Milk Fatty Acids as Biomarkers of Metabolic Diseases in Dairy Cows identified through Thin Layer Chromatography and Gas Chromatographic Techniques (TLC-GC)

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SUMMARY

In the transition period an excessive mobilization of adipose tissue in high milk production dairy cows predisposes to metabolic diseases as subclinical ketosis. The aim of this research was to identify the association between the concentration of milk fatty acids and the elevated plasmatic value of Non Esterified Fatty Acid (NEFA) for the diagnosis of excessive lipomobilization in dairy cows using Thin Layer Chromatography and Gas Chromatographic Techniques (TLC-GC). Fifty-four multiparous Holstein-Friesian dairy cows in the first phase of lactation were enrolled in the study. Blood samples from the coccygeal vein were collected and Non-Esterified Fatty Acids (NEFA) was evaluated in laboratory of University of Padua. Milk samples (40 mL) were taken at the evening milking from each bovine enrolled in the trial. Animals were divided into two groups on the basis of blood NEFA: healthy animals (NEFA-0) with a value of NEFA ≤ 0.57 mEq/L and sick animals (NEFA-1) with a value of NEFA > 0.57 mEq/L.
Milk fatty acids concentrations have been evaluated in 4 lipid classes: Free Fatty Acids (FFA), Cholesterol Esters (CE), Phospholipids (PL), and Triglycerides (TG). Data were analysed using SAS system software (version 9.4; SAS Institute Inc., Cary, NC, USA). The General Linear Model (GLM) analysis was performed for repeated measurements in order to evaluate the differences in the composition of milk fatty acids related to the four lipid fractions in function of two different NEFA blood concentrations (NEFA-0 vs NEFA-1). The results showed the following statistical significance ($p \leq 0.05$) in the milk lipid classes: two fatty acids were significant in CE, one fatty acid was significant in FFA, nine fatty acids were significant in TG and one fatty acid was significant in PL. These milk fatty acids, with predictive value for the development of metabolic disorder, could be considered valuable new biomarkers.

KEY WORDS

Transition period, non-esterified fatty acid, milk fatty acids, lipid classes, thin layer-gas chromatographic techniques.

INTRODUCTION

In the transition period the energy demand for fetal growth and the lactogenesis, results in an energy deficiency[1]. This energy disequilibrium is expressed through a negative energy balance (NEB) aggravated by the reduced of Dry Matter Intake (DMI). The NEB leads to a release of Non-Esterified Fatty Acids into the blood as a result of an enhancement of lipolysis[2,3]. NEFA are biomarkers of excessive lipid mobilization in high milk production dairy cows. The increased plasma concentration of NEFA is associated with a grater incidence of metabolic disorder such as ketosis and fatty liver[4]. Under normal energy balance conditions, the plasma concentration of NEFA in dairy cows is less than 0.2 mEq/L[5]. In prepartum, the blood concentration of NEFA begins to increase up to a threshold, which should not exceed 0.29 mEq/L. Value of NEFA higher than the cut-off indicates an increased risk of developing metabolic pathologies. After calving the threshold is greater than 0.57 mEq/L[6,7]. Within the first 5-6 weeks of lactation, the plasma concentration of NEFA should stabilize around a value of 0.300 mEq/L in healthy dairy cows[8].
The excessive mobilization of adipose tissue precedes subclinical - clinical ketosis development and mobilized fatty acids (FA) are incorporated in milk fat. Changes in milk FA composition might be an earlier indicator of hyperketonemia[9,10]. The milk lipid concentration higher than 4.8% is indicative of a severe NEB, and consequently of a high blood NEFA concentration[11]. Lipids in milk derive either from the ex novo synthesis at the mammary gland level or from the uptake into bloodstream. The substrates used for the ex-novo synthesis are acetic acid and butyric acid. Short and medium chain fatty acids (C4:0 - C14:0) and 50% of fatty acids with a hydrocarbon chain consisting of 16 carbon atoms, are synthesized in the epithelial cells of mammary gland[12,13]. The uptake of fatty acids by the mammary gland

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from the bloodstream provides part of the fatty acids with 16 carbon atoms and all the Long Chain Fatty Acids (LCFA)[14]. The different plasma fatty acids, mobilized from the lipolysis of the adipose tissue, contribute to composition of the milk lipid profile[15]. NEFA, mostly released into circulation from body reserves, are C16:0, C18:0, and C18:1 ω 9[16,17] and their uptake by the mammary gland is directly proportional to their blood concentration[12].

A percentage variable from 96 to 98% of total milk lipids derives from the class of Triglycerides (TG) which constitute cell membranes. The residual percentage is compound of Phospholipids (PL), Cholesterol (CO) and Cholesterol Esters (CE)[18]. The content of Free Fatty Acids (FFA) is very limited[19]. The milk fatty acid present in high concentration are Saturated (SFA- 70%) and Monounsaturated (MUFA-25%)[20,21]. *Oleic acid* (C18:1 ω 9) is the main MUFA present in milk and is also used as a biomarker of excessive concentration of NEFA in the blood[17]. *Myristic acid* (C14:0), *Palmitic acid* (C16:0) and *Stearic acid* (C18:0) are the SFA present in greater quantities in milk[21]. Polyunsaturated Fatty Acids (PUFA) are more concentrated in the lipid fractions of PL and CE, associated with High Density Lipoprotein (HDL) for transport in the bloodstream. The uptake by the mammary gland of lipids from HDL is very low, and for this reason PUFA have lower concentrations in milk, about 5% of the total[12,20].

Dairy cows after calving, present SFA in lower concentrations but these fatty acids increase up to the twelfth week when the energy balance improves. On the other hand, MUFA, mostly represented by C18:1 ω 9, increase the amount in the first phase of lactation and the decrement occurs with the improvement of the NEB[22,23]. High concentrations of Unsaturated Fatty Acids in milk (MUFA and PUFA) and low concentrations of SFA indicate energy imbalance[23].

Moreover, the lactation stage has a significant impact on the lipid profile of milk[24]. The high uptake of LCFA by the mammary gland in the first lactation phase negatively affects the ex novo synthesis of fatty acids[23]. LCFA, reduce the ex-novo synthesis through the inhibition of the enzyme acetyl-CoA carboxylase[22]. Indeed, the increase in the lipid profile of LCFA and the decrease in Short Chain Fatty Acids (SCFA) in postpartum are indicative of a negative energy balance[23]. The lipid profile of the fatty acids belonging to the 4 lipid classes changes as the energy balance enhance throughout the lactation[25].

The goals of our research were to identify a new method not requiring blood sampling that could be incorporated into the daily milking routine and the identification of new biomarkers for the early diagnosis of excessive lipid mobilization and subclinical-clinical ketosis. In order to achieve these aims we examined the correlation between concentrations of fatty acids in milk with elevated NEFA concentrations during the first phase of lactation. The evaluation of the fatty acids concentrations in milk could allow the implementation of nutritional and management strategies in order to avoid the development of metabolic diseases.

MATERIALS AND METHODS

The data analyzed for this article were collected in the context of the Bovine OMICS Project (supported by the University of Padua), based on the analysis through TLC-GC of the plasma lipid profile and milk lipid profile of dairy cows in the transition period. The aim of Bovine OMICS Project was to identify new biomarkers of metabolic disorder. In the first study of Fiore et al., plasma fatty acids were evaluated on the basis of blood BHB, subsequently Fiore et al., investigated the fatty acids in milk always based on the blood value of BHB[26,27,28]. In this article we analyzed the milk fatty acids considering the value of blood NEFA.

Animals

Fifty-four Holstein Frisian dairy cows enrolled in this study, were the same as those sampled in the research of Fiore et al[27]. The farm, located in Northern Italy (45° 36' N. 11° 40' E. 23 m above sea-level), consisted of a single building, with a free housing system and cubicle surface were equipped with a rubber mattress. The group of animals enrolled were in early postpartum, with an average of 26.5 ± 1.5 days in milk (DIM) as the development of ketosis is more frequent between the second and seventh week of lactation[29]. All animals had a dry period of 55 ± 5 days and steaming-up was not carried out. Total Mixed Ration (TMR) and the chemical composition of the lactation diet were reported in the study by Fiore et al.[26] and water was available ad libitum.

All animals were evaluated by the Veterinarian of University of Padua (Italy) and were clinically healthy. Animals had not developed mastitis during ongoing lactation prior to sampling. Bovine did not present clinical signs of mastitis and SCC (Somatic cell count) was also used to define udder health status[30]. All animals had SCC values lower than 200.000 cells/mL during ongoing lactation.

Experimental design

Blood sampling was collected using coccygeal vein puncture. In order to select cows with metabolic disorders, before the blood sample, the BHB was measured using the Nuova Biomedical Express (Nuova Biomedical, Runcorn, United Kingdom) digital reader with specific BHB test strips (Stat Strip Ket, Nova Biomedical). Three samples were collected with the vacutainer system for each enrolled cow. The first aliquot was taken using a tube containing EDTA (5 ml; Terumo Venoject, Leuven, Belgium) and the other two aliquots were stored in Venosafe tubes containing Clot Activator (9 ml; Terumo Venosafe, Leuvel, Belgium).

Milk samples were taken at the evening milking from each bovine enrolled in the trial. For sterile sampling, the operator was equipped with disposable gloves to minimize any potential risk of contamination and induction of mastitis. After cleaning, drying and disinfection of the teat, the first 2 foremilk were discarded by manual milking. Then, 40 mL was aseptically collected in sterile plastic tubes (50 mL, Vetrotecnica S.r.l., Padova, Italy). The biological material collected was transported to the Clinical Diagnostic Laboratory of the Department of Animal Medicine, Production and Health (MAPS) of the University of Padua (Italy) stored at 4°C. The blood samples containing Clot Activator were centrifuged in the laboratory (Heraeus Labofouge 400, Thermo Scientific, Milan, Italy). Finally, serum and milk samples were stored at -20° for subsequent biochemical analysis.

Blood analysis

Serum biochemistry and plasma gas chromatography (GC) were performed in the Clinical Diagnostic Laboratory of MAPS Department.

The Serum was assessed employing automatic clinical chemistry analyser (BT3500 Biotecnica Instrument SPA, Rome, Italy). Serum NEFA (Non-Esterified Fatty Acids) concentration was measured with the NEFA RX Monza test colorimetric method (Randox, Crumlin, UK).

On the basis of NEFA concentrations obtained in the laboratory, animals were divided into two different groups: Group NEFA-0 had 33 animals with NEFA concentration lower than 0.57 mEq/L and Group NEFA-1 had 21 animals with a NEFA quantity higher than 0.57 mEq/L. This cut-off was chosen based on the work of Ospina (2010); this value indicates excessive lipomobilization and can be used as a threshold for monitoring dairy cows at risk of postpartum diseases[7].

Milk analysis

In order to perform the GC in the milk, the samples underwent three stages of treatment including 1) extraction of lipids from the milk 2) separation of the lipid classes by Thin Layer Chromatography (TLC) 3) methylation of the carbon chain. The method used for the quantification of milk fatty acids was the same used in the studies of Fiore et al.[26,27,28] in accordance with the study of Carnielli et al.[31].

Before starting these procedures, the samples were mixed with the internal standards (C9-C15 or C17) of every lipid class. Finally, for each milk sample 30 fatty acids were obtained per lipid classes: FFA (free fatty acids), PL (phospholipids), TG (triglycerides) and CE (cholesterol esters). The fatty acids methyl esters were separated and quantified in splitless mode by GC using a TRACE GC/MS (Thermo Quest, Milan, Italy) equipped with a flame ionization detector (FID) and a polar fused-silica capillary column (Capillary Column Omegawax, 30 m× 0.25 mm × 0.2 µm film). Helium was used as the carrier gas at a flow rate of 1 ml/min. Data for plasma fatty acid were calculated in mg/dL.

Statistical analysis

Blood biochemical parameters and milk fatty acid methyl ester data were analyzed using the SAS system software (version 9.4; SAS Institute Inc., Cary, NC, USA). The General Linear Model (GLM) analysis was performed for repeated measurements in order to evaluate the differences in the composition of milk fatty acids in the four lipid fractions within the two classes (NEFA-0 - vs NEFA-1).

Statistical significance was set at $p \leq 0.05$.

RESULTS

The mean values (±SEM) regarding NEFA, Day in Milk (DIM), Body Condition Score (BCS), parity and yield milk produced (kg/day) for all enrolled animals based on the two classes of NEFA (NEFA-0 vs NEFA-1) are presented in Table 1. The mean value of NEFA concentration was 0.23 ± 0.10 mEq/L and 0.83 ± 0.43 mEq/L for NEFA-0 and NEFA-1 group, respectively. The different composition of fatty acids in the four lipid classes of FFA, CE, PL and TG was assessed through analyzes performed in TLC-GC technique. The mean value of the 30 milk fatty acid relating to these four classes of lipids (FFA, CE, PL and TG) was compared between the two groups with different blood concentrations of non-esterified fatty acid (NEFA-0 vs NEFA-1).

In the Table 2 are shown the mean values and the standard error (SE) of the different milk fatty acids related to the lipid class of CE. A statistically significant difference between NEFA-0 and NEFA-1 was found for C10:0 ($p = 0.023$) and C20:5 ω 3 ($p = 0.05$).

Table 1 - Mean value (±SEM) of NEFA, days in milk (DIM), body condition score (BCS), parity, and daily milk yield.

Parameters	NEFA-0 (n = 33)	NEFA-1 (n = 21)	Correlation (*p*-value)
NEFA [1] (mEq/L)	0.23 ± 0.10	0.83 ± 0.43	<0.001
DIM [2]	28.34 ± 12.44	25.76 ± 14.96	<0.001
BCS [3]	2.75 ± 0.21	2.89 ± 0.15	<0.001
Parity	2.58 ± 1.86	2.57 ± 1.40	NS [4]
Milk (kg/day)	28.40 ± 7.38	30.71 ± 8.43	<0.001

[1]: non-esterified fatty acid; [2]: day in milk; [3]: body condition score; [4]: not significant.

Table 2 - Milk Fatty Acids mean values with SE related to the lipid class of Cholesterol Ester (TLC-GC).

Free Fatty Acids (CE) mg/dL	NEFA-0 (n = 33)	SE NEFA-0	NEFA-1 (n = 21)	SE NEFA-1	Correlation (*p*-value)
C6	1.72	0.37	1.46	0.42	NS
C8	0.99	0.16	0.80	0.18	NS
C10	1.50	0.17	0.89	0.19	0.023
C12	4.46	0.56	3.38	0.64	NS
C14	1.51	0.24	0.99	0.27	NS
C14:1 ω 5	1.24	0.57	0.68	0.65	NS
C16	3.56	1.15	5.70	1.31	NS
C16:1 ω 7	0.78	0.26	0.68	0.30	NS
C18	1.39	0.19	1.24	0.21	NS
C18:1 ω 9	0.88	0.21	0.85	0.25	NS
C18:2 ω 6	0.31	0.04	0.29	0.05	NS
C18:3 ω 6	0.06	0.03	0.09	0.03	NS
C18:3 ω 3	0.18	0.12	0.14	0.13	NS
C20:2 ω 6	0.11	0.03	0.13	0.03	NS
C20:3 ω 6	0.04	0.01	0.03	0.01	NS
C20:4 ω 6	0.09	0.03	0.05	0.04	NS
C20:3 ω 3	0.18	0.06	0.12	0.07	NS
C20:5 ω 3	0.16	0.04	0.04	0.05	0.05
C22	0.03	0.01	0.03	0.01	NS
C22:1 ω 9	0.09	0.02	0.04	0.03	NS
C22:2 ω 6	0.04	0.02	0.03	0.02	NS
C22:4 ω 6	0.36	0.15	0.53	0.17	NS
C22:5 ω 3	0.06	0.02	0.05	0.02	NS
C22:6 ω 3	0.11	0.04	0.19	0.05	NS
C23	0.19	0.05	0.28	0.06	NS
C24	0.13	0.03	0.11	0.03	NS
C24:1 ω 9	0.04	0.01	0.05	0.01	NS
C16 DMA	8.46	0.40	8.57	0.46	NS
mg/dl	78.40	6.46	70.81	7.37	NS

NS: not significant.

Table 3 - Milk Fatty Acids mean values with SE related to the lipid class of Phospholipids (TLC-GC).

Free Fatty Acids (PL; mg/dL)	NEFA-0 (n = 33)	SE NEFA-0	NEFA-1 (n = 21)	SE NEFA-1	Correlation (*p*-value)
C6	0.40	0.16	0.80	0.20	NS
C8	0.27	0.12	0.25	0.15	NS
C10	1.04	0.33	0.52	0.42	NS
C12	4.42	1.69	2.87	2.19	NS
C14	2.68	0.78	1.83	1.01	NS
C14:1 ω 5	0.12	0.04	0.07	0.06	NS
C16	7.55	1.79	6.44	2.32	NS
C16:1 ω 7	0.07	0.08	0.27	0.10	NS
C18	3.53	0.41	4.57	0.53	NS
C18:1 ω 9	3.70	1.18	3.31	1.52	NS
C18:2 ω 6	0.65	0.18	1.01	0.23	NS
C18:3 ω 6	0.04	0.01	0.03	0.01	NS
C18:3 ω 3	0.07	0.02	0.04	0.03	NS
C20	0.11	0.02	0.17	0.03	NS
C20:1 ω 9	0.03	0.01	0.06	0.01	NS
C20:2 ω 6	0.03	0.04	0.12	0.05	NS
C20:3 ω 6	0.10	0.02	0.20	0.02	NS
C20:4 ω 6	0.10	0.04	0.16	0.05	NS
C20:3 ω 3	0.02	0.003	0.02	0.004	NS
C20:5 ω 3	0.05	0.02	0.06	0.03	NS
C22	0.66	0.12	0.90	0.16	NS
C22:1 ω 9	0.03	0.01	0.01	0.02	NS
C22:2 ω 6	0.01	0.01	0.01	0.01	NS
C22:4 ω 6	0.12	0.05	0.06	0.06	NS
C22:5 ω 3	0.03	0.02	0.07	0.03	NS
C22:6 ω 3	0.06	0.02	0.13	0.03	0.05
C23	0.28	0.07	0.42	0.09	NS
C24	0.64	0.08	0.82	0.10	NS
C24:1 ω 9	0.24	0.16	0.07	0.21	NS
C16 DMA	1.14	0.16	1.30	0.21	NS
mg/dl	41.87	9.70	39.21	12.53	NS

NS: not significant.

Table 4 - Milk Fatty Acids mean values with SE related to the lipid class of Free Fatty Acids (TLC-GC).

Free Fatty Acids (FFA; mg/dL)	NEFA-0 (n= 33)	SE NEFA-0	NEFA-1 (n= 21)	SE NEFA-1	Correlation (*p*-value)
C6	2.65	0.72	3.09	0.88	NS
C8	6.92	1.07	5.75	1.32	NS
C10	8.64	1.77	5.45	2.16	NS
C12	9.25	1.24	7.77	1.52	NS
C14	7.35	1.42	6.29	1.74	NS
C14:1 ω 5	0.25	0.07	0.15	0.09	NS
C16	1.92	0.46	1.12	0.56	NS
C16:1 ω 7	23.99	4.27	22.82	5.23	NS
C18	6.15	0.80	5.85	0.98	NS
C18:1 ω 9	3.16	1.25	2.29	1.53	NS
C18:2 ω 6	0.38	0.16	0.23	0.20	NS
C18:3 ω 6	0.11	0.03	0.06	0.04	NS
C18:3 ω 3	0.07	0.05	0.01	0.06	NS
C20	0.09	0.01	0.07	0.02	NS
C20:1 ω 9	0.02	0.01	0.04	0.02	NS
C20:2 ω 6	0.13	0.03	0.07	0.03	NS
C20:3 ω 6	0.14	0.03	0.11	0.04	NS
C20:4 ω 6	0.05	0.02	0.07	0.02	NS
C20:3 ω 3	0.02	0.01	0.02	0.01	NS
C20:5 ω 3	0.04	0.01	0.02	0.01	NS
C22	0.07	0.03	0.05	0.03	NS
C22:1 ω 9	0.02	0.01	0.01	0.01	NS
C22:2 ω 6	0.38	0.12	0.08	0.15	NS
C22:4 ω 6	0.16	0.05	0.03	0.06	0.05
C22:5 ω 3	0.09	0.04	0.01	0.05	NS
C22:6 ω 3	0.21	0.07	0.21	0.09	NS
C23	0.29	0.06	0.28	0.08	NS
C24	0.08	0.01	0.06	0.01	NS
C24:1 ω 9	0.05	0.01	0.04	0.01	NS
C16 DMA	2.38	0.29	1.97	0.35	NS
mg/dl	75.43	11.63	64.23	14.25	NS

NS: not significant.

Table 3 shows the mean values and the standard error (SE) of the different milk fatty acids related to the lipid class of PL. A statistically significant difference between NEFA-0 and NEFA-1 was found for C22:6 ω 3 ($p = 0.05$).

Table 4 shows the mean values and the standard error (SE) of the different milk fatty acids related to the lipid class of FFA. Comparing the two groups NEFA-0 and NEFA-1 only C22:4 ω 6 was statistically different ($p = 0.05$).

Data of the mean values and the standard error (SE) of the different milk fatty acids related to the lipid class of TG are presented in Table 5. A statistically significant difference between NEFA-0 and NEFA-1 was found for C14:1 ω 5 ($p = 0.043$), C16:0 ($p = 0.05$), C16:1 ω 7 ($p = 0.013$), C18:1 ω 9 ($p = 0.05$), C18:3 ω 3 ($p = 0.05$), C20:1 ω 9 ($p = 0.05$), C22:0 ($p = 0.05$) and C22:4 ω 6 ($p = 0.011$).

DISCUSSION

In order to identify, among milk fatty acids, new biomarkers with predictive function in the development of metabolic pathologies, we divided the recruited animals according to plasmatic NEFA. Plasma NEFA concentrations are considered to be important risk factors for the development of metabolic disease[32]. Mann et al. (2016), assessed the variation in milk fatty acid concentrations in dairy cows with different blood NEFA values, using as threshold value 1.0 mmol/L. The main biomarkers selected in the study mentioned above were *Pentadecanoic* acid (C15:0), *Palmitoleic acid* (C16:1 ω 9), C18:1 ω 9 and also the ratio C16:1 ω 9/ C15:0 and C18:1 ω 9/ C15:0[10]. Differently from Mann et al., our study, for the first time, evaluated the difference between the fatty acids distinguished by lipid

Table 5 - Milk Fatty Acids mean values with SE related to the lipid class of Triglycerides (TLC-GC).

Free Fatty Acids (TG; mg/dL)	NEFA-0 (n= 33)	SE NEFA-0	NEFA-1 (n= 21)	SE NEFA-1	Correlation (*p*-value)
C6	29.21	4.30	31.13	5.37	NS
C8	28.26	3.75	27.53	4.67	NS
C10	51.70	6.70	48.13	8.36	NS
C12	39.09	5.98	46.92	7.46	NS
C14	92.19	7.50	87.16	9.36	NS
C14:1 ω 5	4.37	0.69	6.65	0.86	0.043
C16	275.82	24.59	352.24	30.67	0.05
C16:1 ω 7	11.73	1.93	19.70	2.41	0.013
C18	96.57	13.13	110.07	16.37	NS
C18:1 ω 9	205.30	32.62	311.04	40.69	0.05
C18:1 ω 7	13.26	1.64	14.91	2.05	NS
C18:2 ω 6	25.51	3.14	29.73	3.92	NS
C18:3 ω 6	0.96	0.11	1.03	0.14	NS
C18:3 ω 3	3.64	0.43	4.88	0.54	0.05
C20	0.96	0.19	1.22	0.23	NS
C20:1 ω 9	0.66	0.25	1.35	0.31	0.05
C20:2 ω 6	0.45	0.05	0.48	0.06	NS
C20:3 ω 6	0.71	0.09	0.66	0.11	NS
C20:4 ω 6	1.61	0.27	2.21	0.33	NS
C20:3 ω 3	0.12	0.02	0.12	0.03	NS
C20:5 ω 3	2.17	0.59	1.00	0.74	NS
C22	0.38	0.07	0.15	0.09	0.05
C22:1 ω 9	0.07	0.02	0.07	0.02	NS
C22:2 ω 6	0.02	0.01	0.03	0.01	NS
C22:4 ω 6	0.20	0.04	0.02	0.05	0.011
C22:5 ω 3	0.39	0.06	0.45	0.07	NS
C22:6 ω 3	0.19	0.04	0.11	0.05	NS
C23	0.58	0.18	1.02	0.23	NS
C24	0.13	0.02	0.11	0.03	NS
C24:1 ω 9	0.16	0.02	0.15	0.03	NS
C16 DMA	1.25	0.20	1.16	0.25	NS
mg/dl	943.37	95.13	1170.45	118.65	NS

NS: not significant.

class of belonging: CE, PL, FFA and TG. The variation of fatty acid concentrations within the lipid fractions of milk, could be used as a marker to predict a state of hyperketonemia[9].

In table 1, statistically significant difference ($p < 0.001$) was highlighted between healthy (NEFA-0) and sick animals (NEFA-1) with regards to concentration of plasmatic NEFA (0.23 ± 0.10 mEq/L vs 0.83 ± 0.43 mEq/L); while no statistically significant difference was detected regarding the number of lactations between the two groups (2.58 ±1.86 vs 2.57 ±1.40).

Animals with an excessive BCS have a more reduction in the voluntary ingestion of dry matter (DMI) during the transition period[33]. The reduction of DMI leads to a greater activation of the biochemical process of lipolysis and an increase of plasma fatty acids concentrations[33]. As expected, BCS score was significantly ($p < 0.001$) higher in dairy cows with high blood concentrations of NEFA (NEFA-1, 2.89 ± 0.15) than in healthy cows (NEFA-0, 2.75 ± 0.21). In the study of Gillund et al. (2010), dairy cows that developed a metabolic disorder in post-partum, such as ketosis, had a greater BCS score at calving than a healthy bovine[34]. In our study, repeated measures of BCS to calculate the loss of nutritional status have not been evaluated, but we expected a higher progressive reduction of the BCS score in NEFA-1 group than bovine with normal value of NEFA. Indeed, BCS drops abruptly the first 42 DIM and it is relatively constant thereafter in animals with normal concentration of BHB. In ketotic bovine the BCS continues a negative decrease until around 90 DIM[34].

Milk production generated a significant difference ($p < 0.001$) between the healthy group (NEFA-0, 28.40 ± 7.38 kg/day) and sick animals (NEFA-1, 30.71 ± 8.43 kg/day). When the energy demand for milk production exceeds the available energy, the lipolysis is activated with the release of NEFA into the bloodstream[35].

Subsequently, we analyzed the acid composition of each lipid class comparing the two groups of animals (NEFA-0 *vs* NEFA-1).

In **CE** lipid class, a reduction of milk fatty acids has been observed in cows with blood NEFA concentrations higher than 0.57 mEq/L compared to healthy animals. Table 2 showed that total fatty acids decrease their concentrations as blood NEFA increase (NEFA-0 = 78.40 mg/dL; NEFA-1 = 70.81 mg/dL). The ruminants present a decrease of the enzyme Lecithin Cholesterol Acyl-Transferase (LCAT) after calving[36,37]. LCAT is a serum enzyme, synthesized in the liver, that catalyzes the transfer of fatty acids from lecithin to cholesterol producing esterified cholesterol (CE)[37].

In the study of Nakagawa and Katoh (1998), animals in ketosis presented a greater decrease in blood concentrations of LCAT and CE compared to healthy cows[38]. The decrease in blood concentrations could explain their reduced uptake by the mammary gland[12]. Also, in our data, C10:0 ($p = 0.023$) and C20:5 ω 3 ($p = 0.05$), significant milk fatty acids, show decreasing concentrations as the blood NEFA increase.

The milk lipid fraction of **PL** is predominantly constitute of saturated LCFA and saturated medium chain fatty acids (MCFA) such as *Lauric acid* (C12:0), C14:0, C16:0 and C18:0 and unsaturated LCFA like C18:1 ω 9 and *Linoleic acid* (C18:2 ω 6)[39]. Also, in our study a higher concentration of these fatty acids was noted in the lipid fraction of PL. Furthermore, dairy cows with a normal NEFA value (NEFA-0) have higher concentrations of these fatty acids than cows with excessive mobilization of adipose tissue (NEFA-1) (Table 3).

HDL and Low-Density Lipoproteins (LDL) transport PL into the bloodstream[12]. Dairy cows with fatty liver in post-partum, resulting from excessive mobilization of fat reserves, have lower concentrations of PL associated with HDL compared to animals with normal fat infiltration in the liver[40]. This further reduces uptake by the mammary gland[12]. Therefore, the decrease in milk fatty acids in PL lipid class was expected in cows with greater alteration of lipid metabolism (NEFA-1). Contrary, in our data, healthy animals had total fatty acid values equal to 41.87 mg/dL compared to bovine with high concentrations of NEFA where the value was 39.21 mg/dL.

In BEN, the plasma fatty acids increase their concentrations as blood BHB and NEFA concentrations increase[27]. Contrary to plasma concentrations, our study showed that milk fatty acids, belonging to **FFA** lipid class, tended to decrease with in-

creasing blood NEFA. In table 4, the total fatty acids in healthy animals (NEFA-0, 75.43 mg/dL) were greater than the total fatty acids present in the NEFA-1 group (65.23 mg/dL). It is essential to note how concentration of *Arachidonic acid* (C22: 4 ω 6), necessary precursor for the endogenous synthesis of inflammatory mediators, decreased significantly ($p = 0.05$) between healthy cows (0.16 mg/dL) to animals in a negative state of excessive lipid mobilization (0.03 mg/dL). Further studies are needed to evaluate the variation of fatty acids in the FFA lipid fraction especially what concerns eicosanoids, precursors of inflammation mediators[41].

A variable concentration from 96 to 98% of the milk lipid composition is constituted by **TG**[18]. Total concentrations of **TG** in the NEFA-0 group were 943.37 mg/dL whereas, NEFA-1 group had a value of 1170.45 mg/dL. Analyzing the lipid fraction of **TG**, we observed that most of the significant fatty acids were higher in the group of animals affected by excessive lipid mobilization (NEFA-1) with respect to the NEFA-0 group (healthy animals). The increase of these lipid fractions is indicative of an energy imbalance[11].

In the present study 5 out of the six MUFA and PUFA (TG class) increased their concentrations with increasing of plasma NEFA (Table 5) in accordance to the study of Vrankovic et al. (2017), in which milk concentrations of MUFA and PUFA are higher in dairy cows with higher NEB[23]. *Myristoleic acid* (C14:1 ω 5, $p = 0.043$), *Palmitic acid* (C16:1 ω 7, $p = 0.013$), α-*Linolenic acid* (C18:3 ω 3, $p = 0.05$) e *Gondoic acid* (C20:1 ω 9, $p = 0.05$) had higher concentrations in the NEFA-1 group than in the NEFA-0 group. Specifically, *Oleic acid* (C18:1 ω 9) had a statistically different ($p \leq 0.05$) concentration of 205.30 mg/dL in the NEFA-0 group while in sick animals the quantity is equal to 311.04 mg/dL. *Oleic acid* in milk fat is a good indicator of NEB[10,17,42] both in the early lactation phase and in any other period in which the animal is anorexic or suffering from ketosis[9]. Indeed, *Oleic acid* is the main fatty acid contained in the adipose tissue and released during the periods of NEB[33]. Dairy cows, affected by subclinical ketosis, often present high quantities of C18:1 ω 9 in milk fat, which could potentially correlate with a deficiency of the enzyme acyl-CoA dehydrogenase[9].

Moreover, animals in subclinical ketosis and in NEB have lower concentrations of Medium Chain Saturated Fatty Acids (MCSFA) and SFA in milk than healthy cows[9,23]. In our study C22:0 decreased significantly ($p = 0.05$) with increasing NEFA value (NEFA-0, 0.38 mg/dL; NEFA-1, 0.15 mg/dL), as in available literature, whereas C16:0 had higher concentrations ($p = 0.05$) in the NEFA-1 group (352.24 mg/dL) compared to the NEFA-0 group (275.82 mg/dL)[23]. The higher level of concentration of C16:0 could be explained by the increase of mobilization of fat reserves that occurred in dairy cows with a negative energy balance[17].

Moreover, in the present study was examined the different chemical composition of significant fatty acids, evaluating the presence of double bounds. Predictive values include 3 SFA and 9 UFA, that were divided in four MUFA and five PUFA.

According to Vrankovi et al. (2017), although not distinguishing fatty acids for individual lipid fractions, showed that the concentration of all MUFA increased in dairy cows affected by a more intense NEB[23]. In our study C14:1 ω 5, C16:1 ω 7, C18:1 ω 9 and C20:1 ω 9, fatty acids with predictive function for the development of metabolic disorder, had higher concentrations in the NEFA-1 group and belonged to the TG lipid class.

As in available literature, PUFA, C20:6 ω 3 and C18: 3 ω 3, showed an increasing trend when the concentrations of blood NEFA increase[23]. Conversely, C22:4 ω 6 (FFA class and TG class) and C22:5 ω 3 (CE class) exhibited an anomalous trend, decreasing their concentrations with increasing lipid mobilization. This abnormality in the PUFA class could be caused due to different composition of the diet of recruited animals. Feeding largely affects the content of LCFA in milk[21,43]. Diets enriched with rapeseed oil or linseed oil lead to an increase in PUFA in milk[44]. In the study of Rutkowska et al. (2015), soybeans increase the content of PUFA ω 3 and PUFA ω 6 whereas rapeseed seeds cause an increase in the proportion of C18:2 ω 9 and C18:1 ω 9[45]. Therefore, a limitation of the present study was the diet of enrolled animals.

CONCLUSIONS

In our study, the trend of milk fatty acids in four lipid classes (CE, PL, FFA and TG) with different blood concentrations of Non-Esterified Fatty Acid was comprehensively described and 12 predictive parameters were identified. The variation in the composition of plasma fatty acids in the first lactation phase confirmed the applicability of TLC-GC as a new method, not requiring blood sampling, that could be incorporated into the daily milking routine. Finally, we concluded that the main fatty acids with high predictive power for the diagnosis of excessive lipomobilization were MUFA belong to TG lipid fraction such as C14:1 ω 5, C16:1 ω 7, C18:1 ω 9 and C20:1 ω 9.

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Ethical approval

Ethics statement approved by Animal Care and Use Committee of University of Padua (number 91/2019 - "BovineOmics" Projects).

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