J. Dairy Sci. 109:3075–3092
https://doi.org/10.3168/jds.2025-27695

A multivariate approach to exploring interrelationships among milk fatty acids across ruminant species

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ABSTRACT

The milk fatty acid (FA) profile is influenced by complex interactions among animal species, physiology, feeding, and management, resulting in modification to the nutritional and functional properties of milk fat. This study aimed to identify latent biological and nutritional factors influencing milk FA composition in ruminants using multivariate factor analysis (MFA), applied across 4 species: dairy cows (Holstein-Friesian and Brown Swiss), sheep (Sarda and Massese), Mediterranean buffaloes, and goats (Saanen). A total of 1,960 mid-lactation animals (150 ± 16 DIM for cows; 90 ± 5 DIM for sheep; 162 ± 14 DIM for buffalo; 86 ± 3 DIM for goats), raised under commercial farm conditions and subjected to diverse feeding regimens, were included in the study. All milk samples were analyzed by the same laboratory using GC under consistent analytical conditions. Factor analysis was carried out on 49 individual FA. An ANOVA on the factor scores was performed to assess the effects of species and feeding strategies, specifically lipid supplementation in cow milk and n-3 supplementation in sheep milk. The MFA was able to extract 7 latent factors with specific biologic meaning: synthesis of odd- and branched-chain fatty acids from ruminal bacteria (factor 1: "OBCFA"), de novo FA synthesis via acetate in mammary gland (factor 2: "Mammary activity"), biohydrogenation of linoleic acid (factor 3: "BH_1"), milk fluidity regulation (factor 4: "Milk fluidity"), mitochondrial Beta-oxidation of FA (factor 5: "Beta-oxidation"), biohydrogenation via vaccenic acid (factor 6: "BH_2"), ruminal 16:1c7 elongation (factor 7: "16:1c7 elongation"), regulation of branched (factor 8: "BCFA") and odd (factor 9: "OCFA") chain FA, n-6 metabolism (factor 10: "n-6 FA"), and synthesis of short-chain FA (factor 11: "SCFA"). According to a previous study on MFA, a variable was considered to be associated with a specific factor if the absolute value of its correlation with the factor was ≥0.60. Several factors reflected established metabolic processes, such as "OBCFA," "Mammary activity," and "BH_1." Others, such as "Beta-oxidation" and "16:1c7 elongation," captured novel pathways not previously described in single-species analyses. Importantly, this multispecies MFA approach revealed both conserved and species-specific metabolic signatures. For example, whereas core factors such as "SCFA" and "OCFA" appeared across all species and aligned with prior bovine-focused studies, certain factors (e.g., "n-6 FA" and "BCFA") showed species-dependent variation, likely reflecting differences in animal diet and ruminal microbial ecosystems. The extracted factor scores were useful to evaluate the effects of species (cow, goat, sheep, and buffalo) and different feeding regimens (applying the scores only to Holstein-Friesian cows for lipid supplementation and Sarda sheep for n-3 source supplementation). This approach allowed us to assess the influence of these dietary interventions on the latent factors affecting milk fatty acid composition. The resulting factors offer biologically meaningful, synthetic variables that can be used for future phenotypic, genetic, or nutritional modeling. Moreover, this approach enhances our understanding of mammary lipid metabolism across species and underscores the value of integrative multivariate methods in dairy science. These findings have implications for animal selection, feeding practices, and management strategies aimed at improving the nutritional quality of milk fat.

Key words: multivariate factorial analysis, mammary metabolism, biohydrogenation, dietary modulation, feeding

Received September 30, 2025.
Accepted November 25, 2025.
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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

INTRODUCTION

In recent years, there has been increasing interest from both the scientific community and consumers in the nutritional and health-related properties of milk fat. The fatty acid (**FA**) composition of ruminant milk is influenced by both intrinsic factors, such as species, breed, and genotype (Cesarani et al., 2019; Gebreyesus et al., 2019; Correddu et al., 2021), parity and lactation stage (De La Fuente et al., 2009), and extrinsic factors, including feeding and management (Conte et al., 2017) and environmental conditions (Sevi et al., 2002). The majority of milk FA (~60%) are synthesized de novo in the mammary gland from acetate and butyrate, whereas the remainder originates from dietary FA or fat mobilization (Palmquist et al., 1993).

Several studies have evaluated the effects of intrinsic factors on the milk FA profile of ruminants (Kay et al., 2005; Mele et al., 2007, 2009; Soyeurt et al., 2007; Stoop et al., 2008). However, extrinsic factors, particularly the feeding regimen, explain the largest proportion of variance in milk FA composition. Diet significantly influences the milk FA profile by supplying higher levels of FA, modulating the availability of acetate and butyrate for mammary de novo synthesis, and affecting rumen biohydrogenation (**BH**) processes as well as the availability of BH intermediates and products transported to the mammary gland via bloodstream (Buccioni et al., 2012; Shingfield et al., 2013; Mele et al., 2016). Furthermore, certain BH intermediates can affect the FA profile within the mammary gland (Buccioni et al., 2012; Shingfield et al., 2013). In recent decades, various strategies have been developed to enhance the health benefits of milk lipids by modifying animal feeding practices, including lipid supplementation, different forage inclusion, and the use of rumen-protected fats (Dewhurst et al., 2006; Toral et al., 2010; Nudda et al., 2014). These strategies can indeed influence the variability of milk lipid composition. Specifically, by altering the diet, the FA profile in milk can vary depending on the type and amount of lipid supplementation or forage provided.

Understanding the metabolic pathways of FA and the factors influencing their modulation is crucial for defining and improving the nutritional properties of milk. In particular, the complex correlations among individual milk FA complicate the interpretation of lipid metabolic pathways in the mammary gland (Cecchinato et al., 2019). Studies conducted on commercial farms often reveal considerable variation in feeding practices and management strategies, including forage types (silage, hay, or fresh biomass); lipid supplementation (Toral et al., 2020); ration types (TMR or component feeding); methods of concentrate delivery (autofeeders or TMR); and housing systems (freestalls or tiestalls).

Under such conditions, evaluating simultaneous variation in groups of FA, rather than individual FA, may provide more informative insights into the animal's metabolic pathway and nutrients (i.e., FA). To address this, multivariate statistical methods that reduce dimensionality are effective in elucidating the metabolic pathway that shapes the milk fat profile (Conte et al., 2022). Multivariate statistics offer a range of techniques to detect the covariance structure within complex variable patterns. One such technique, multivariate factor analysis (**MFA**), can reduce the number of variables while explaining the maximum amount of (co)variance among the original variables (Morrison, 1976).

The MFA is particularly useful for examining and understanding complex multivariate systems by extracting a small number of latent factors with clear technical and biological meaning, as demonstrated in previous studies on milk FA composition (Conte et al., 2016, 2022; Mele et al., 2016; Correddu et al., 2017, 2021; Palombo et al., 2020, 2021). Furthermore, factor scores can be used as new phenotypes for further analyses, such as feeding regimen effect on milk FA (Conte et al., 2016; Mele et al., 2016) or SCC effect on lipid metabolism (Turini et al., 2020). All previous studies have primarily focused on the lipid profiles of single species: bovine (Conte et al., 2016; Mele et al., 2016; Palombo et al., 2020, 2021), ovine (Correddu et al., 2021; Conte et al., 2022), and Italian Mediterranean buffalo (Correddu et al., 2017). However, this approach has overlooked the interspecific differences, leading to an incomplete understanding of ruminant mammary metabolism. In the present study, we simultaneously conducted an MFA on the detailed FA profile of a large number of milk samples from 4 ruminant species (cow, sheep, goat, and buffalo), obtained by GC analysis.

The overarching hypothesis of this work is that the mammary lipid metabolism can be interpreted through a common set of metabolic factors, which can be encompassed by the milk FA profile across ruminant species. Despite physiological and productive differences among species, it is expected that shared metabolic patterns underlie lipid synthesis and secretion in the mammary gland. Using MFA, we aimed to define a set of factors capable of explaining the main sources of variation in milk FA composition. These factors were then proposed as new variables to assess the relationship between milk FA profiles and diet composition across ruminant species. By adopting this approach, the study intends to provide a unifying framework for interpreting mammary metabolism that encompasses different ruminant species—an aspect that, to our knowledge, has not yet been addressed in the literature.

MATERIALS AND METHODS

Study Design, Animals, and Diet Composition

The study was conducted on 1,960 animals (one sample per animal) reared in several Italian commercial dairy farms from 2004 to 2020, distributed as follows: 770 cows (27 ± 5 cows per farm: 550 Holstein-Friesian [**HF**] and 220 Brown Swiss [**BS**]); 751 sheep (38 ± 5 sheep per farm: 307 Massese breed [**Ma**] and 444 Sarda breed [**S**]); 230 Italian Mediterranean buffalo (54 ± 6 buffalo per farm); and 209 goats (25 ± 4 animals per farm, Saanen breed). All animals were pluriparous and in mid-lactation (150 ± 16 DIM for cows; 90 ± 5 DIM for sheep; 162 ± 14 DIM for buffaloes; and 86 ± 3 DIM for goats). The data used in this study refer exclusively to the Italian context, as all samples were collected from farms located in Italy. This focus was chosen because national data were readily available and internally consistent. Moreover, all analyses were performed in the same laboratory by the same operators, thereby minimizing potential sources of variability that could have complicated the interpretation of the results. The milk yields (L/d) were 28.0 ± 2.3 for cows; 1.5 ± 0.1 for sheep; 8.0 ± 0.9 for buffaloes; and 1.5 ± 0.1 for goats. Details regarding the composition of the diets are provided in Supplemental Tables S1–S3, see Notes. Furthermore, HF cows were divided into 4 groups, each receiving a different diet: (1) diet containing maize silage (**M**; 138 cows), (2) diet containing triticale silage (**T**; 138 cows), (3) M with sunflower oil supplementation (**MSu**; 137 cows), and (4) M with soybean oil supplementation (**MSo**; 137 cows). Sarda sheep were divided into 3 dietary groups (148 sheep per group): no pasture (**NP**), pasture (**P**), and no pasture with linseed supplementation (**L**).

Fatty Acid Composition

Milk FA were extracted and derivatized following the procedure described by Conte et al. (2016) over a 10-yr period. The FA profile was determined by GC with a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD) equipped with a flame ionization detector and a highly polar fused-silica capillary column (Chrompack CP-Sil88 Varian, Middelburg, the Netherlands; 100 m, 0.25 mm i.d.; film thickness 0.20 mm). Hydrogen was used as the carrier gas at a flow of 1 mL/min. Split/splitless injector was used with a split ratio of 1:80. Each sample was injected under the following GC conditions: the oven temperature was initially set to 40°C and maintained for 1 min, then increased to 173°C at a rate of 2°C/min and held at this temperature for 30 min. The temperature was then increased to 185°C at 1°C/min and held for 5 min, and finally increased to 220°C at a rate of 3°C/min, with a final hold for 19 min. The injector temperature was set at 270°C, and the detector temperature was set at 300°C. Individual FA methyl esters (**FAME**) were identified by comparison with a standard mixture of 52 Component FAME Mix (Nu-Chek Prep Inc., Elysian, MN). The identification of isomers of 18:1 was based on commercial standard mixtures (Supelco, Bellefonte, PA) and published isomeric profiles (Kramer et al., 2008). Nonanoic and nonadecanoic methyl esters were used as internal standards for short- and medium-long-chain FA, respectively. Milk FA composition was expressed as grams per 100 g of total FA.

Statistical Analysis: Multivariate Factor Analysis

The aim of MFA is to characterize the (co)variance structure of a system described by n traits (y1, ..., yn), measured across observational units, by deriving a smaller set of p latent (p <n), unobservable variables (X1, ..., Xp), known as common latent factors. This multivariate approach assumes that the variance of each original variable can be divided into 2 components: one shared among all variables (referred to as communality) and one specific for each individual variable (referred to as uniqueness) as follows:

$$\mathbf{S} = \mathbf{BB}' + \mathbf{\Psi},$$

where $\mathbf{BB}'$ and $\mathbf{\Psi}$ are the communality and the uniqueness (co)variance matrices, respectively (Morrison, 1976). According to the (co)variance model, the measured traits can be represented as a combination of p unobservable common factors (X) plus a unique variable (e):

$$y_1 = b_{11}X_1 + \ldots + b_{1p}X_p + e_1,$$

$$y_n = b_{n1}X_1 + \ldots + b_{np}X_p + e_n,$$

where X_j is the jth common factor, and b_{ij} are factor coefficients (or loadings, i.e., correlations between the jth common factor and the ith trait; Morrison, 1976). Loadings are the elements of the **B** matrix used in the factor model. Common factors generate covariances between original variables, whereas the remaining specifically contribute only to the individual variation. The MFA was carried out on the correlation matrix of 49 FA measured in the 1,960 animals using JMP Pro 18 software of SAS (SAS Institute Inc., Cary, NC).

To evaluate the adequacy of datasets used for the MFA, the Kaiser measure of sampling adequacy (**MSA**) was calculated. This coefficient represents the difference between Pearson and partial correlations (Kaiser and Rice, 1974). The number of factors to be considered was

based on their eigenvalues (>1), their interpretability in terms of relationships with the original variables, and the amount of explained variance (Macciotta et al., 2015). Factor readability was improved through a VARIMAX rotation. A variable was associated with a specific factor if the absolute value of its loading was ≥0.60 (Macciotta et al., 2015). Factor scores were calculated for each animal according to the following formula:

$$\mathbf{x}' = \mathbf{y}' \times (\mathbf{B}\mathbf{B}' + \mathbf{\Psi})^{-1} \times \mathbf{B},$$

where $\mathbf{x}'$ is the row vector of factor scores, and $\mathbf{y}'$ is the row vector of standardized [(value − mean)/SD] traits. Standardized values, instead of raw values, were used because analyzed traits had different units of measurement and scales.

ANOVA

Individual factor scores were used as new phenotypes and analyzed with the 2 linear models. The first model was used to evaluate the effect of species, and it was applied to the whole dataset:

$$y_{ij} = \mu + species_i + \varepsilon_{ij}, \quad [1]$$

where y_{ij} is the factor score (11 extracted factors); μ is the overall mean; $species_i$ is the fixed effect of the ith species (i = cow, sheep, goat, buffalo); and ε_{ij} is the random residual term.

To estimate the effect of diet, we applied the following linear model to the S sheep and HF cows' subsets:

$$y_{ij} = \mu + diet_i + \varepsilon_{ij}, \quad [2]$$

where y_{ij} is the factor score (11 extracted factors); μ is the overall mean; $diet_i$ is the fixed effect of the ith diet: 1) i = M, T, MSo, MSu for HF cows, 2) = NP, P, L for S sheep; and ε_{ij} is the random residual term.

To test the linearity of the 2 models, normality of the residuals was tested using the Shapiro–Wilk test, and homoscedasticity was assessed using Bartlett's test. All statistical tests were 2-tailed and evaluated at a significance level of $\alpha = 0.05$.

All the analyses were carried out with JMP software of SAS version (SAS Institute Inc., Cary, NC). Differences were declared significant with *P*-value <0.05.

RESULTS AND DISCUSSION

The results presented here reflect the complex metabolic adaptations that support milk synthesis in different species. The lactating mammary gland's ability to use FA from multiple sources—including dietary lipids, mobilized triacylglycerols from adipose tissue, and de novo synthesis—varies depending on species, nutritional status, and physiological stage, such as early lactation (Barber et al., 1997; Conte et al., 2017). Understanding these dynamics is essential to interpret the variations observed in milk lipid composition across our dataset.

Descriptive statistics of the milk FA profile are reported in Table 1. The SFA represented the most abundant class, followed by MUFA and PUFA. Medium-chain FA (**MCFA**) accounted for over 47% of the total, followed by long-chain FA (**LCFA**) and short-chain FA (**SCFA**). As shown in Table 1, all species exhibited high levels of SCFA and MCFA, with lower concentrations of very-long-chain PUFA (i.e., >20 carbon atoms). Across all species, the most prevalent FA (g/100 g of FA) were 16:0 (~26.5 ± 3.2), 18:1 *c*9 (~20.1 ± 4.6), 18:0 (~8.8 ± 2.3) and 18:2n-6 (~2.5 ± 1.0). The PUFA, such as 22:6n-3, 20:5n-3 and 20:4n-6, often associated with beneficial effects on human health, were present in low amounts (<0.1%). The concentrations of total SFA, as well as those of the most representative individual compounds (14:0, 16:0, and 18:0), were consistent with findings from previous studies on all 4 species: bovine and caprine (Ceballos et al., 2009; Heck et al., 2009; Devle et al., 2012), ovine (Conte et al., 2022), and buffalo (Correddu et al., 2017; Pegolo et al., 2017).

Multivariate Factorial Analysis

Several studies have demonstrated the challenges in managing and interpreting relationships between multiple variables used to define nutritional and technological quality of milk (Ikonen et al., 2004; Vallas et al., 2010; Macciotta et al., 2012). This represents a limitation for the large-scale implementation of selection and management strategies aimed at improving the quality of milk.

In addition, Bolormaa et al. (2010) reported that, in the case of correlated traits, sampling errors tend to be also correlated, further complicating the interpretation of the results. Therefore, reducing the complexity of the system through factor analysis can be of significant practical interest. A few uncorrelated variables, or those with a low degree of correlation, can serve as reliable indicators of different aspects of milk quality. Pearson and partial correlations between the variables considered are shown in Figure 1. The partial correlation values were significantly lower than the Pearson correlations, suggesting that the relationship between 2 variables was mediated by other variables in the dataset. This difference indicates the presence of a latent correlation structure. A measure of this reduction is the Kaiser MSA, which was ~0.87, higher than the value (0.80) typically considered the optimal threshold for

Table 1. Descriptive statistics of fatty acid profile of the 4 species (g/100 g of fatty acids)

	Cow			Sheep			Goat			Buffalo		
Fatty acid[1]	Mean	Median	CV	Mean	Median	CV	Mean	Median	CV	Mean	Median	CV
4:0	2.69	2.67	37.04	2.64	2.74	45.26	3.12	3.10	12.07	5.48	5.68	20.51
6:0	1.62	1.68	34.38	1.64	1.74	37.66	3.03	3.07	10.02	1.33	1.29	27.13
8:0	0.92	0.83	36.89	1.47	1.58	41.01	3.35	3.42	11.13	1.84	1.69	37.79
10:0	2.16	2.10	33.61	4.47	4.69	42.70	12.47	12.91	14.73	1.62	1.63	26.59
10:1 *c*9	0.25	0.22	41.06	0.18	0.17	24.80	0.15	0.14	21.57	0.14	0.15	15.34
11:0	0.05	0.04	63.29	0.06	0.08	38.79	0.08	0.08	36.15	0.03	0.02	60.33
12:0	2.61	2.64	29.54	2.80	2.87	36.52	8.14	8.31	18.58	2.19	2.19	25.09
13:0 *iso*	0.04	0.04	55.47	0.04	0.04	68.15	0.02	0.02	26.69	0.01	0.01	11.23
13:0 *anteiso*	0.01	0.01	21.88	0.01	0.01	15.05	0.01	0.01	12.65	0.01	0.01	10.45
13:0	0.10	0.10	43.20	0.07	0.06	40.44	0.08	0.08	28.33	0.09	0.09	35.44
14:0	11.14	11.29	13.39	9.34	9.45	22.62	9.27	9.50	14.22	9.97	10.08	17.79
14:0 *iso*	0.09	0.07	51.21	0.12	0.11	48.49	0.10	0.09	34.68	0.18	0.19	21.57
14:1 *c*9	1.21	1.21	37.91	0.19	0.17	44.36	0.18	0.17	37.73	0.88	0.89	24.56
15:0	1.37	1.36	25.14	1.14	1.14	26.41	1.00	1.00	17.61	1.18	1.18	19.67
15:0 *iso*	0.28	0.29	18.14	0.32	0.28	47.37	0.21	0.21	22.67	0.41	0.42	8.43
15:0 *anteiso*	0.55	0.53	22.92	0.55	0.51	35.03	0.36	0.37	18.83	0.65	0.66	21.90
16:0	30.04	30.23	14.89	23.73	24.25	13.93	23.42	23.57	11.98	29.40	29.81	15.30
16:0 *iso*	0.24	0.20	44.89	0.30	0.28	34.96	0.24	0.23	24.78	0.38	0.38	19.70
16:1 *t*6–7	0.04	0.03	52.84	0.07	0.04	69.77	0.30	0.31	34.67	1.87	1.91	22.29
16:1 *t*9	0.06	0.06	33.34	0.20	0.12	62.04	0.05	0.05	38.52	1.53	1.56	29.61
16:1 *c*7	0.43	0.45	54.30	0.41	0.39	36.44	1.00	1.01	32.29	0.41	0.42	33.45
16:1 *c*9	1.63	1.53	37.85	1.00	1.01	27.87	0.66	0.64	23.74	2.06	2.10	36.01
17:0 *anteiso*	0.58	0.57	21.45	0.49	0.47	32.21	0.43	0.44	15.33	0.42	0.43	18.48
17:0	0.66	0.67	20.15	0.73	0.72	28.01	0.66	0.65	18.93	0.54	0.55	21.13
17:0 *iso*	0.35	0.36	9.64	0.43	0.39	47.50	0.38	0.40	10.45	0.31	0.31	37.01
17:1 *c*9	0.31	0.29	38.01	0.31	0.33	35.99	0.32	0.32	32.18	0.24	0.24	23.09
18:0	7.62	7.60	27.58	10.27	10.58	20.63	6.40	6.30	20.06	10.99	10.98	21.35
18:1 *t*6–8	0.40	0.35	42.80	0.38	0.28	61.42	0.13	0.12	36.21	0.12	0.09	68.60
18:1 *t*9	0.32	0.29	40.41	0.37	0.29	59.34	0.16	0.15	31.21	0.25	0.24	45.00
18:1 *t*10	1.51	1.50	61.97	0.88	0.52	64.04	0.19	0.17	45.75	0.26	0.21	71.57
18:1 *t*11	1.26	1.19	34.12	2.86	2.36	65.42	0.58	0.54	47.55	1.59	1.27	59.01
18:1 *t*12	0.64	0.60	64.04	0.63	0.51	56.23	0.47	0.48	65.46	0.21	0.17	84.91
18:1 *c*9	22.00	22.36	17.82	22.94	23.37	21.13	17.69	16.88	24.74	18.56	18.83	17.05
18:1 *c*11	0.99	1.01	37.82	0.71	0.71	36.57	0.53	0.51	27.18	0.37	0.35	20.72
18:1 *c*12	0.42	0.40	49.71	0.53	0.28	69.71	0.22	0.22	23.18	0.24	0.20	65.44
18:1 *c*13	0.10	0.09	64.83	0.08	0.08	73.58	0.20	0.20	45.82	0.05	0.04	65.36
18:1 *t*16	0.39	0.39	36.23	0.52	0.53	40.49	0.19	0.19	17.92	0.41	0.42	34.68
18:2 *t*11,*c*15	0.29	0.21	66.93	0.45	0.29	66.03	0.05	0.04	35.00	0.34	0.34	68.33
18:2n-6	2.61	2.58	25.70	2.90	2.82	35.85	2.18	2.15	20.50	2.06	1.68	54.96
20:0	0.13	0.13	33.38	0.29	0.29	32.86	0.20	0.20	17.55	0.20	0.20	26.55
18:3n-3	0.72	0.71	33.54	1.19	1.22	29.58	0.77	0.77	23.82	0.25	0.24	31.26
18:2 *c*9,*t*11	0.76	0.72	35.46	1.62	1.59	53.70	0.45	0.43	43.43	0.63	0.56	47.98
22:0	0.07	0.07	20.62	0.16	0.16	33.17	0.07	0.07	21.62	0.07	0.07	18.21
20:4n-6	0.15	0.14	37.77	0.17	0.16	44.07	0.19	0.19	25.11	0.08	0.08	21.08
20:5n-3	0.05	0.05	36.25	0.08	0.08	32.83	0.05	0.05	27.69	0.02	0.02	38.94
24:0	0.03	0.03	49.50	0.04	0.03	69.88	0.05	0.04	61.77	0.05	0.05	36.17
22:5n-3	0.10	0.10	34.86	0.15	0.14	37.18	0.14	0.14	27.95	0.05	0.05	25.93
22:6n-3	0.01	0.01	59.42	0.07	0.05	63.91	0.03	0.03	40.79	0.02	0.02	67.14
SFA	63.35	63.51	11.27	61.12	62.50	11.91	73.09	74.08	7.80	67.35	67.93	9.45
MUFA	31.96	31.97	18.14	32.26	31.15	21.80	23.03	22.11	21.06	29.20	29.07	14.79
PUFA	4.69	4.52	25.02	6.63	6.35	29.51	3.87	3.81	18.47	3.45	3.00	42.00
SCFA	4.31	4.35	35.18	4.28	4.48	39.42	6.14	6.18	9.65	6.81	6.97	16.36
MCFA	55.12	55.12	11.75	48.43	49.19	15.98	62.89	63.92	9.74	56.35	56.92	13.32
LCFA	40.57	40.53	14.85	47.28	46.33	20.74	30.97	29.90	19.45	36.84	36.12	20.53

[1]*c* = *cis*; *t* = *trans*; SCFA = short-chain fatty acids (chain shorter than 7 carbon atoms); MCFA = medium-chain fatty acids (chain of length between 7 and 17 carbon atoms); LCFA = long-chain fatty acids (chain longer than 17 carbon atoms).

the suitability of the dataset for multivariate analysis (Cerny and Kaiser, 1977).

The MFA was able to identify 11 common latent factors (factors with eigenvalue >1 as reported in Tables 2 and 3) from the correlation matrix of the milk data. Varimax rotated factor loadings, representing the correlations between latent factors and original attribute measurements, are shown in Tables 2 and 3. Variables with loading values greater than 0.60 were considered as the most representative of the variance explained by each factor.

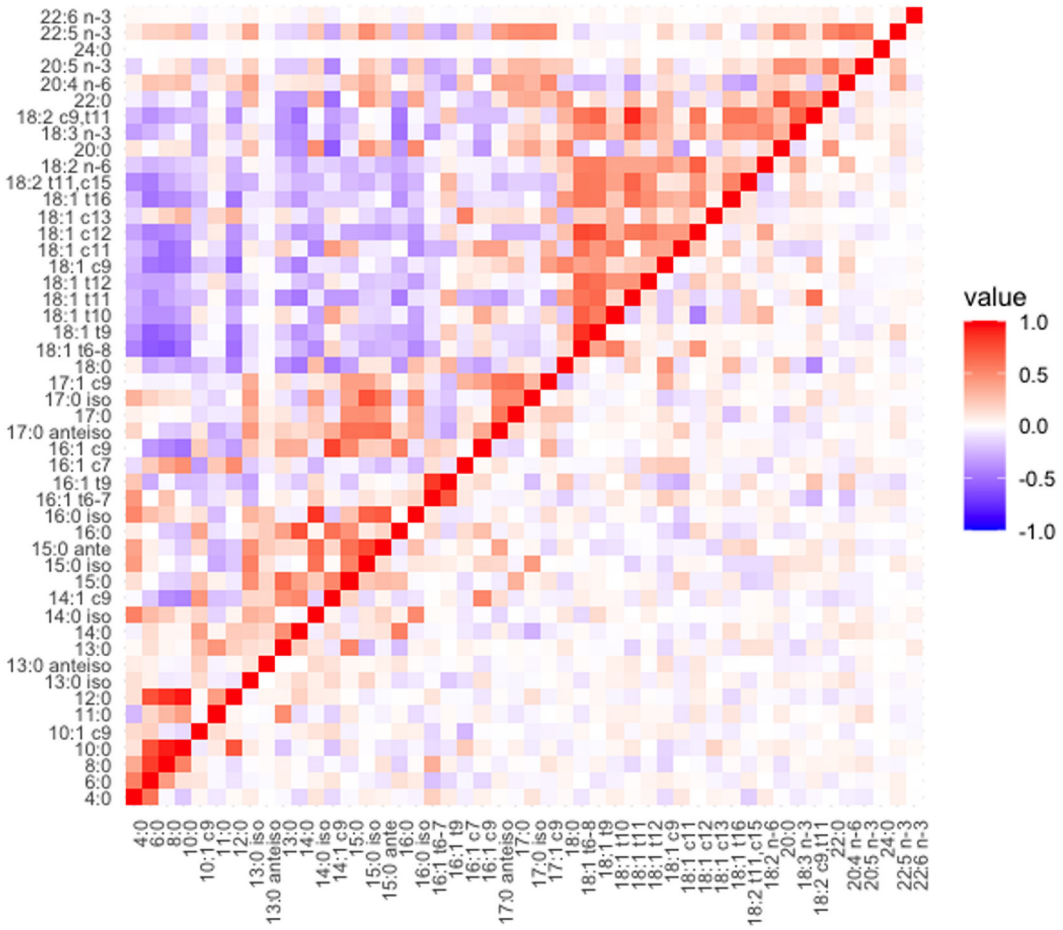

Figure 1. Pearson (above the diagonal) and partial (under the diagonal) correlation among selected 49 FA of milk samples.

The distribution of explained variance among the factors was fairly balanced, with a small, expected predominance of factor 1 (eigenvalue = 12.44), whereas the eigenvalues of the other 10 factors ranged between 1.23 and 12.08 (Tables 2 and 3). This is a distinctive feature of MFA compared with other multivariate dimensionality reduction techniques, such as principal component analysis, where the first component typically explains a much larger proportion of variance than the subsequent components (Jombart et al., 2009).

The first latent factor was named "OBCFA" because it was positively correlated with the odd- and branched-chain FA (**OBCFA**): 13:0 *iso*, 15:0, 15:0 *iso*, 15:0 *anteiso*, 16:0 *iso*, 17:0, 17:0 *anteiso*, 17:0 *iso*, and 17:1 *c*9. A similar factor was identified by Mele et al. (2016). Isobranched-chain FA (i.e., 13:0 *iso*, 14:0 *iso*, 15:0 *iso*, Toral et al., 2020) are mainly produced in the rumen by cellulolytic bacteria; therefore, their content in milk is positively correlated with the amount of forage in the diet (Vlaeminck et al., 2006). On the contrary, linear odd-chain FA are associated with amylolytic bacteria and therefore correlated with higher levels of concentrates in the diet (Vlaeminck et al., 2006; Toral et al., 2020). Given the increasing emphasis on enteric methane emissions from ruminants and their environmental effect, understanding rumen microbial populations and their metabolic products is crucial for developing mitigation strategies (Hristov et al., 2013; Knapp et al., 2014). Because the production of branched-chain FA (**BCFA**) is closely linked to cellulolytic bacterial activity in the rumen, dietary interventions that modulate fiber content may also influence methane emissions (Beauchemin et al., 2020). Strategies such as increasing forage quality or supplementing with additives targeting rumen microbiota have shown promise in reducing methane production while maintaining animal productivity (Patra, 2012). Thus, the biological role of the first factor observed in this study could have important implications for methane mitigation efforts.

The second latent factor was positively associated with MCFA (14:0, 16:0, 10:1 *c*9, 14:1 *c*9, 16:1 *c*9) and negatively associated with 18:0, 20:0, and 22:0. All FA

Table 2. Rotated factor (F) pattern and proposed factor name, for F1 through F6[1]

Item[2]	F1, OBCFA	F2, Mammary activity	F3, BH_1	F4, Milk fluidity	F5, Beta-oxidation	F6, BH_2
15:0 *iso*	**0.87**	−0.06	−0.15	−0.04	0.18	0.04
17:0 *anteiso*	**0.78**	0.20	0.05	−0.17	−0.16	−0.18
15:0 *anteiso*	**0.78**	0.06	−0.18	−0.10	0.10	0.05
17:0 *iso*	**0.75**	−0.16	−0.08	0.14	−0.08	0.18
15:0	**0.70**	0.46	0.01	−0.06	−0.03	−0.09
17:0	**0.66**	−0.19	−0.07	−0.12	−0.13	−0.11
16:0 *iso*	**0.65**	−0.26	−0.31	0.06	0.18	−0.01
13:0 *iso*	**0.61**	0.10	−0.03	−0.01	−0.20	−0.01
17:1 *c*9	**0.60**	0.03	0.03	−0.16	−0.05	−0.14
14:1 *c*9	0.10	**0.88**	0.17	−0.24	0.01	−0.16
16:1 *c*9	0.25	**0.71**	0.15	−0.36	0.25	−0.10
16:0	0.05	**0.66**	−0.21	−0.14	0.05	−0.26
14:0	0.021	**0.61**	−0.20	0.14	−0.09	−0.27
10:1 *c*9	−0.02	**0.60**	−0.02	0.03	−0.23	−0.06
22:0	0.40	**-0.61**	0.03	−0.10	−0.01	0.17
20:0	0.34	**-0.70**	−0.10	−0.02	0.06	0.04
18:0	−0.04	**-0.70**	0.05	−0.42	0.19	−0.02
18:1 *t*6–8	−0.16	0.01	**0.87**	−0.31	−0.11	0.26
18:1 *t*9	−0.16	−0.11	**0.76**	−0.29	0.06	0.34
18:1 *t*10	0.06	0.24	**0.76**	−0.08	0.00	−0.04
18:1 *c*12	−0.31	−0.18	**0.69**	−0.24	0.01	0.27
18:1 *t*12	−0.07	−0.02	**0.61**	−0.10	−0.07	0.27
18:1 *c*11	0.15	0.27	**0.61**	−0.34	−0.22	−0.11
18:2n-6	−0.12	−0.27	**0.61**	−0.11	0.01	0.14
18:1 *t*16	−0.03	−0.31	**0.61**	−0.31	0.06	0.31
10:0	−0.06	−0.20	−0.27	**0.88**	−0.08	−0.04
8:0	−0.02	−0.16	−0.36	**0.85**	0.14	−0.07
12:0	−0.14	0.04	−0.28	**0.85**	−0.07	−0.12
6:0	0.06	0.01	−0.37	**0.73**	−0.14	−0.09
18:1 *c*9	0.00	−0.37	0.23	**-0.68**	−0.08	−0.01
16:1 *t*7	−0.07	0.05	−0.11	0.06	**0.94**	−0.11
16:1 *t*9	−0.18	−0.05	0.01	−0.15	**0.91**	0.20
18:2 *t*11,*c*15	−0.19	−0.12	0.44	−0.13	0.12	**0.63**
18:2 *c*9,*t*11	−0.02	−0.35	0.37	−0.17	0.02	**0.81**
18:1 *t*11	−0.19	−0.37	0.38	−0.17	0.08	**0.71**
18:3n-3	0.15	−0.43	0.40	0.01	−0.20	**0.66**
24:0	0.01	−0.01	0.02	0.00	0.00	0.05
16:1 *c*7	0.00	0.00	0.03	0.30	0.05	−0.15
18:1 *c*13	0.05	0.08	0.32	0.27	0.00	0.00
14:0 *iso*	0.45	−0.24	−0.28	0.09	0.22	−0.01
13:0	0.14	0.47	0.02	0.06	0.03	−0.21
11:0	−0.19	−0.11	0.04	0.31	−0.09	0.05
20:4n-6	0.42	−0.08	−0.08	0.16	−0.18	−0.15
4:0	0.28	0.02	−0.25	0.22	0.37	−0.07
20:5n-3	0.31	−0.45	−0.03	0.02	−0.21	0.13
22:5n-3	0.40	−0.32	0.01	0.17	−0.18	0.06
22:6n-3	0.04	−0.06	0.02	0.00	0.00	0.03
13:0 *anteiso*	0.09	0.11	0.01	0.00	0.00	0.02
Eigenvalue	6.09	5.92	5.37	4.81	2.57	2.53
% of variance explained	12.44	12.08	10.96	9.81	5.25	5.17
% of cumulative variance	12.44	24.52	35.48	45.29	50.53	55.70

[1]Values above 0.6 are in bold.

[2]*c* = *cis*; *t* = *trans*.

positively correlated with the second factor share a common metabolic origin, being synthesized de novo in the mammary gland from acetate by the enzyme fatty acid synthase (**FAS**; Conte et al., 2017), with the exception of 14:0 and 16:0, which may also partly derive from the diet. Interestingly, these FA are either substrates (14:0 and 16:0) or products (14:1 *c*9 and 16:1 *c*9) of stearoyl CoA desaturase (**SCD**), which catalyzes the desaturation in Δ9 position of the carbon chain of FA in the mammary gland (Ntambi, 1999). In contrast, FA that showed negative scores are not directly linked to mammary metabolism. As is well known, animals can synthesize FA up to 16:0. The transition from 16:0 to 18:0 is rate-limited by elongase ELOVL6, which controls the elongation of 16:0 to longer chain FA (Shi et al., 2017). Evidence indicates that ELOVL6 is expressed and functionally active

Table 3. Rotated factor (F) pattern and proposed factor name, for F1 through F6[1]

Item[2]	F7, 16:1 *c*7 elongation	F8, BCFA	F9, OCFA	F10, n-6 FA	F11, SCFA	Communality
15:0 *iso*	−0.15	0.14	−0.14	0.09	0.01	0.89
17:0 *anteiso*	0.20	0.01	−0.08	−0.04	0.12	0.80
15:0 *anteiso*	−0.18	0.33	0.00	−0.18	0.07	0.84
17:0 *iso*	−0.01	0.05	−0.11	−0.07	0.09	0.68
15:0	−0.04	0.08	**0.60**	−0.06	−0.04	0.87
17:0	0.25	−0.04	0.11	0.03	0.00	0.70
16:0 *iso*	−0.20	**0.62**	−0.09	−0.03	0.14	0.84
13:0 *iso*	−0.16	0.16	0.04	0.30	−0.07	0.62
17:1 *c*9	0.46	−0.09	0.04	0.39	−0.01	0.75
14:1 *c*9	0.05	0.15	−0.01	−0.04	0.02	0.92
16:1 *c*9	0.10	0.03	−0.02	0.19	−0.10	0.84
16:0	−0.15	0.18	0.09	0.10	−0.01	0.65
14:0	−0.20	0.10	0.18	0.02	−0.20	0.64
10:1 *c*9	−0.28	0.37	0.04	0.01	0.19	0.51
22:0	−0.15	0.06	−0.02	0.28	−0.25	0.73
20:0	−0.13	0.20	−0.01	0.22	−0.15	0.74
18:0	−0.10	0.21	−0.05	−0.08	0.09	0.78
18:1 *t*6–8	0.01	−0.02	−0.03	−0.06	−0.06	0.97
18:1 *t*9	0.05	0.03	−0.03	0.04	−0.07	0.83
18:1 *t*10	0.06	−0.09	−0.01	−0.02	−0.10	0.67
18:1 *c*12	0.01	0.02	0.03	0.18	0.04	0.76
18:1 *t*12	0.16	0.00	0.08	−0.06	−0.03	0.50
18:1 *c*11	0.21	−0.26	0.04	0.04	0.19	0.80
18:2n-6	0.00	0.01	0.03	**0.68**	0.07	0.69
18:1 *t*16	−0.05	−0.11	0.00	−0.23	−0.04	0.77
10:0	0.28	−0.02	0.10	0.06	−0.04	0.99
8:0	0.17	−0.02	0.07	0.07	0.15	0.96
12:0	0.31	0.11	0.15	0.00	−0.03	0.97
6:0	0.05	0.12	0.03	0.07	**0.67**	0.94
18:1 *c*9	0.38	0.06	−0.08	0.30	0.09	0.91
16:1 *t*7	0.08	0.03	−0.02	−0.04	0.11	0.94
16:1 *t*9	−0.07	0.04	−0.03	−0.08	−0.01	0.94
18:2 *t*11,*c*15	−0.01	−0.07	0.03	−0.27	−0.26	0.61
18:2 *c*9,*t*11	−0.06	0.05	−0.01	0.01	−0.08	0.95
18:1 *t*11	−0.12	0.03	−0.03	−0.14	−0.09	0.89
18:3n-3	−0.01	−0.10	−0.06	0.13	−0.22	0.70
24:0	−0.01	0.00	−0.01	0.00	0.01	0.00
16:1 *c*7	**0.74**	−0.15	0.07	0.04	−0.04	0.69
18:1 *c*13	**0.60**	−0.05	0.03	0.02	−0.05	0.55
14:0 *iso*	−0.23	**0.62**	−0.03	0.04	0.14	0.85
13:0	0.04	0.08	**0.77**	−0.08	0.07	0.91
11:0	0.10	−0.04	**0.69**	0.10	−0.14	0.68
20:4n-6	0.19	−0.09	0.07	**0.68**	0.02	0.56
4:0	−0.20	0.16	−0.14	0.02	**0.64**	0.83
20:5n-3	0.00	−0.04	0.07	0.20	−0.18	0.45
22:5n-3	0.16	−0.08	−0.02	0.34	−0.03	0.65
22:6n-3	0.00	0.02	0.04	0.03	0.03	0.01
13:0 *anteiso*	−0.01	0.40	0.03	−0.01	−0.01	0.18
Eigenvalue	2.31	1.57	1.46	1.43	1.23	
% of variance explained	4.72	3.19	2.98	2.93	2.51	
% of cumulative variance	60.42	63.61	66.59	69.52	72.02	

[1]Values above 0.6 are in bold.

[2]*c* = *cis*; *t* = *trans*.

in the mammary gland of ruminants, contributing to FA elongation in vivo (Shi et al., 2017). Therefore, mammary epithelial cells use 18:0 present in the bloodstream, which originates from the diet, adipose tissue, or ruminal biohydrogenation activity (Conte et al., 2017). Although the quantitative contribution of ELOVL6 to FA elongation in bovine mammary tissue may vary, current data support its biological relevance in this tissue, consistent with its established role in nonruminant species. Fatty acids 20:0 and 22:0 are derived from the elongation of 18:0 and are thus strictly linked to this FA, which justifies their negative score. For this reason, the factor has been called "Mammary activity;" a similar factor having already been identified in bovine milk (Conte et al., 2016; Mele et al., 2016; Palombo et al., 2021). However, it is important to note that, according to current literature, the

de novo elongation of long-chain SFA such as 20:0 and 22:0 within the bovine mammary gland is likely limited (Palmquist and Jenkins, 1980; Bauman and Griinari, 2001). These FA may primarily originate from elongation in other tissues, such as the liver, or be derived directly from the circulation.

The third latent factor was named "BH_1" because it was positively correlated with linoleic acid and some products of its ruminal biohydrogenation (18:1 *c*12, 18:1 *t*6–8, 18:1 *t*9, 18:1 *t*10, 18:1 *t*12, 18:1 *c*11, and 18:1 *t*16). However, vaccenic acid (18:1 *t*11), the major product of linoleic acid biohydrogenation, did not score highly on this factor and was instead included in factor 6. Linoleic acid is often the predominant FA in dietary lipids and is actively biohydrogenated by rumen bacteria to 18:0 with an estimated extent of 75% and 90% (Doreau and Ferlay, 1994; Lock and Bauman, 2004; Jenkins et al., 2008). During this process, a wide range of *cis* (***c***) and *trans* (***t***) isomers of 18:1 is produced in the rumen, including 18:1 *t*6 to 8, 18:1 *t*9, 18:1 *t*10, 18:1 *t*16, and 18:1 *c*12 (Shingfield et al., 2010). Therefore, the content of these FA in milk fat would be closely related to the amount of 18:2n-6 in the diet and the extent of rumen biohydrogenation. The same factor was obtained when FA composition of only bovine milk was considered (Conte et al., 2016; Mele et al., 2016; Palombo et al., 2021).

The fourth latent factor was associated with the activity of the mammary gland in regulating milk fluidity, as it was positively correlated with 6:0 and MCFA and negatively correlated with C18:1 *c*9. This pattern is partly explained by the common origin of 6:0 and MCFA, which are synthesized de novo in the mammary gland from circulating acetate by the enzymes acetyl-CoA carboxylase (**ACC**) and FAS (Chilliard et al., 2001). The observed negative correlation with 18:1 *c*9, which is extensively synthesized in the mammary gland from 18:0 by SCD (Enjalbert et al., 1998), can be explained by the mammary gland's ability to regulate the melting point of milk fat. By converting saturated stearic acid (18:0) into monounsaturated oleic acid (18:1 *c*9), the mammary gland maintains the melting point of milk fat within a physiological range, ensuring proper fluidity and function. Indeed, both SCFA synthesis and Δ9 desaturation can mutually compensate for the melting point of milk fat (Toral et al., 2013; Chilliard et al., 2014). However, feedback inhibition of Δ9 desaturase is an important regulatory mechanism that modulates oleic acid synthesis and thus milk fat fluidity. Moreover, dietary factors, such as supplementation with high-oleic acid feeds, may influence this balance, although this aspect was not directly assessed in our dataset and warrants further investigation. According to Toral et al. (2015), the selective esterification of SCFA and 18:1 *c*9 at the same position on the glycerol backbone (sn-3) is an important mechanism for controlling milk fat uniformity. This model is also supported by the results reported by Fievez et al. (2003), who, through principal component analysis (**PCA**) of milk FA composition, found an inverse correlation on the PC1 loading plots between de novo synthesized FA and the products of Δ9 desaturase, particularly 18:1 *c*9. Similar observations have been reported in previous studies on bovine milk (Conte et al., 2016; Mele et al., 2016; Palombo et al., 2021). However, it is important to consider that oleic acid uptake by the mammary gland may affect de novo FA synthesis at both the transcriptional and enzymatic levels, highlighting the complex regulatory interactions underlying milk fat composition.

The fifth factor includes 16:1 *t*7 and 16:1 *t*9, which showed highly positive scores. These FA are the β-oxidation products of 18:1 *t*9 and 18:1 *t*11, respectively (Roe et al., 2001). Hepatic studies in dairy cows indicate that the expression of β-oxidation-related genes (e.g., CPT1A, ACOX1) is upregulated during early lactation or in conditions of lipid mobilization (Schlegel et al., 2012). Moreover, reduced hepatic β-oxidation capacity has been associated with higher hepatic triglyceride content and lower milk production (Grum et al., 2002). Although direct tracing of *trans*-18:1 isomers and their conversion in the liver remains sparse, these findings support the plausibility that hepatic FA oxidation and lipid handling might influence the pool of FA available for milk fat synthesis and secretion.

Factor 6 is positively correlated with 18:3 n-3, 18:2 *t*11,*c*15, 18:1 *t*11, and C18:2 *c*9,*t*11. The 18:3 n-3 is also derived from the ruminant diet, such as 18:2 n-6. Rumenic acid is not an intermediate product of the biohydrogenation of α-linolenic acid (Shingfield et al., 2010; Toral et al., 2024). Its presence in this factor, together with 18:3 n-3, 18:1 *t*11, and 18:2 *t*11,*c*15 may be due to the relationship between rumenic acid and vaccenic acid for endo-mammary biosynthesis by SCD. Several studies have shown that over 80% of rumenic acid in milk is due to mammary desaturation of 18:1 *t*11, which results from ruminal biohydrogenation of dietary PUFA (Shingfield et al., 2013). Interestingly, the products of SCD activity were associated with 2 different latent factors: factor 2 for de novo synthesized FA (10:1 *c*9, 14:1 *c*9, and 16:1 *c*9) and factor 6 for rumenic acid. A similar result was observed in previous works on bovine milk (Conte et al., 2016; Mele et al., 2016). These results suggest that the chain length and degree of unsaturation of the substrate could influence the activity of the SCD enzyme.

Factor 7 is positively correlated with 16:1 *c*7 and 18:1 *c*13. These FA are linked by rumen biohydrogenation and elongation processes, which can lead to the synthesis of 18:1 isomeric forms (such as 18:1 *c*13) from 16:1 *c*7, as reported in yaks by Xie et al. (2022). Although this mechanism was described in yaks—ruminants adapted to high-

altitude environments—a similar metabolic relationship may also occur in other ruminant species. For this reason, the factor has been called "16:1 *c*7 elongation."

The eighth factor was named "BCFA" because it is positively correlated with the main iso BCFA, 14:0 *iso* and 16:0 *iso*. Branched-chain FA are primarily produced in the rumen by cellulolytic bacteria; therefore, their content in milk is positively correlated with the amount of forage in the diet (Vlaeminck et al., 2006). These FA are derived from branched-chain AA (valine, leucine, and isoleucine) and their corresponding short-chain branched carboxylic acids (isobutyric acid, isovaleric acid, and 2-methyl butyric acid; Fievez et al., 2003).

In contrast, factor 9 is positively associated with 11:0, 13:0, and 15:0; therefore, it was named "OCFA" (**odd-chain FA**). These FA are synthesized by amylolytic rumen bacteria and are associated with a diet rich in grains and concentrates (Vlaeminck et al., 2006). Odd-chain FA can also originate from the elongation of propionic and valeric acid in the mammary gland (Vlaeminck et al., 2006).

It should be noted that in the MFA, OCFA, and BCFA were separated into 2 different factors, despite having a common origin in the metabolism of rumen bacteria. Fievez et al. (2003) also showed that the loading diagrams of the first 2 principal components based on milk FA concentrations revealed a cluster of BCFA and OCFA of rumen microbial origin, completely separated from the other milk FA groups. However, the rumen bacterial population contains different amounts of OCFA and BCFA, with cellulolytic bacteria responsible for the presence of iso and anteiso FA, whereas amylolytic bacteria primarily produce linear OCFA (Fievez et al., 2003). It is likely that the greater number of data processed in the present study, compared with the study of Fievez et al. (2003), allowed for the separation of these 2 classes of FA. A similar result was obtained by Conte et al. (2016), who worked exclusively on bovine milk.

Factor 10 was associated with linoleic acid and its main elongation product, 20:4n-6. In milk fat, arachidonic acid is mainly found in the phospholipid fraction, which represents less than 2% of total lipids (Jensen, 2006). It can also be produced in the mammary gland by elongation of dietary linoleic acid (Bionaz and Loor, 2008). High scores for this factor may indicate an animal that is more efficient at promoting linoleic acid elongation. For this reason, the factor was named "n-6 FA."

Finally, factor 11 is mainly related to 4:0 and 6:0; therefore, it has been named "SCFA." Saturated FA with chain lengths between 4:0 and 14:0 are also endogenously synthesized in the mammary gland by the enzymes ACC and FAS (Chilliard et al., 2000). Interestingly, FA with lengths between 8:0 and 14:0 are associated with factor 4, suggesting that there may be differences in the endogenous synthesis of even-chain FA based on carbon chain length. Unlike MCFA (8:0–14:0), SCFA can be partially synthesized in the mammary gland through metabolic pathways that are not dependent on ACC (Chilliard et al., 2007). With MFA, we were able to highlight this metabolic difference by extracting 2 different latent variables, one representing SCFA metabolism and the other representing MCFA metabolism. The same factor has been detected in previous studies that evaluated the FA profile of bovine milk with a multivariate approach (Conte et al., 2016; Mele et al., 2016).

Table 4. Comparison of factors extracted with those reported in literature

Factor	Name	Species	Reference
F1	OBCFA	Cow	Palombo et al., 2020
		Sheep	Correddu et al., 2021
		Buffalo	Correddu et al., 2017
F2	Mammary activity	Cow	Palombo et al., 2020
			Palombo et al., 2021
		Sheep	Correddu et al., 2021
		Buffalo	Correddu et al., 2017
F3	BH_1	Cow	Palombo et al., 2021
		Sheep	Correddu et al., 2021
			Conte et al., 2022
		Buffalo	Correddu et al., 2021
F4	Milk fluidity	Cow	Palombo et al., 2020
			Palombo et al., 2021
		Sheep	Correddu et al., 2021
			Conte et al., 2022
		Buffalo	Correddu et al., 2017
F5	Beta-oxidation		
F6	BH_2	Cow	Palombo et al., 2020
F7	16:1 *c*7 elongation		
F8	BCFA	Cow	Palombo et al., 2020
F9	OCFA	Cow	Palombo et al., 2020
		Cow	Palombo et al., 2021
		Buffalo	Correddu et al., 2017
F10	n-6 FA	Sheep	Correddu et al., 2021
F11	SCFA	Cow	Palombo et al., 2020
		Cow	Palombo et al., 2021
		Sheep	Conte et al., 2022

The literature review has shown that, to date, the MFA on the FA profile has been conducted on milk FA profile of the same species: bovine (Mele et al., 2016; Conte et al., 2016; Palombo et al., 2020, 2021), sheep (Correddu et al., 2021; Conte et al., 2022), and buffalo (Correddu et al., 2017). To our knowledge, this is the first study to apply MFA to the milk FA profile across ruminant species, offering a comprehensive and integrative perspective on ruminant mammary metabolism as the diet changes. The following 2 sections address effects of species and diets on the extracted factors, respectively. Table 4 shows a comparison between the factors extracted in this study and those reported in the literature, to understand how consistent and therefore consolidated the metabolic pathways are. Most of the extracted factors were also found in previous studies using the same approach but conducted on a single species: "OBCFA," "Mammary ac-

Table 5. Effect of species on the 11 extracted factors

Factor	Name	Cow	Buffalo	Goat	Sheep	SEM	*P*-value
F1	OBCFA	0.00^{B}	-0.20^{C}	-0.56^{D}	0.13^{A}	0.06	<0.001
F2	Mammary activity	1.02^{A}	0.51^{B}	-0.17^{C}	-0.68^{D}	0.04	<0.001
F3	BH_1	0.17^{A}	-0.72^{D}	-0.44^{C}	0.00^{B}	0.05	<0.001
F4	Milk fluidity	-0.45^{C}	-0.44^{C}	2.12^{A}	-0.04^{B}	0.04	<0.001
F5	Beta-oxidation	-0.41^{C}	4.56^{A}	-0.03^{B}	-0.08^{B}	0.01	<0.001
F6	BH_2	-0.31^{B}	-0.46^{B}	-0.39^{B}	0.32^{A}	0.05	<0.001
F7	C16:1 *c*7 elongation	-0.01^{B}	-0.38^{C}	1.69^{A}	-0.29^{C}	0.05	<0.001
F8	BCFA	0.16^{A}	0.01^{B}	0.03^{B}	-0.13^{C}	0.05	<0.001
F9	OCFA	0.02^{A}	-0.34^{B}	-0.02^{A}	0.01^{A}	0.06	0.007
F10	n-6 FA	-0.26^{B}	-0.35^{B}	0.10^{A}	0.15^{A}	0.05	<0.001
F11	SCFA	0.26^{B}	0.41^{A}	0.18^{B}	-0.25^{C}	0.05	<0.001

$^{A–D}$Values with different superscripts within a row are significantly different between groups at $P < 0.01$.

tivity," "BH_1," "Milk fluidity," "OCFA," and "SCFA" factors (Table 4). Other factors extracted in this study were detected only in some works with specific species: the "BH_2" and "BCFAiso" factors were detected only in bovine milk (Palombo et al., 2020), and the "n-6 FA" factor was found only in sheep milk (Correddu et al., 2021). Conversely, the "Beta-oxydation" and "16:1 *c*7 elongation" factors were not found in any of the previously published works. These results demonstrate that the multispecies approach allows to detect different pathways that would otherwise not emerge when working with a single animal species.

Effect of the Species on the Extracted Factor

The results shown in Table 5 reveal highly significant differences ($P < 0.01$) among the 4 ruminant species for all 11 extracted factors, each representing distinct metabolic pathways or compositional traits of milk. The data satisfied the assumptions of residual normality and homoscedasticity, as indicated by *P*-values greater than 0.05 for the Shapiro–Wilk and Bartlett tests, respectively.

Factor 1 ("OBCFA"), predominantly representing OBCFA derived from ruminal microbial fermentation, was significantly higher in sheep (0.13), followed by cow (0.00), buffalo (−0.20), and goat (−0.56; $P < 0.001$). These results highlight interspecies differences in ruminal microbial activity, likely influenced by variations in fermentation pathways and microbial community composition. Among the species studied, sheep exhibited the most active OBCFA-producing microbial ecosystem, suggesting a greater predisposition toward the synthesis of rumen-derived FA (Buccioni et al., 2012; Ebrahimi et al., 2017). In contrast, goats showed a markedly lower association with microbial OBCFA production. In addition, the endogenous mammary synthesis of odd-chain FA appears to be more pronounced in cows, goats, and buffaloes (Alonso et al., 1999; Ran-Ressler et al., 2014; Bainbridge et al., 2016). The elevated OBCFA values observed in sheep therefore support the interpretation that this factor is primarily indicative of ruminal microbial activity.

Cows exhibited the highest scores for factor 2 (Mammary activity; 1.02), followed by buffaloes (0.51), goats (−0.17), and sheep (−0.68; $P < 0.001$). Notably, cows and buffaloes showed greater de novo mammary synthesis activity compared with small ruminants. Ruminant milk fat is typically rich in MCFA (C8-C16), which are synthesized de novo in the mammary epithelial cell. Consequently, the proportion of these FA in milk serves as an indicator of the mammary gland's contribution to overall milk fat content. This factor likely reflects the metabolic intensity of the mammary gland and is consistent with established interspecies differences in milk production potential and selective breeding pressures, particularly those concerning high-yielding bovine breeds.

The 2 factors related to the biohydrogenation process (factor 3 "BH_1" and factor 6 "BH_2") revealed distinct interspecies strategies. Factor BH_1, associated with the alternative pathway of ruminal biohydrogenation, showed the highest scores in cows (0.17), with markedly lower values in sheep (0.00), goats (−0.44), and buffaloes (−0.72; $P < 0.001$). Conversely, sheep exhibited the highest activity for factor BH_2, which reflects biohydrogenation via vaccenic acid as the main intermediate. These differences likely originate from species-specific variations in ruminal lipid metabolism and the composition and activity of the microbial community. Sheep appear to preferentially activate the α-linolenic acid biohydrogenation pathway, possibly due to greater access to fresh forage by grazing or diets enriched with linseed, both rich sources of this FA. As a result, sheep milk becomes a particularly rich source of CLA (Conte et al., 2017). In contrast, cows showed significantly higher values for factor 3, indicating a greater reliance on the alternative biohydrogenation pathways that lead to the formation of 18:1 *trans* isomer other than vaccenic acid. Notably, goats and buffaloes exhibited negative scores for this factor, suggesting reduced biohydrogenation activity compared with both sheep and cattle.

Goat milk exhibited significantly higher values for factor 4 ("Milk fluidity"; 2.12), in contrast to the negative or near-zero values in buffaloes (−0.44), cows (−0.45), and sheep (−0.04; $P < 0.001$). These findings suggest that goat milk contains a higher proportion of MCFA, which are known to enhance its fluidity and digestibility (Büyükkılıç Beyzi et al., 2020).

Factor 5 values were highest in buffalo milk (4.56), with significantly lower scores observed in cow (−0.41), goat (−0.03), and sheep (−0.08; $P < 0.001$). This pattern suggests a possible higher capacity for mitochondrial β-oxidation in the buffalo mammary gland, potentially linked to the species' generally elevated milk fat content and energetic density. Although recent studies have highlighted the role of mitochondrial regulatory genes such as PPARGC1A in buffalo mammary epithelial cells, which influence mitochondrial function and lipid metabolism (Hosseini et al., 2024), direct comparative data on mitochondrial β-oxidation activity in mammary tissue across ruminant species remain limited. In other species, such as the cow, mitochondrial β-oxidation in the mammary gland appears to be lower than in other tissues such as the liver, favoring lipid synthesis instead (Rawson et al., 2012). Therefore, further research is warranted to clarify the extent and significance of mitochondrial β-oxidation in ruminant mammary glands and its species-specific variations.

Factor 7 ("16:1 *c*7 elongation") was notably higher in goat milk (1.69), with substantially lower values observed in cow (−0.01), buffalo (−0.38), and sheep (−0.29; $P < 0.001$), highlighting the unique FA elongation in the goat rumen environment.

The BCFA, associated with microbial protein metabolism, were more abundant in cow milk (0.16), followed by goat (0.03), buffalo (0.01), and sheep (−0.13; $P < 0.001$), confirming interspecies variation in microbial-derived milk fat components. Significant species differences were also observed for factor "OCFA" ($P = 0.007$), with cow (0.02), sheep (0.01), and goat (−0.02) showing higher values than buffalo (−0.34). The OCFA are also microbial in origin and reflect differences in ruminal fermentation dynamics and microbial population structures.

Goat (0.10) and sheep (0.15) milk exhibited higher levels of factor "n-6 metabolism" compared with cow (−0.26) and buffalo (−0.35; SEM = 0.05, $P < 0.001$). Goats and sheep possess smaller milk fat globules, which results in a proportionally higher phospholipid-to-triglyceride ratio compared with other species (Pisanu et al., 2013). This characteristic may influence the requirement for linoleic acid absorption, as well as the synthesis of its elongation and saturation products (Argov-Argaman et al., 2016).

Finally, factor "SCFA" levels were highest in buffalo (0.41), followed by cow (0.26), goat (0.18), and lowest in sheep (−0.25; $P < 0.001$). The SCFA are ruminally produced and contribute significantly to the energetic and sensorial profile of milk.

These results underscore clear interspecies differences in milk lipid metabolism, rumen microbial activity, and mammary gland function. The distinct profiles across the 11 factors highlight the metabolic plasticity of ruminants, suggesting the potential for species-specific valorization of milk in terms of nutritional quality, technological applications, and human health implications.

Effect of the Diet on the Extracted Factor

Effect of the Lipid Supplementation. In this study, we evaluated the effect of different silage types and lipid supplementation on the extracted factors (Table 6). Four dietary treatments were considered: M, T, MSo, and MSu, all offered as TMR. These treatments differed in their lipid content, with the first 2 groups (M and T) serving as controls without lipid supplementation, whereas the latter 2 (MSo and MSu) included lipid supplements (5% sunflower oil and 5% soybean oil, respectively). The data satisfied the assumptions of residual normality and homoscedasticity, as indicated by *P*-values greater than 0.05 for the Shapiro–Wilk and Bartlett tests, respectively.

The factors used in this analysis were extracted from a multispecies dataset as explained previously, providing a general framework to interpret metabolic responses to lipid supplementation in cows. The use of MFA-derived factors allows testing whether the metabolic dimensions identified across ruminants can effectively capture diet-induced variations within a single species.

Factor 1 (OBCFA), factor 8 (BCFA), and factor 9 (OCFA), which reflect microbial FA profiles in the rumen, showed a significant reduction in both MSo and MSu treatments compared with M and T ($P < 0.001$). The lower levels of OBCFA in the lipid-supplemented groups (MSo and MSu) likely result from a shift in microbial populations, which are influenced by the increased intake of unsaturated fats. These lipids can inhibit certain microbial activities (Vlaeminck et al., 2006). This suggests that lipid supplementation, particularly with sunflower and soybean oils, can alter ruminal fermentation processes, leading to a decrease in the production of OBCFA and BCFA.

Factor 2 (Mammary activity) was significantly lower in the lipid-supplemented treatments (MSo and MSu), with values of 1.20 and 1.10, respectively, compared with 1.29 and 1.24 in the control treatments ($P < 0.001$). This result proposes that lipid supplementation may influence mammary gland activity, potentially by altering the availability of FA for milk fat synthesis or by shifting metabolic processes that redirect energy partitioning away from milk production (Chilliard et al., 2000).

Table 6. Effect of lipid supplementation on the 11 extracted factors[1]

Factor	Name	M	T	MSo	MSu	SEM	*P*-value
F1	OBCFA	0.50^{A}	0.48^{A}	0.17^{B}	0.15^{B}	0.04	<0.001
F2	Mammary activity	1.29	1.24	1.20	1.10	0.07	<0.091
F3	BH_1	0.48^{B}	0.38^{B}	0.82^{A}	0.92^{A}	0.09	<0.001
F4	Milk fluidity	−0.70^{B}	−0.65^{B}	−0.23^{A}	−0.22^{A}	0.04	<0.001
F5	Beta-oxidation	−0.28	−0.36	−0.30	−0.33	0.02	0.155
F6	BH_2	−0.22^{A}	−0.20^{A}	−0.35^{B}	−0.36^{B}	0.05	0.005
F7	C16:1 *c*7 elongation	0.68^{A}	0.64^{A}	0.25^{B}	0.29^{B}	0.06	<0.001
F8	BCFA	−0.23^{A}	−0.32^{A}	−0.58^{B}	−0.69^{B}	0.05	<0.001
F9	OCFA	0.46^{A}	0.47^{A}	−0.46^{B}	−0.48^{B}	0.11	<0.001
F10	n-6 FA	−0.19	−0.19	−0.16	0.06	0.06	0.211
F11	SCFA	0.23^{A}	0.20^{A}	−0.16^{B}	−0.19^{B}	0.05	<0.001

^{A,B}Values with different superscripts within a row are significantly different between groups at $P < 0.01$.

[1]M = maize silage group; T = triticale silage group; MSo = maize silage + soybean oil supplementation; MSu = maize silage + sunflower oil supplementation.

Factors associated with biohydrogenation (BH_1 and BH_2; F3 and F6) exhibited clear dietary effects. The BH_1 was significantly higher in the lipid-supplemented groups (MSo and MSu) compared with M and T ($P < 0.001$), whereas BH_2 showed a significant decrease in MSo and MSu ($P = 0.005$). These changes suggest that lipid supplementation may shift the biohydrogenation pathways in the rumen, particularly influencing the hydrogenation of unsaturated FA. The increased biohydrogenation (BH_1) could be a result of an alternative biohydrogenation pathway related to the higher level of 18:2 n-6 in the MSo and MSu diets, which favors the synthesis of *trans* isomers 18:1, different from vaccenic acid (Shingfield et al., 2010). This effect may indicate an incomplete or altered pathway in the primary hydrogenation process, where 18:1 *t*11 acts as the principal intermediate (Shingfield et al., 2010).

Milk fluidity (F4) showed a significant decrease in the lipid-supplemented groups (MSo and MSu) compared with M and T, indicating a change in milk fat composition. The less negative values for fluidity in MSo and MSu ($P < 0.001$) suggest that the addition of oils in animal diets may have increased the content of unsaturated FA in the milk, which are known to affect the physical properties of milk fat (Jensen, 2006). This could imply that the mammalian system may favor higher activity of sn-3 esterification of SFA.

Factor 5 (Beta-oxidation) did not show significant differences between treatments ($P = 0.155$), indicating that lipid supplementation did not significantly affect mitochondrial FA oxidation processes. This suggests that the observed changes in milk fat composition were likely more related to rumen fermentation dynamics than to postabsorptive metabolic pathways. Moreover, this result supports the idea that, in ruminants, the mammary gland primarily directs long-chain FA toward esterification and milk fat synthesis, whereas oxidation plays a relatively minor energetic role.

"16:1 *c*7 elongation" (F7) was significantly reduced in both lipid-supplemented treatments, MSo and MSu ($P < 0.001$), indicating a decreased synthesis of long-chain FA in the rumen. This suggests that the increased availability of unsaturated FA from the lipid supplements may inhibit FA elongation in the rumen, potentially due to shifts in microbial community composition or metabolic pathways (Vlaeminck et al., 2006).

The SCFA (F11) showed significantly higher values in the control groups (M and T) compared with the lipid-supplemented groups (MSo and MSu; $P < 0.001$). This further supports the notion that lipid supplementation modifies ruminal fermentation, specifically reducing the microbial production of these FA.

Finally, factor 10 (n-6 FA) did not show significant differences ($P = 0.211$), suggesting that the n-6 PUFA content in milk was not notably affected by the lipid supplementation. This finding contrasts with other studies where lipid supplementation significantly altered the n-6/n-3 ratio in milk (Palmquist and Griinari, 2006), possibly due to compensatory increases in other FA pathways or differences in the types of lipids used in supplementation.

Effect of the n-3 PUFA Supplementation. We also evaluated the effect of different n-3 sources (herbage and linseed) on the 11 extracted factors. This comparison was made to evaluate whether the source of α-linolenic acid affects mammary metabolism. Indeed, previous studies have reported a greater inhibitory effect on lipid metabolism when the diet includes plant oil supplementation (Rego et al., 2009). One of the main sources of α-linolenic acid is linseed; therefore, we aimed to determine whether it exerts a different effect on the factors compared with an α-linolenic-acid-rich diet based on pasture. The results revealed significant differences in several factors related to rumen fermentation, biohydrogenation, and milk composition (Table 7). The data satisfied the assumptions of residual normality and homoscedasticity, as indicated

Table 7. Effect of n-3 source on the 11 extracted factors[1]

Factor	Name	NP	P	L	SEM	*P*-value
F1	OBCFA	0.66^{A}	−0.39^{B}	−0.43^{B}	0.07	0.003
F2	Mammary activity	−0.28^{A}	−0.28^{A}	−0.67^{B}	0.04	<0.001
F3	BH_1	−0.05^{A}	−0.05^{A}	−0.53^{B}	0.03	<0.001
F4	Milk fluidity	0.50^{A}	0.09^{B}	−0.12^{C}	0.07	<0.001
F5	Beta-oxidation	−0.32^{B}	−0.29^{B}	−0.02^{A}	0.02	<0.001
F6	BH_2	−0.79^{B}	−0.56^{B}	0.58^{A}	0.11	<0.001
F7	C16:1 *c*7 elongation	−0.76^{B}	−0.67^{B}	−0.28^{A}	0.05	<0.001
F8	BCFA	0.72^{A}	−0.45^{B}	−0.44^{B}	0.06	<0.001
F9	OCFA	0.38^{A}	−0.04^{B}	0.08^{B}	0.05	<0.001
F10	n-6 FA	1.29^{A}	1.45^{A}	−0.32^{B}	0.09	<0.001
F11	SCFA	−0.31^{A}	−0.48^{A}	−0.98^{B}	0.09	<0.001

$^{A–C}$Values with different superscripts within a row are significantly different between groups at $P < 0.01$.

[1]NP = no pasture group; P = pasture group; L = linseed supplementation.

by *P*-values greater than 0.05 for the Shapiro–Wilk and Bartlett tests, respectively.

The factors applied in this section are the same as those extracted from the multispecies dataset, providing a common basis to interpret the metabolic response to n-3 PUFA supplementation in sheep. Using these MFA-derived factors allows us to test their biological consistency and their ability to capture shared lipid metabolic mechanisms across ruminants.

Factors 1, 8, and 9, which correspond to the production of OBCFA, BCFA, and OCFA in the rumen, respectively, were significantly higher in NP compared with both P and L ($P = 0.003$). Specifically, OBCFA levels were 0.66 in NP, compared with −0.39 in P and −0.43 in L. The lower OBCFA levels observed in the n-3 supplemented diets (P and L) suggest that n-3 PUFA, whether from herbage or linseed, may inhibit the microbial production of these FA, likely due to changes in the rumen microbial community (Vlaeminck et al., 2006). This reduction in OBCFA is consistent with previous findings that n-3 FA can modulate rumen microbial activity (Buccioni et al., 2012).

Factor 2 (Mammary activity) showed a significant decrease in the L group compared with both NP and P ($P < 0.001$). Whereas NP and P had similar values for mammary activity (−0.28), the L diet resulted in a more negative value (−0.67), indicating reduced mammary activity and lipid synthesis in sheep fed with linseed. This suggests that n-3 supplementation, particularly from linseed, may alter the partitioning of nutrients away from the mammary gland, potentially affecting milk fat synthesis (Chilliard et al., 2000). Such an effect is likely mediated by shifts in ruminal biohydrogenation of UFA—leading to increased production of 18:1 *t*10 and other bioactive intermediates—that inhibit mammary de novo FA synthesis through downregulation of key lipogenic enzymes (e.g., ACC, FAS) and transcription factors such as SREBP1 (Shingfield et al., 2010; Meignan et al., 2017). The net result is often a decrease in SCFA and MCFA produced in the mammary gland and an increase in LCFA in the milk fat profile. However, oilseed inclusion can also reduce DMI (Chilliard et al., 2007; Glasser et al., 2008), which may partially confound the interpretation of milk fat responses.

Factors related to biohydrogenation (BH_1 and BH_2; F3 and F6) showed clear differences across the diets. The BH_1 was significantly lower in the NP and P groups (−0.50 and −0.51, respectively) compared with L (−0.03; $P < 0.001$). This suggests that n-3 supplementation, particularly from linseed, reduced the extent of the alternative biohydrogenation pathway in the rumen, related to the conversion of linoleic acid through the *trans*-10, *cis*-12 CLA intermediate, producing 18:1 *t*10 rather than 18:1 *t*11 (Shingfield et al., 2010). Activation of this pathway, often under low rumen pH or high-unsaturated-fat conditions. The higher values observed in NP and P indicate greater hydrogenation of unsaturated FA, whereas the reduction in L is likely related to the specific fatty acid profile of linseed, characterized by its high α-linolenic acid content and, to a lesser extent, linoleic acid. These PUFA can alter the ruminal biohydrogenation pathways, leading to a lower overall hydrogenation extent (Harfoot and Hazlewood, 1997; Shingfield et al., 2010). In contrast, BH_2 showed the opposite trend, with the L diet resulting in significantly higher values (0.58) compared with NP (−0.79) and P (−0.56; $P < 0.001$). This suggests that linseed supplementation may alter the hydrogenation of unsaturated FA in a way that favors more complete hydrogenation.

Milk fluidity (F4), which reflects the physical properties of milk fat, was significantly reduced in the n-3 groups. The L diet showed the lowest value (−0.12), followed by P (0.09) and NP (0.50; $P < 0.001$). The more negative fluidity values in the n-3 PUFA supplemented diets suggest that the inclusion of n-3 PUFA increases the proportion of unsaturated FA in the milk, which typically results in lower milk fat fluidity (Jensen, 2006). This change in milk fat properties aligns with the known effects of PUFA on milk fat composition.

Beta-oxidation (F5) showed a significant increase in the L group (−0.02) compared with both NP (−0.32) and P (−0.29; $P < 0.001$). This result suggests that n-3 supplementation may be associated with enhanced FA oxidation, which likely occurs primarily in the liver rather than in the mammary gland. The higher oxidative activity observed in the L group could reflect either an upregulation of hepatic β-oxidation or an increased availability of oxidizable substrates, possibly resulting from alterations in ruminal biohydrogenation pathways induced by the linseed diet (Lock and Bauman, 2004).

"16:1 *c*7 elongation" (F7) was significantly higher in the L diet compared with both NP and P ($P < 0.001$). This increase in ruminal elongation suggests that n-3 supplementation may reduce the synthesis of LCFA in the rumen, potentially due to changes in microbial fermentation or direct effects on FA biosynthesis pathways (Vlaeminck et al., 2006).

Factor 10 (n-6 FA) showed a significant increase in both P and L diets (1.45 and 1.29, respectively) compared with NP (−0.32; $P < 0.001$). This suggests that n-3 supplementation reduces the proportion of n-6 FA in milk fat, likely due to the complex interactions between n-3 and n-6 PUFA in the rumen and the subsequent effect on milk fat composition (Palmquist and Griinari, 2006). This shift in the n-6 to n-3 ratio aligns with previous studies on n-3 supplementation in dairy cattle (Palmquist and Griinari, 2006). However, it is interesting to note that this effect was only observed with L diet, whereas P group was similar to NP.

Finally, SCFA (F11) was significantly lower in the L group (−0.98) compared with both NP (−0.31) and P (−0.48; $P < 0.001$). This suggests that linseed supplementation may reduce the production of SCFA in the rumen, possibly due to changes in microbial populations or fermentation pathways (Shingfield et al., 2010).

CONCLUSIONS

The MFA approach used in this study provided several advantages when applied to a large dataset that included multiple species. One key benefit was the reduction of numerous variables into a few latent factors with biological significance, factors that were not extracted in previous studies where MFA was applied to a single species. The statistical approach effectively separated FA groups with similar origins and functions, reflecting common or related metabolic pathways. The scores for these latent factors were consistently influenced by various productive environments and individual animal factors, in line with current knowledge. As such, this approach proves to be a valuable tool for studying the effects of different production systems, such as varying lipid source supplementation or different n-3 PUFA supplementation, such as herbage and extruded linseed. The nutritional and metabolic information inherent in the milk FA profile can be effectively leveraged using this approach. Finally, some of the results of this study could serve as a valuable tool for guiding animal management and feeding practices, as well as supporting future breeding strategies, due to the identification of specific and differentiated approaches among the species analyzed.

NOTES

This study was carried out within the Agritech National Research Center and received funding from the European Union program NextGenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA [PNRR]—MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4–D.D. 1032 17/06/2022, CN00000022) Spoke 5, Task 5.2.2, and Spoke 9, Task 9.11-1.3. The authors sincerely thank all the farms involved in the work, which willingly cooperated during milk collection and remained available to provide any information needed. This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them. During the preparation of this work the author(s) did not use any artificial intelligence or artificial intelligence-assisted technologies. Supplemental material for this article is available at https://doi.org/10.17632/gs36xhsgpy.1. Only data from milk samples collected from animals not used for scientific purposes were used. Milk sampling was carried out on farms during routine performance recording. Therefore, this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: ACC = acetyl-CoA carboxylase; BCFA = branched-chain FA; BH = biohydrogenation; BS = Brown Swiss; *c* = *cis*; FA = fatty acid; FAME = FA methyl esters; FAS = fatty acid synthase; HF = Holstein-Friesian; L = no pasture with linseed supplementation; LCFA = long-chain FA; M = maize silage; MA = Massese; MCFA = medium-chain FA; MFA = multivariate factor analysis; MSA = Kaiser measure of sampling adequacy; MSo = M with soybean oil supplementation; MSu = M with sunflower oil supplementation; NP = no pasture; OBCFA = odd- and branched-chain FA; OCFA = odd-chain FA; P = pasture; PCA = principal component analysis; S = Sarda; SCD = stearoyl CoA desaturase; SCFA = short-chain FA; T = triticale silage; *t* = *trans*.

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