0%; 2 farms and 9 milk samples). On all farms (including the GRG group), cows were fed TMR formulated to cover the herd's nutritional requirements (avalilable energy and protein) based on the NRC standard [27]. All forages were produced on the farms - average rations for the five FG (% on DM) and their diet proximate compositions (% on DM) are reported in Table 1.

Milk and TMR sampling.

In 2018, five raw bulk milk samples were collected on each farm over 5 sampling rounds (March, May, July, September and December), giving a total of 70 samples. Since it is not uncommon for farms to alter diet ingredients depending on seasonal supply of feeds, some farms changed TMR formulation over the experimental period, essentially changing group. One original MMS farm changed twice, once into MCS and once into HAY, and one GRG farm changed once into a HAY diet. However, according to Rego, *et al.*[17], we ensured at least three weeks between the diet change and milk sampling. At each sampling, the current TMR were collected and formulations recorded. The milk and TMR samples were refrigerated and carried to the laboratory for analysis immediately after the sampling and milk sub-samples for FA profile frozen at −20 °C until analysis.

TMR and milk chemical analysis.

After collection, each TMR sample was kept frozen at -20 °C and after thawing, they were analysed for dry matter (DM), crude protein (CP), ether extract (EE), ash and starch by official chemical methods, and for neutral detergent fiber (aNDF) and acid detergent fiber (ADF) by ANKOM chemical methodology [28].

The milk proximate composition (crude protein, casein, fat, lactose) and chemical traits (urea, pH) were recorded by a Fourier transform mid-infrared (FT-MIR) spectroscopy technique using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark). Additionally, the somatic cell count (SCC, 100,000/ml) was performed by a Fossomatic 5000 (Foss Electric A/S, Hillerød, Denmark) and reported as SCC score calculated with the following formula [log2 (SCC/100,000) + 3].

FA analysis.

For each milk sample 2 replicates of approximately 35 g were freeze-dried, mixed until a fine homogenous powder and transfered to suitable vials. The lyophilized samples were methylated and esterified to prepare for gas chromatography (GC), using the method described by Chilliard, *et al.*[29], and Stergiadis, *et al.*[30]. The chemicals used for extraction of FA, correction factors for SCFA, analytical standards and identification of peaks followed the methodology of and are described by Stergiadis, *et al.*[31]. To optimize peak separation, modifications to the chromatographic conditions from the original method by Chilliard, *et al.*[29], were updated as described by Stergiadis, *et al.*[31]. FA results are expressed as g/100 g of the whole FA profile. Values for individual FA were used to calculate total saturated FA (SFA), short chain (£ C10) FA (SCFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA), conjugated linoleic acids (CLA), highly unsaturated FA (HUFA), odd chain FA (OCFA), n-3 (omega-3 FA), n-6 (omega-6 FA), HUFAn-3 as well as n-3:n-6 and n-6:n-3 ratio.

Statistical analysis.

All analyses were carried out using the SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) and XLStat (Addinsoft, release 2016, New York, USA). Milk chemical and FA profile data were analysed using a linear mixed model that included the fixed effects of feeding group (FG: i-v) and the random effect of the farm (SAS PROC MIXED). Pairwise comparisons among levels of the FG factor were performed using Bonferroni correction. The hypotheses of the linear model on the residuals were graphically assessed.

The dataset of the FA was subjected to supervised multivariate factorial discriminant analysis (FDA), considering the FG as the predictor factor. The FDA split the total variance in four main canonical functions; F1-F4. The outcomes of the FDA were plotted to classify the five FG according to the first two main canonical functions F1 and F2. The correlation coefficients (with absolute value greater than 0.20) between the original FA and F1 and/or F2 were also plotted in the FDA-scattergram. The reliability of the FDA classification model was assessed by a leave one out cross-validation (SAS PROC DISCRIM). A confusion matrix was built throughout the results of the procedure and the classification performance was assessed using accuracy, precision, sensitivity, specificity and Matthews correlation coefficient (MCC), as reported in Segato, *et al.*[32].

A multiple stepwise regressions (SAS PROC REG) were preformed on the four main forages (maize silage, mixed crop silages, grass and lucerne hays, fresh grass) on some FA and their derived chemical classes (SFA, MUFA, PUFA, CLA, HUFAn-3, OCFA). The regression coefficients were estimated. The most discriminative FA selected by the FDA were graphically represented by some box-whisker plots across the five FG.

Results And Discussion

Husbandry management, herd productive performances and milk quality.

All the farms produced their own fodders in the investigated lowland area. Maize silage (late variety, such as FAO class 600-700) and the permanent meadows (mix of perennial ryegrass, meadow fescue with a minor presence of red and white clover) were produced in optimal pedoclimatic and irrigated conditions with an average annual yield of 21 and 10 t DM/ha, respectively. The third main fodder, used in the MCS group, was a mix of ensiled forages such as sorghum (25%), lucerne (25%), wheat (20%), perennial grass (15%) and Italian ryegrass (15%), with a medium-high productive yield.

The herd characteristics and productive performances of the five experimental FG are reported in Table 1. Although no statistical analysis was carried out on this data, the highest herd daily DMI was observed in the MMS and MCS groups (24.4 and 23.8 kg/cow, respectively), meanwhile the lowest in the GRG group (21.8 kg/cow) and, as expected, the milk yield was strongly linked to intake. However, the potential production of the maize-silage groups might have been reduced as a strategy to enhance milk quality because also these farms destinated their milk to Protected Designation of Origin (PDO) hard cheese production.

Milk proximate composition and chemical traits characterizing the five FG are reported in Table 2, and they show only little differences among them. The feeding system affected CP, casein and lactose content with the lowest ($p < 0.05$) values recorded for GRG. The lower CP and casein in GRG samples may be due to an inbalance in ruminal degradability between the highly fermentable fiber and N-sources, which are a typical metabolic condition in the case of early-stage grass intake [33]. A similar metabolic process may occur in MCS cows as they too had lower values of both CP and casein compared to the maize silage-fed cows (HMS and MMS), although it may be also related to the higher FPCM milk yield for this group. As reported by Riuzzi, *et al.* [25], a lower lactose content was found in milk from GRG cows probably because of both their lowest recorded intake and the lower level of energetic concentrates, potentially leading to less ruminal propionate synthesis compared with other groups. Since proprionate is the main precursor of gluconeogenesis in ruminants, this can lead to a decrease in glucose and hence lactose [34].

FA profile.

All the 74 profiled FA are reported in a supplementary table and they had already been detected in similar studies [22]. Table 3 includes only the FA that are abundant in milk and that were expected to be influenced by the four roughage sources considered in this study.

Major differences between the FG existed for the concentration of most nutritionally relevant FA, driven by the amount and type of forage in the diets. These often reached significance between GRG and HMS milk, although in some cases GRG milk also differed from MMS, MCS and/or HAY groups with no

consistency in the pattern of variation seen for the individual FA. VA, CLA9, C20:5n-3 *c*-5, *c*-8, *c*-11, *c*-14, *c*-17 (EPA), total CLA concentrations were higher ($p < 0.05$) for GRG than HMS milk and concentrations of SFA and SCFA were lower ($p < 0.05$). These differences also reached significance in comparing GRG and MMS milk for CLA9, total CLA and SCFA whereas for CLA9, GRG milk was significantly higher than for all other groups except HAY and SFA concentrations were less than all groups except MMS. Differences were also significant in comparing GRG with MCS and HAY milk, where C16:0 (palmitic acid, PA) was lower and PUFA concentrations were higher in GRG milk. Other differences also existed when comparing HAY milk with the other groups; C18:2n-3 (linoleic acid, LA) had a tendancy ($p = 0.066$) to be lower than in GRG milk, and milk from the HAY group had more ($p < 0.05$) C18:3n-3 (a-linolenic acid, ALA) and total n-3 than HMS milk and the ratio n-6:n-3 was lower ($p < 0.05$).

One noticeable outcome from our study is a significant ($p < 0.05$) effect of hay and fresh-grass based diets on the concentrations of total CLA, CLA9 and its precursor VA in milk (especially if compared to HMS), in line with previous studies [10,11,19,35]. Indeed, the use of a dairy cow feeding based on fresh grass as the main forage source led to significantly higher concentrations in VA, CLA9 and total CLA [36]. The concentration of CLA in milk is enhanced thanks to intake of a polyphitic forage rich in LA and ALA as precursors, which undergo less extensive hydrogenation to the intermediate vaccenic acid rather than fully into C18:0 (stearic acid, SA). Both these hydrogenation products (VA and SA) are subsequently desaturated in the mammary gland secreting CLA9 and C18:1 *c*-9 (oleic acid, OA) respectively into milk [16,37]. A study by Akbaridoust, *et al.* [15], confirmed that the partial replacement of lowland grass grazing with maize silage leads to a decrease of CLA9, as in the present study.

Both HAY and GRG forages in this study originated as polyphitic vegetation from permanent meadow also leading to high concentrations of ALA, EPA and n-3 compared with other diets, although differences between GRG and other groups do not always reach significance. Many strong linear relationaships between the content of PUFAn-3 and specific botanical families of lowland permanent meadow have been reported, such as those observed for the consumption of forage with an high proportion of Fabaceae (legume) and Ranunculaceae [10] compared to those with a prevalence of Poaceae such as Timothy and perennial ryegrass [16,20]. Also Stergiadis, *et al.* [14], report high concentrations of n-3 in milk from cows grazing legume dominated pastures.

The use of maize silage as the dominant roughage source caused higher concentrations of SCFA (C4:0 and C6:0) and PA content, resulting in a higher amount of total SFA in milk along with a higher ratio of n-6:n-3. The elevated n-6:n-3 ratios found here (mostly driven by less n-3 rather than more n-6) is in common with other studies using maize silage [12,23]. However not all findings here are in line with previous studies. With respect to C4:0 and C6:0, Yang, *et al.* [38], report maize silage diets lead to lower concentrations of these FA but another study by Coppa, *et al.* [21], highlighted a decrease of C4:0 with an increase of fresh grass in the cows' diet. Moreover, this latter study observed a significant increase in the proportion of SCFA (from C8:0 to C12:0) with the increase of maize silage in the TMR. Other studies using maize silage in the diet resulted also in higher secretion of PA in milk [16,17,22]. Short chain and some

medium chain FA are mainly produced by *de novo* synthesis in the mammary gland, using acetate and butyrate from the ruminal fibrolytic bacteria activity, even if they can also derive from the diet, especially PA, which is reported to increase with the use of maize silage. With the exception of the HAY group, all cows in the study ate diets with a very similar in NFD content (an indication of digestible forages), varying by less than 1%age unit across groups. This might explain why SCFA were not lower with maize silage but does not explain the apparent slightly higher *de novo* synthesis compared with other forages.

Factorial Discriminant Analysis.

The main purpose of this study was to evaluate the influence of the dairy cow feeding system on the lipidic fingerprinting considering TMR based on five main roughage sources. Thus, a factorial discriminant analysis (FDA) was carried out using the 70 milk FA profiles to have an insight into the changes occurring due to the replacement of maize silage with a mix of ensiled, dried or fresh forages. The FDA resulted in two main significant functions (F1 and F2; Wilks's $\lambda = 0.002$), which accounted for 59.0% and 20.1% of the total variance, respectively. The FDA results are based on 9 most significantly ($p < 0.05$) discriminative FA: C9:0, C10:0, C16:1 *c*-9 (for brevity, only C16:1 as in Table 3), C17:0, C17:1 *c*-9, C18:0 (SA), C18:3n-3 (ALA), C18:2 *c*-9, *t*-11 (CLA 9), C20:0. These FA also have a correlation coefficient in absolute value greater than 0.25 with at least one of the two main functions (F1 and F2). Figure 1 shows a scattergram of the FDA model based on F1 and F2 along with the 9 most discriminative FA. The FA that contributed the most to the separation among FG were found to be only partially in agreement with the significant differences outlined by the univariate statistical analysis; indeed, only ALA and CLA9 are shared with the multivariate targeted FDA. As reported in Figure 1, the GRG and HAY milk FA profiles clearly differ from those from the silage-based TMR and between each other, meanwhile there are considerable overlaps among HMS, MMS and MCS samples. HMS and MCS seemed to be similar and only partially overlapping with the MMS group. The analysis of Figure 1 confirmed that HAY-milk samples are correlated with ALA (C18:3 n-3 on the chart), which proves once more to be a specific strong biomarker of hay-based diets [39], with a minor contribution of the long chain SFA C20:0. GRG, instead, seemed to be characterized by higher contents of CLA9 and C17:0, even if these FA can also be used to discriminate the HAY samples. Both of them had already been identified as specific biomarkers of fresh grass-based milk by Butler, *et al.* [36], Stergiadis, *et al.* [14], and Paredes, *et al.* [19], respectively. As mentioned, the finding of CLA9 having discriminative capacity is due to the transformation of dietary LA and ALA, through the metabolic pathways in the rumen and mammary gland. As regards the odd chain FA C17:0, it derives largely from the ruminal microbial activity and its transfer into milk is reported to be enhanced in cows fed hays and fresh grass rich in C18 FA, such as the grass and legume species that were used to formulate the HAY and GRG diet in our study [19,40]. Whereas, C16:1 can be indentified as a weak lipidic biomarker of MCS and HMS milk samples, even if it has only a minor discriminative capacity and is only slightly correlated with F1. As for C17:1 *c*-9, it appears to be associated with both MCS and GRG because of its spatial position along the positive F1 axis and negative ones of F2. Identifying the reasons for their feeding discriminative role is not an easy task. From the literature, C16:1 seems to indicate both the use

of maize-based diets [22] and the adoption high concentrates diets [39]. C17:1 *c*-9 was reported to be associated with the use of fresh grass by Coppa, *et al.* [22], as it is the results of the D9-desaturation of C17:0 in the mammary gland. The samples of the three FG receiving silages tended to have similar FDA loadings that make them spatially overlap into one cluster located in the left-centre of the scattergram, associated with C9:0, C10:0 and SA. However, MMS group is slightly separated from the other two groups (along the negative F2 axis) because of the influence of C10:0 and SA. Compared to rations based on a large inclusion of grass, feeding strategies involing silages, as highly nutritional forages, seemed to significantly increase the proportion of SFA, such as C10:0 [23,39] and SA [15], due to a higher ruminal biohydrogenation rate.

The cross-validation used to assess the reliability of the FDA confirmed the accuracy of this supervised targeted model for a correct classification of milk from HAY and GRG groups (Matthews correlation coefficient values of 1.00), meanwhile there was a noticeable misclassification rate (7 out of 49) of the silage-based milk samples, especially between MCS and HMS (Table 4). However, if the silage samples were considered as one cluster, as suggested by the FDA, the predictive performances would be enhanced, thus proving the effective role of FA profile to trace the dairy products according to the adopted feeding system. Extending this approach to the large-scale distribution, it may be effective to consider a labelling system of dairy products based on at least the three feeding strategies investigated in the present study: ensiled (HMS, MMS, MCS) vs. dried (HAY) vs. fresh (GRG) forages; even if they are all produced in the same intensive lowland area. Indeed, milk FA profile can be a powerful, reliable and accurate metabolomics tool to discriminate among rations including high amounts of cereal-derived silages or a mix of grass and legume-derived hays, which affect the milk nutritional value (incidence of beneficial FA), contamination risk (i.e., presence of clostridium bacteria) and the degree of sustainability of the farm (ratio between input and output of human edible energy).

Prediction of milk FA composition with stepwise regression models based on the main dietary roughage source. T

able 5 reports the multiple linear regressions of the most predictive FA according to the four forage sources (maize silage, other silages, hays, fresh grass). The findings discussed in the previous sections are mostly confirmed by the predictive equations. Indeed, although the silages (both maize and others) slightly influence the individual FA, they significantly increased the total SFA and, consequently, reduced PUFA, especially ALA and CLA9. As regards dried forages, they are correlated positively with C17:0 and CLA9 and negatively with SA, resulting in higher levels of PUFA. Also feeding hays seems to play a role in the increase of SFA, even though their effect is weaker than with silages, especially the mixed-crop ones. Feeding fresh grass seems to mildly modify the FA profile, even if it too contributes to higher concentrations of two beneficial FA - C17:0 and CLA9. In the case of OCFA there was no significant predictive capacity by any of the roughage sources.

By looking at these results, it seems the main consequence of replacing maize or other crop silages with hay or fresh grass is the increase in PUFA that are beneficial for human health especially CLA and ALA. However, as shown in figure 2, the levels of ALA and total PUFA do vary a lot within milk from the HAY group, probably because of the variability of botanical composition and phenological stage at the time of harvesting across the investigated farms. Furthermore, the use of silages, especially from cereals other than maize, seem to increase the SFA content of milk, more than feeding hays do, as shown by their higher regression coefficients. Conversely to HAY and GRG groups, the silage-based diets are characterised by greater uniformity within their groups, as showcased in the boxplots of figure 2. The feeding system that has the greatest effect on milk FA composition is GRG, which leads to an increase in beneficial FA, such as CLA and C17:0, despite the highest presence of outliers within this group (Figure 2) probably due to the stronger impact of fresh grass on the ruminal activities.

Conclusions

This study proved it is possible to assess the changes in milk FA composition according to the main dietary roughage for highly productive dairy cows. The factorial discriminant analysis (FDA) chemometric approach highlights substantial differences in milk FA composition from silage diets compared to hays (HAY) and fresh grass (GRG) feeding , which both lead to higher concentrations of FA beneficial for health (e.g. C17:0, C18:3n-3, C18:2 *c*-9, *t*-11). The cross-validation confirmed the accuracy of the FDA modelling approach to discriminate the HAY and GRG milk samples, meanwhile it showed a mild misclassification rate among the different silage-based diets. Compared with maize silage, poliphytic forages from perennial swards seems to be characterised by a higher variability refected in milk FA profiles. To summarise, replacing maize silage with hays and/or fresh grass improves the milk nutritional value by both reducing SFA and increasing CLA and long chain PUFAn-3, potentially improving the nutritional sustainability of the dairy products from intensive lowland areas. The study also identified which FA could benchmark biomarkers to distinguish the feeding systems, especially in the comparison with the use of maize silage.

Declarations

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Author Contributions

G.R. planned the trial, collected the milk and feed samples, carried out the fatty acid analysis and prepared the manuscript. D.H. organized the fatty acid analysis and helped revise the manuscript. I.L. helped prepare the data sets and revise the manuscript. G.B. prepared the manuscript and contributed to the funding of this work. B.C. performed the statistical analyses. F.G. planned and oversaw the trial, helped revise the manuscript and obtained funding for this work. S.S. collected the milk and feed samples, advised on the statistical analyses, prepared the manuscript and contributed to the funding of this work.

Competing interests

The authors declare no competing interests.

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Tables

Table 1

Herd descriptive statistics (average ± S.D.) and lactating cows performance; diet formulation (%) and proximate composition (% on DM) of the five feeding groups according to the main roughage source. FPCM = fat protein corrected milk (4.0% fat and 3.3% true protein); other silages = sorghum, wheat, lucerne, grass, ryegrass; energetic concentrates = maize products, sorghum mash, barley meal; protein concentrates = soybean products, sunflower meal; residual = straw, bran, beet pulps, salts, vit- and min-mix; aNDF = neutral detergent fibre; ADF = acid detergent fibre.

	High Maize Silage	**Medium Maize Silage**	**Mixed Crop Silages**	**Grass and lucerne hays**	**Green Grass**
	HMS	MMS	MCS	HAY	GRG
Herd descriptive statistics					
Lactating cows (n)	96 (± 44)	122 (± 41)	68 (± 16)	71 (± 16)	50 (± 7)
Average Days In Milk (d)	198 (± 29)	177 (± 27)	172 (± 17)	165 (± 21)	189 (± 24)
Calving interval (d)	434 (± 31)	408 (± 17)	410 (± 24)	399 (± 17)	403 (± 17)
Lactating cows performance					
Dry matter intake (kg/d)	23.2 (± 0.8)	24.4 (± 1.1)	23.8 (± 2.0)	22.7 (± 1.3)	21.8 (± 1.8)
Milk (kg/d)	29.5 (± 5.4)	33.3 (± 3.6)	31.0 (± 3.3)	28.8 (± 2.1)	25.6 (± 2.7)
FPCM yield (kg/d)	29.7 (± 4.2)	32.7 (± 3.9)	30.1 (3.9)	28.1 (± 1.7)	24.1 (± 2.3)
Diet ingredients (% DM)					
Maize Silage	35	23	0	0	0
Other silages	6	15	41	6	11
Permanent meadow hay	8	8	8	35	19
Lucerne hay	3	4	2	13	6
Fresh grass	0	0	0	0	26
Energetic concentrates	27	27	34	35	28
Protein concentrates	16	19	11	8	9
Residual	5	4	4	3	1

	High Maize Silage	Medium Maize Silage	Mixed Crop Silages	Grass and lucerne hays	Green Grass
Diet composition (% DM)					
DM (%)	54.8 (± 5.3)	56.6 (± 5.1)	55.8 (± 6.5)	69.5 (± 5.8)	64.7 (± 8.6)
Crude protein	14.0 (± 0.5)	14.1 (± 0.6)	14.1 (± 0.6)	14.0 (± 1.1)	13.5 (± 1.3)
Ether extract	2.7 (± 0.4)	2.8 (± 0.4)	2.7 (± 0.5)	2.6 (± 0.4)	2.5 (± 0.7)
Ash	8.1 (± 0.7)	7.6 (± 0.5)	8.3 (± 0.4)	7.9 (± 0.6)	7.8 (± 0.4)
aNDF	36.8 (± 1.9)	37.2 (± 2.1)	37.7 (± 3.1)	40.7 (± 4.4)	37.6 (± 4.0)
ADF	21.7 (± 1.6)	22.2 (± 1.3)	22.3 (± 1.9)	23.9 (± 1.6)	20.3 (± 2.9)
Starch	22.6 (± 1.6)	22.4 (± 2.3)	21.7 (± 3.1)	19.4 (± 1.1)	20.6 (± 4.7)

Table 2

Effect of dietary roughage source on milk proximate composition (g/100g), somatic cell count (SCC) score as $\log_2$ (SCC/100,000) + 3, urea and pH. HMS = high maize silage; MMS = medium maize silage; MCS = mixed crop silages; HAY = grass and lucerne hays; GRG = green grass; SEM = standard error of the means. $^{a-b}$LSMeans in a row without a common superscript differ ($p < 0.05$).

	Dietary forage group					SEM	p-value
	HMS (*n* = 20)	MMS (*n* = 18)	MCS (*n* = 11)	HAY (*n* = 12)	GRG (*n* = 9)		
Crude protein (g/100 g)	3.52^{a}	3.48^{a}	3.46^{ab}	3.47^{a}	3.32^{b}	0.05	**0.014**
Casein (g/100 g)	2.71^{a}	2.69^{ab}	2.65^{ab}	2.67^{ab}	2.50^{b}	0.05	**0.025**
Fat (g/100 g)	4.21	3.91	3.99	3.87	3.84	0.12	0.083
Lactose (g/100 g)	4.79^{ab}	4.82^{a}	4.78^{ab}	4.74^{ab}	4.71^{b}	0.03	**0.034**
SCC score (units)	3.98	3.74	3.75	3.98	4.37	0.20	0.140
Urea (mg/dL)	24.0	24.6	25.6	24.9	20.5	1.9	0.360
pH	6.65	6.67	6.65	6.65	6.65	0.01	0.147

Table 3
Effect of dietary roughage source on milk fatty acid (FA) profile (g/100 g of fatty acids). HMS = high maize silage; MMS = medium maize silage; MCS = mixed crop silages; HAY = grass and lucerne hays; GRG = green grass; SEM = standard error of the means; $^{a-b}$LSMeans in a row without a common superscript differ ($p < 0.05$).

Fatty acids	**Dietary forage group**					**SEM**	**p-value**
	HMS ($n = 20$)	MMS ($n = 18$)	MCS ($n = 11$)	HAY ($n = 12$)	GRG ($n = 9$)		
C4:0	3.20^{a}	2.97^{ab}	3.01^{ab}	3.10^{ab}	2.73^{b}	0.108	**0.024**
C6:0	2.30^{a}	2.20^{ab}	2.21^{ab}	2.18^{ab}	2.11^{b}	0.046	**0.045**
C8:0	1.33	1.30	1.30	1.26	1.21	0.036	0.213
C10:0	2.99	3.03	2.92	2.76	2.70	0.102	0.122
C12:0	3.54	3.55	3.47	3.27	3.19	0.134	0.233
C14:0	11.6	11.8	11.7	11.6	11.2	0.260	0.468
C14:1 *c*-9	0.876	0.925	0.969	0.908	0.913	0.048	0.463
C15:0	1.09	1.17	1.22	1.13	1.09	0.065	0.384
C16:0 (PA)	32.7^{ab}	32.2^{ab}	33.3^{a}	33.2^{a}	31.2^{b}	0.663	**0.026**
C16:1	1.67	1.65	1.71	1.71	1.84	0.095	0.505
C17:0	0.409	0.422	0.473	0.460	0.502	0.028	0.117
C18:0 (SA)	9.55	9.62	9.44	9.83	10.3	0.488	0.532
C18:1 *c*-9 (OA)	19.5	19.3	19.0	19.5	20.8	0.504	0.090
C18:1 *c*-12	0.268^{a}	0.298^{a}	0.243^{ab}	0.167^{b}	0.219^{ab}	0.024	**0.002**
C18:1 *t*-11 (VA)	0.702^{b}	0.787^{ab}	0.782^{ab}	0.954^{ab}	1.074^{a}	0.085	**0.021**
C18:1 *t*-12, *t*-13, *t*-14	0.344^{a}	0.315^{ab}	0.266^{ab}	0.241^{b}	0.246^{ab}	0.029	**0.037**
C18:2 *c*-9, *t*-11 (CLA9)	0.366^{b}	0.409^{b}	0.378^{b}	0.442^{ab}	0.619^{a}	0.042	**0.001**
C18:2n-3 *c*-15, *t*-11	0.067^{b}	0.094^{ab}	0.080^{ab}	0.106^{a}	0.110^{a}	0.012	**0.041**

Fatty acids abbreviations: PA = palmitic acid; SA = stearic acid; OA = oleic acid; VA = vaccenic acid; CLA = conjugated linoleic acid; ALA = α-linolenic acid; EPA = eicosapentaenoic acid (C20:5n-3 *c*-5, *c*-8, *c*-11, *c*-14, *c*-17); DHA = docosahexaenoic acid (C22:5n-3 *c*-4, *c*-7, *c*-10, *c*-13, *c*-16, *c*-19); DPA = docosapentaenoic acid (C22:5n-3 *c*-7, *c*-10, *c*-13, *c*-16, *c*-19).

Fatty acids	Dietary forage group					SEM	p-value
C18:2n-6 *c*-9, *c*-12 (LA)	1.97^{ab}	1.95^{ab}	1.88^{ab}	1.62^{b}	2.13^{a}	0.162	0.066
C18:3n-3 (ALA)	0.343^{b}	0.430^{ab}	0.365^{ab}	0.513^{a}	0.487^{ab}	0.048	**0.045**
C20:0	0.112	0.135	0.128	0.142	0.131	0.013	0.395
C20:4n-6 *c*-5, *c*-8, *c*-11, *c*-14	0.141^{ab}	0.165^{a}	0.160^{a}	0.116^{b}	0.140^{ab}	0.009	**0.007**
EPA	0.090^{b}	0.097^{ab}	0.091^{ab}	0.124^{a}	0.127^{a}	0.010	**0.021**
C22:0	0.073	0.075	0.067	0.097	0.091	0.010	0.148
DHA	0.046	0.043	0.041	0.047	0.035	0.005	0.456
DPA	0.092	0.113	0.077	0.079	0.091	0.011	0.187
C23:0	0.034^{b}	0.061^{a}	0.051^{ab}	0.050^{ab}	0.051^{ab}	0.007	**0.051**

Fatty acids abbreviations: PA = palmitic acid; SA = stearic acid; OA = oleic acid; VA = vaccenic acid; CLA = conjugated linoleic acid; ALA = α-linolenic acid; EPA = eicosapentaenoic acid (C20:5n-3 *c*-5, *c*-8, *c*-11, *c*-14, *c*-17); DHA = docosahexaenoic acid (C22:5n-3 *c*-4, *c*-7, *c*-10, *c*-13, *c*-16, *c*-19); DPA = docosapentaenoic acid (C22:5n-3 *c*-7, *c*-10, *c*-13, *c*-16, *c*-19).

Table 3(continued).

Effect of dietary roughage source on milk fatty acid (FA) profile (g/100 g of fatty acids). HMS = high maize silage; MMS = medium maize silage; MCS = mixed crop silages; HAY = grass and lucerne hays; GRG = green grass; SEM = standard error of the means; $^{a-b}$LSMeans in a row without a common superscript differ ($p < 0.05$).

Calculated values	Dietary forage group					SEM	p-value
	HMS ($n = 20$)	MMS ($n = 18$)	MCS ($n = 11$)	HAY ($n = 12$)	GRG ($n = 9$)		
SFA	69.6^{a}	69.1^{ab}	69.9^{a}	69.6^{a}	66.9^{b}	0.645	**0.003**
MUFA	25.7^{ab}	25.8^{ab}	25.5^{b}	25.9^{ab}	27.7^{a}	0.547	**0.022**
PUFA	4.72^{ab}	5.07^{ab}	4.56^{b}	4.55^{b}	5.21^{a}	0.216	**0.046**
HUFA	0.391	0.447	0.459	0.409	0.436	0.025	0.281
n-3	0.898^{b}	1.08^{ab}	0.979^{ab}	1.219^{a}	1.162^{ab}	0.068	**0.003**
n-6	2.66	2.72	2.57	2.26	2.85	0.183	0.053
n-3:n-6	0.353^{b}	0.436^{ab}	0.401^{ab}	0.570^{a}	0.416^{ab}	0.052	**0.018**
n-6:n-3	3.04^{a}	2.53^{ab}	2.67^{ab}	2.11^{b}	2.60^{ab}	0.245	**0.044**
HUFAn-3	0.209	0.235	0.246	0.251	0.249	0.019	0.382
CLA	0.629^{b}	0.687^{ab}	0.558^{b}	0.684^{ab}	0.868^{a}	0.048	**0.002**
SCFA	9.97^{a}	9.60^{a}	9.56^{ab}	9.42^{ab}	8.83^{b}	0.205	**0.004**
OCFA	2.09	2.34	2.20	2.06	2.30	0.151	0.611

Fatty acids abbreviations: SFA = saturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; HUFA = highly unsaturated FA (double bonds ≥ 4); n-3 = omega-3 fatty acids; n-6 = omega-6 fatty acids; HUFAn-3, highly unsaturated FA n-3; CLA = conjugated linoleic acids; SCFA = short chain FA (≤ C10); OCFA = odd chain FA.

Table 4
Descriptive statistics of the cross-validation based on the leave-one-out criteria of the factorial discriminant analysis (FDA). Bold values represent the samples classified correctly. HMS, high maize silage; HMS = high maize silage; MMS = medium maize silage; MCS = mixed crop silages; HAY = grass and lucerne hays; GRG = green grass; MCC = Matthews correlation coefficient.

	Actual				
Predicted	HMS	MMS	MCS	HAY	GRG
HMS	**19**	1	5	0	0
MMS	0	**17**	0	0	0
MCS	1	0	**6**	0	0
HAY	0	0	0	**12**	0
GRG	0	0	0	0	**9**
Total	20	18	11	12	9
Sensitivity	0.95	0.94	0.55	1.00	1.00
Specificity	0.88	1.00	0.98	1.00	1.00
Accuracy	0.90	0.99	0.91	1.00	1.00
Precision	0.76	1.00	0.86	1.00	1.00
MCC	0.79	0.96	0.64	1.00	1.00

Table 5

Multiple linear regression equations of the most discriminant milk fatty acids (FA) and their derived chemical classes based on the dietary forage sources (% DM). Each equation (data of FA are as g/100 g of fatty acids) is presented in the following format: intercept of the model (± standard error) and regression coefficient of the forages, when significant (*p < 0.05; †p < 0.10; ns = p > 0.10). The p-value refers to the significance of the regression model. For the fatty acids abbreviations see Table 3.

Fatty acids	Intercept	Regression coefficients of the forages				p-value
		Maize silage	Other silages	Hays	Fresh grass	
C16:1	1.53 (± 0.23)	ns	ns	ns	ns	0.229
C17:0	0.35 (± 0.06)	0.0018†	ns	0.0024*	0.0026*	0.001
C18:0	11.4 (± 1.0)	ns	ns	-0.011†	-0.009†	0.078
C18:3n-3	0.51 (± 0.11)	-0.0043*	-0.0033*	ns	ns	0.005
C18:2 c-9, t-11	0.53 (± 0.12)	-0.0026†	-0.0025†	0.0027*	0.0041*	0.003
SFA	65.5 (± 1.4)	0.068*	0.084*	0.054*	ns	0.055
MUFA	28.7 (± 1.3)	ns	ns	-0.045*	ns	0.034
PUFA	5.77 (± 0.47)	-0.018*	-0.035*	0.019*	ns	0.001
CLA	0.72 (± 0.13)	-0.0035*	-0.0049*	ns	ns	0.001
OCFA	2.13 (± 0.40)	ns	ns	ns	ns	0.699

Figures

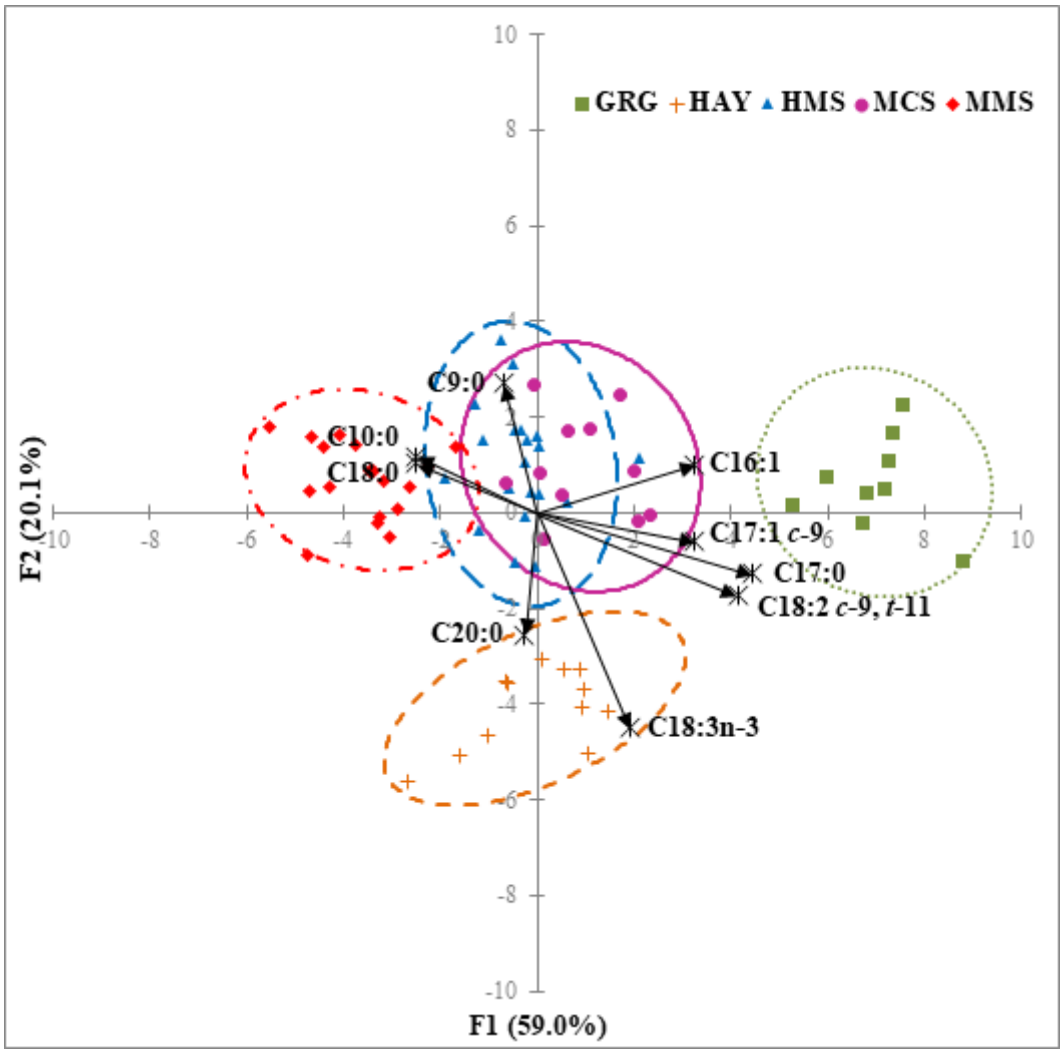

Figure 1

FDA scatterplot of the milk samples according to the five feeding groups based on the fatty acid profiles. F1 and F2 accounted for 59.0% and 20.1% of the total variance, respectively. The 0.95 confidence ellipses are drawn around each centroid of groupings. High maize silage (HMS): blue dotted line and ▲; Medium maize silage (MMS): red dotted-pointed line and •; Mixed crop silages (MCS): solid purple line and •; Grass and lucerne hays (HAY): orange dotted line and +; Green grass (GRG): green pointed line and ■. The black arrows indicate the most significant ($p<0.05$) discriminative FA that had correlation coefficient values higher than 0.20 with at least either F1 or F2 (for graphic purposes these significant correlations coefficients were multiplied 10 times according to the maximun value of F1 and F2).

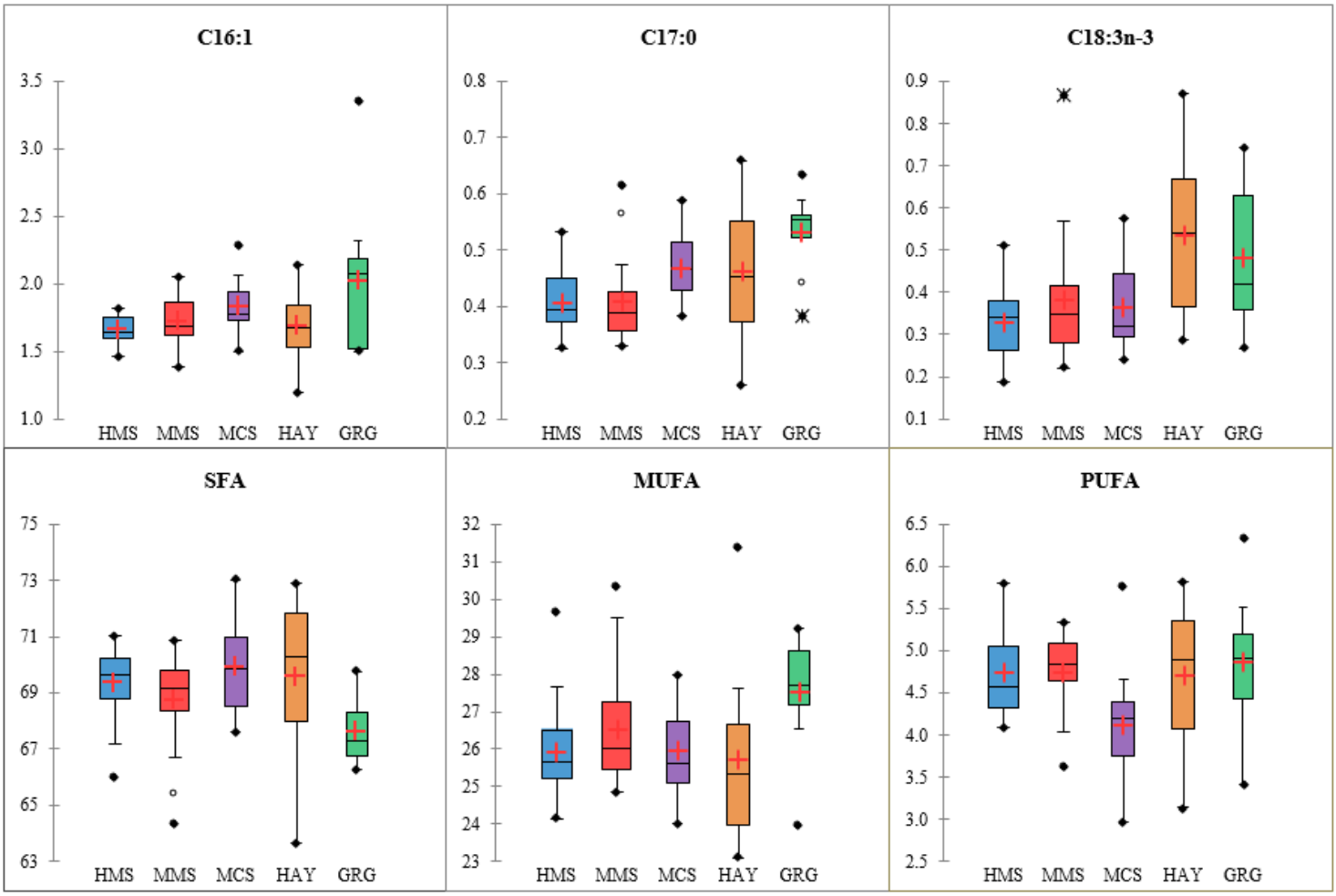

Figure 2

Box–Whisker plots of fatty acids (g/100 g of total fatty acids) according to the five feeding groups. HMS = high maize silage; MMS = medium maize silage; MCS = mixed crop silages; HAY = grass and lucerne hays; GRG = green grass. The box plots represent the following descriptive statistics: median (bar in box), mean (+, red cross), 25% (Q1) and 75% (Q3) quartile (bottom and top end of the box), minimum [Q1 - 1.5 × (Q3 - Q1)] and maximum [Q1 + 1.5 × (Q3 - Q1)] whiskers (lines outside), minimum and maximum values (•, full black circles), outliers with a distance to box of 1.5–3.0 times interquartile range (°, empty circles) or higher than 3 times interquartile range (*, asterisks). For the fatty acids abbreviations see Table 3.

Supplementary Files

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- TableS1.docx