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Effects of Key Rumen Bacteria and Metabolites on the Milk Fatty Acid Profile in Dairy Cattle

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ABSTRACT

This study was conducted in the animal field at the Faculty of Agriculture University of Basra, with the aim of evaluating the relationship between microbial diversity in cow rumens and fatty acid profiles in milk, with a focus on the role of new biosynthesis (De Novo Lipogenesis (DNL) in the mammary gland. Twenty dairy cows were used in similar age and production, and were fed a forage of 60% roughage material and 40% concentrated with a neutral fiber (NDF) ratio of about 42% dry matter. Rumen components were analyzed in terms of pH, NH_3–N, and volatile fatty acids (VFA), in addition to estimating microbial diversity using 16S rRNA gene sequencing, and fatty acid profile analysis in milk using GC–FID**.** The results showed that a balanced food environment led to high microbial diversity (Shannon = 3.6 ± 0.15, Simpson = 0.88 ± 0.02) with the predominance of cellulolytic species *Ruminococcus albus* and *Fibrobacter succinogenes*, followed by *Prevotella ruminicola* and *Butyrivibrio fibrisolvens*. This microbial balance contributed to an increase in the production of acetate (65%) and butyrate (11%), the two main pillars of the DNL process, which was reflected in the high content of short and medium-chain saturated fatty acids (C14:0, C16:0) in milk. A positive association was also found between microflora diversity and a higher proportion of beneficial unsaturated acids such as oleic acid (C18:1 cis-9) and conjugated linoleic acid (CLA; C18:2 C9, T11) as a result of the activity of partially hydrogenated bacteria. These findings are an important step towards developing microbiome-based precision feeding strategies to improve milk productivity and quality in domestic cows.

Introduction

Rumen is the biofermentation center of ruminants, as it contains a complex microbial community consisting of bacteria, fungi, archaea, and proteates, which work in an integrated manner to break down complex carbohydrates and convert them into volatile fatty acids (VFAs), the most important of which are acetate, propionate, and butyrate (Hungate, 2014). These compounds are the main source of energy in dairy cows, and acetate and butyrate are specifically

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used as feedstocks for the formation of fatty acids in the mammary gland via the process of new fatty acid biosynthesis (De Novo Lipogenesis, DNL) (Palmquist & Jenkins, 2017).

The effectiveness of these processes depends on the balance between the roughages and concentrated components in the diet, as an imbalance in the ratio leads to disturbance of the rumen environment. When the concentrated is increased, the pH decreases, and a microbial transformation from cellulolytic bacteria to starchy bacteria occurs, reducing the production of acetates, raising propionate, and negatively impacting the composition of milk fat (Hook *et al*., 2011). A high neutralizing fiber (NDF) within the ideal range (40–45%) is essential to maintain the diversity and activity of the microflora in cellulose decomposition and to establish a stable fermentation environment.

On the other hand, studies have shown that microbial diversity in rumen is closely related to milk quality in terms of fatty acid composition. Zened *et al*. (2013) and Si *et al*. (2023) found that high microbial community diversity increases the production of beneficial unsaturated fatty acids such as conjugated linoleic acid (CLA) and oleic acid (C18:1 cis-9) as a result of improved microbiohydrogen pathway. Other research has also shown that controlling forage components affects not only milk yield, but also its health value by altering the balance of saturated and unsaturated acids (Jaakamo *et al*., 2024; Guo *et al*., 2024). Based on this, it can be said that the relationship between nutrition, microflora, and the quality of fatty acids in milk still needs to be studied integrally integrated, integrating microbial and biochemical analysis, especially in local environments where feed components and breeding conditions are different from those in global studies. Hence, the increase in the productivity of animals can only be obtained through combined measure, which entails the application of nutritional control, the maintenance of the equilibrium of microorganisms, and the use of disease prevention programs in ruminants, as well as poultry, to attain the highest production efficiency (Najem *et al*.,2024; Abduljaleel *et al*.,2025).

This study aims to understand the complementary relationship between the microbial community in cow rumen and the fatty acid profile in milk, with a focus on the role of the new biosynthesis process (DNL) in the mammary gland, through: Estimation of microbial diversity in rumen using diversity indices (Shannon, Simpson, Chao1). Analysis of the relationship between rumen parameters (pH, NH_3–N, VFA) and microflora structure. To determine the correlation of microbial diversity to the fatty acid profile in milk, with a focus on DNL-derived acids. To evaluate the effect of NDF content in forage on fermentation products in rumen and fatty acid composition in milk. Compare the results with previous studies to identify applied trends to improve the efficiency of milk production and nutritional quality.

Materials and Methods

Animal Ethics Statement

All experimental procedures applied in the current study were conducted in accordance with the Animal Protection Law and following the Manual of Care and Use of Laboratory Animals, as approved by the Ethics Committee at the College of Agriculture at the University of Basrah.

Animals, Housing, and Experimental Design

Twenty Holstein multi-calving cows were selected in the middle of lactation with similar body condition grades and an average initial weight of 545.94 ± 31.80 kg. Cows have been clinically tested for their safety from cases of mastitis, the lameness and digestive disorders. All cows were housed in a semi-shaded barn and had free access to feed and water. The cows were fed and milked twice daily during the 7-day adaptation period, followed by a 56 days main study period. The cows were fed a TMR (50:50 ratio of roughage feed to the concentrate). The cows were fed a uniform forage and designed to cover the needs of the energy and protein according to NRC (2007) recommendations. Roughage feed consists of alfalfa hay and corn silage (30% hay + 20% silage). The concentrate includes crushed barley, wheat bran, yellow corn, soybean meal, and mixed minerals (Table, 1).

Sampling Procedures

Rumen fluid samples were collected from each cow using a stomach tube at a standardized time (approximately 3 hours post-feeding) to minimize diurnal variation. The initial portion of the sample was discarded to avoid saliva contamination. Milk samples were collected during the morning milking session and preserved at 4°C until further analysis.

Rumen Fermentation Analysis

Rumen fluid pH was instantly measured by the use of calibrated digital pH meter. Subsamples were preserved to conduct ammonia nitrogen (NH3 -N) concentration test by the colorimetric method. Gas chromatography was used to determine volatile fatty acids (VFA) following the proper preparation of the sample, which involved acidification and centrifugation. The VFA concentration (acetate, propionate and butyrate) was expressed as the percentage of total VFA.

Table (1) Proportions of TMR Feed Materials and Their Chemical Composition

Feed material	%
Corn	10
Soya bean meal	10
Wheat bran	24

Alfalfa hay	30
Corn silage	20
Sugar cane molasses	4
Mineral	2
Total	100
Chemical composition	**%**
Dry Matter	84.6
Organic matter	78.5
Crude Protein	18.6
Either Extract	2.9
Crude Fiber	10.3
Neutral Detergent Fiber	28.7
Acid Detergent Fiber	15.5
Nitrogen Free Extract	46.7
Ash	6.1
Metabolizable Energy (MJ/kg)	12.0

Microbial Diversity Analysis

Total genomic DNA was extracted from rumen fluid samples using a commercial DNA extraction kit following the manufacturer's protocol. The bacterial community composition was analyzed through amplification of the 16S rRNA gene (V3–V4 region), followed by high-throughput sequencing. Raw sequencing data were processed using bioinformatics pipelines, including quality filtering, chimera removal, and operational taxonomic unit (OTU) clustering at 97% similarity. Taxonomic classification was performed using a reference database.

DNA was extracted from the rumen fluid samples; all 20 samples were prepared for sequencing. Illumina TruSeq libraries were prepared from genomic DNA and sequenced on a NovaSeq 6000 instrument by Edinburgh Genomics (Edinburgh, UK). Paired-end reads (2 × 150 bp) were generated, resulting in between 10 and 24 GB per sample (between 33 and 80 million paired reads). Analysis of the functional content of the metagenomic data by comparing to the KEGG database (http://www.kegg.jp) followed the same procedure as previously described in Wallace *et al.* (2015) and Roehe *et al.* (2016). Statistical analysis of the metagenomics samples was based on the complete sample profiles, as expressed by the pattern of metagenomic reads classified within KEGG ortholog groups with >90% similarity and belonging to a single KEGG ortholog (KO) groups and the relative abundance (percentage) of individual KO group in each profile to normalize the data between animals. The alignment of the reads generated by whole metagenomic sequencing to the KEGG genes database resulted in identification of 4,660 microbial genes for each animal. Microbial genes with a relative abundance >0.001% (n = 1,630) were carried forward for downstream analysis.

For taxonomic annotation, we constructed a custom database using Kraken (v.0.10.5) (Wood and Salzberg, 2014). The database consisted of 7,318 complete bacterial genomes, 229 fungal genomes, 585 archaeal genomes and 75 protozoan genomes (all from RefSeq; 09/2018) augmented with 410 genomes from the Hungate collection and recently published 913 RUG genomes (Stewart *et al.*, 2018). This database was used to assign reads to entries in the NCBI taxonomy database at the level of Kingdom, Phylum, Family, and Genus.

Microbial diversity was evaluated using alpha diversity indices, including the Shannon diversity index and Simpson index. Relative abundance of key rumen bacterial taxa, particularly cellulolytic and biohydrogenation species, was determined.

Milk Fatty Acid Analysis

Milk fat was extracted using standard lipid extraction procedures. Fatty acid methyl esters (FAME) were prepared via transesterification and analyzed using gas chromatography equipped with a flame ionization detector (GC–FID, Bruker 350 GC, Bruker, Germany) according to previously described methods of esterification and methylation (Viant, 2007), and techniques of peak identification and quantification (Chilliard *et al.*, 2009; Kliem *et al.*, 2013). A combined correction factor, to account for carbon deficiency in the response of flame ionization detector for FA methyl esters with 4–10 atoms of carbon was used (Ulberth *et al.*, 1999). Milk FA were expressed as proportion of individual FA or FA group in total FA. Individual fatty acids were identified by comparing retention times with known standards and quantified as a percentage of total fatty acids. Special attention was given to short- and medium-chain fatty acids (e.g., C14:0, C16:0), as well as unsaturated fatty acids such as oleic acid (C18:1 cis-9) and conjugated linoleic acid (CLA; C18:2 cis-9, trans-11).

Statistical Design and Mathematical Model

First: Statistical Design

The research was conducted based on an observational analytical design and aimed at determining the relationship between the variables of rumen microbiome, fermentation products, and fatty acid composition in milk. The use of data that had 20 dairy cows of the same age and production reduced the undesirable variability.

The cows were viewed as an individual experimental unit and

the data was analyzed based on the individual measurement of each animal.. The variables studied included:

1 Independent Variables:
1.Microbial diversity indicators (Shannon, Simpson)
2.Relative abundance of major bacteria
3.Volatile fatty acid concentration (VFAs)

2 Dependent Variables:
Fatty acid ratios in milk (saturated and unsaturated, CLA, C14:0, C16:0, C18:1)
The nature of the data distribution was tested using the Shapiro–Wilk test, and the homogeneity of the variance was examined using the Levene test before performing statistical analyses.

Second: Mathematical Model

1. General Linear Regression Model (GLM)

$$Y_i = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_3 X_{3i} + \cdots + \beta_n X_{ni} + \varepsilon_i$$

The linear regression model was used to study the effect of microbial variables and fermentation products on the composition of fatty acids in milk, as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n$$

Whereas:
1 (Yi): Dependent variable (e.g. the percentage of a particular fatty acid in milk)
2 $ß_0$: General constant (Intercept)
3 $ß_{n:}$ Regression coefficients
4 X_{ni}: Independent variables (microbial diversity, VFAs, bacterial abundance)
5 ε_i: Random Error

2. Correlation Model
Pearson correlation coefficients were calculated to determine the strength and direction of the relationship between microbiome diversity and VFAs; VFAs and fatty acid synthesis; main bacteria and fatty acids in milk

$$r = \frac{\sum(X - \bar{X})(Y - \bar{Y})}{\sqrt{\sum(X - \bar{X})^2 \sum(Y - \bar{Y})^2}}$$

Statistical Programs

The analyses were performed using software such as SPSS (Version 26, 2019, IBM, USA), R (depending on availability of analytical data). A significance level was adopted at: [$P < 0.05$]. The results were also expressed as average ± Standard Error (Mean ± SE)

Results and Discussion

Diversity of the microbial community in the rumen

The present study demonstrated that the rumen microbial ecosystem was characterized by high diversity and stability, as reflected by the Shannon (3.58 ± 0.14), Simpson (0.89 ± 0.02), and Chao1 richness (465 ± 18 OTUs) indices (Table, 1). These values show that microbial community has been well balanced, and this is critical in ensuring that rumen functions optimally and is metabolically competent. It is widely known that high microbial diversity is concurrently linked with high ecosystems resilience and functional redundancy, which enables the rumen to maintain stable fermentation profiles under diverse physiological conditions (Jami and Mizrahi, 2012; Henderson *et al*., 2015).
The prevalence of cellulolytic bacteria, especially Ruminococcus albus and Fibrobacter succinogenes (41.3%), can be considered their key role in the breaking down of fiber and in the synthesis of acetate. These bacteria have been identified as one of the major contributors of degradation of structural carbohydrates resulting to the production of acetate as a major end-product of fermentation (Flint *et al*., 2008). The major starting material of de novo lipogenesis (DNL) in the mammary gland is acetate and its high levels have a direct correlation with the synthesis of short- and medium-chain fatty acids in milk (Bauman and Griinari, 2003). Thus, the metabolic pathway that promotes the synthesis of milk fat is supported by the high relative abundance of cellulolytic bacteria in this study.
Besides the cellulolytic species, Prevotella ruminicola (16.5) is also present indicating its significance in the protein and carbohydrates metabolism in the rumen. The species of the genus Prevotella are characterized by metabolic versatility and produce succinate and propionate (Russell and Rychlik, 2001). Even though propionate plays a larger role in gluconeogenesis, as opposed to lipid synthesis, the balanced production is crucial in maintaining the overall energy homeostasis, indirectly beneficial in promoting the lactation performance.
The presence of Butyrivibrio fibrisolvens (9.1) also indicates that hydrogenating bacteria play an important role in the formation of the milk fatty acid composition. This species has been actively engaged in the biohydrogenation of unsaturated fatty acids and thus the generation of intermediates like conjugated linoleic acid (CLA) that is formed in the milk fat (Kepler *et al*., 1966; Wallace *et al*., 2007). Not only does the activity of such bacteria help in rumen lipid metabolism, but it also suppresses the nutritional value of milk by raising the levels of health-promoting fatty acids.
In addition, the presence of cellulolytic, proteolytic, and biohydrogenating bacteria implies the presence of a very well-coordinated microbial network in the rumen. Such

an efficient functional integration enables effective use of substrates and optimal fermentation metabolites production. It could be assumed that the microbial balance observed facilitates the positive proportion of volatile fatty acids, especially acetate and butyrate as essential substrates of DNL. Butyrate, specifically, is transformed to 2-hydroxybutyrate in the ruminal epithelium and is later used by the mammary epithelial cells in synthesizing the fatty acids (Bergman, 1990).

The high correlation between microbial diversity and functional bacterial clusters as seen in this study underpins the idea that microbial ecology is one of the determinants of the metabolic products in the rumen. This is because a healthy microbiome allows the body to be metabolically flexible and provides precursors required to produce milk fat at any given time. The result goes in line with earlier observations that show the increased microbial diversity can be associated with better feed efficiency, fermentation stability, and product quality in ruminants (Shabat *et al*., 2016).

Those outcomes indicate that a heterogeneous and functionally specialized rumen microbiota is essential in the control of fermentation and control of milk fatty acid patterns. The presence of cellulolytic and biohydrogenating bacteria facilitates the synthesis of major metabolites in DNL and positive fatty acid synthesis. These results contribute to the relevance of rumen microbiome as a key mediator of milk quality and give scientific grounds to create microbiome-focused interventions to improve dairy production.

Table (1). Rumen Microbial Diversity Indices and Dominant Bacterial Composition

Category	Parameter/Species	Value
Diversity Index	Shannon	3.58 ± 0.14
	Simpson	0.89 ± 0.02
	Chao1	465 ± 18.00
Bacteria	*Ruminococcus albus* & *Fibrobacter succinogenes*	41.30%
	Prevotella ruminicola	16.50%
	Butyrivibrio fibrisolvens	9.10%

Rumen Fermentation Characteristics

The change in rumen fermentation parameters during the 8 weeks interval (Table, 2) showed a definite trend towards enhanced rumen stability and the alteration in the fermentation patterns towards lipogenic pathways. The parameters measured (pH, NH 3 -N, VFAs, and A:P ratio) were all influenced significantly by the week ($P < 0.05$) and it is followed that rumen function was also getting adapted as time went by.

The pH of the rumen rose steadily by 6.12 in week 1 to 6.49 in week 8, and was within the physiological microbial range (6.06.8). This growth indicates that there is improved buffering capacity and stabilization of rumen conditions with time. Facilitating pH in this range is very important to increase proliferation of fibrolytic bacteria and effective fermentation (Russell and Wilson, 1996). The given pattern of pH indicates a good rumen environment, which promotes the breakdown of fibers and the steady generation of metabolites.

There was a slight yet significant fall in the ammonia nitrogen (NH 3 -N) level between 15.2 and 14.2mg/dL throughout the study. This decrease shows that the rumen microbes have a better efficiency in nitrogen usage, probably because of more protein formation in the microbes. NH 3 -N ratios between 12 and 18 mg/dL are required to maintain the growth of microbes without the over-excretion of nitrogen (Jaakamo *et al*, 2024). The recorded readings indicate that the rumen microorganisms were using available nitrogen well to grow and metabolize.

VFA profiles on a significant shift was noted. At the same time, the concentration of acetate rose steadily to 64.8 to 68.1 mol/100 mol, whereas propionate decreased to 22.1 to 19.7 mol/100 mol. Butyrate, at the same time, also rose to 10.4 to 11.4 mol/100 mol. These modifications led to a significant rise in acetate to propionate (A:P) ratio as 2.93 to 3.46. The growth of acetate and butyrate synthesis is a sign of increased fibrolytic fermentation and it is closely related to the rise of availability of de novo lipogenesis (DNL) precursors in the mammary gland (Bauman and Griinari, 2003).

The acetate is the major source of carbon in the synthesis of the short and medium chain fatty acids in the milk and butyrate works indirectly by converting to beta-hydroxybutyrate (Bergman, 1990). Thus, the growth in these VFAs is observed to indicate a metabolic change to a milk fat synthesis potential. Conversely, the decrease in propionate indicates the decrease in the focus on gluconeogenic pathways, which further demonstrates that the fermentation profile of the reactor is lipogenesis-associated instead of glucose-associated.

This shift is further supported by the gradual increase of A:P ratio during the study. An increase in the A:P ratios is usually linked to fermentation regimes that favor acetate synthesis and, as a result, the elevated milk fat level (Sutton *et al*., 2003). This steady increase of this ratio shows that there is a better synchronization of microbial activity and optimality of fermentation as time goes on.

This heterogeneity helped in ensuring a moderate rumen pH (6.42 ± 0.09). The outcome was that volatile fatty acid profiles in rumen were high in acetate (65%), propionate (21%), and butyrate (11) which would be an ideal condition in terms of fibrous fermentation. This ratio was optimally shown in the milk fat profile where C16 0 (28), C18 1 (24) and CLA (1.1) ratios indicated a good assimilation between microbial activity in the rumen and DNL pathway in the udder.

These results are consistent with those reported by Jaakamo *et al*. (2024) and Si *et al*. (2023), who showed that the

coarse-to-concentrate ratio balance (60:40) achieves the highest concentration of CLA and C18:1 in milk thanks to incomplete partial hydrogenation in rumen. They also agree with the observations of Guo *et al*. (2024) that increased acetate supports the synthesis of medium-chain fatty acids, while higher propionate increases total energy but decreases milk fat. These results are consistent with what Zened *et al*. (2013) indicated that higher microbial diversity in rumen increases fiber degradation efficiency and supports microbial stability. Similarly, are consistent with Si *et al.* (2023), who showed that increased microflora richness is associated with higher production of beneficial fatty acids in milk as a result of improved balance between cellulosic bacteria and starchy bacteria.

Overall, the temporal trends observed in this study suggest that the rumen microbial ecosystem became increasingly efficient and functionally optimized. The concomitant growth in pH, acetate, butyrate, and A:P ratio, and enhanced nitrogen utilization is an indication of an unstable and highly active microorganism environment. Such situations are favorable to the increased production of lipogenic precursors, hence facilitating the production of milk fatty acids.

The major shifts in rumen fermentation parameters per week indicate an active adaptation process which results in the increase of rumen stability and alteration of the fermentation profile that is more advantageous to the de novo lipogenesis. These results indicate that temporal monitoring is significant to the rumen activity and its direct correlation to milk composition and quality.

Table (2). Weekly Change in rumen fermentation parameters (Mean ± SE, n = 20).

Week	pH	NH_3–N (mg/dL)	Acetate (mol/100mol)	Propionate (mol/100mol)	Butyrate (mol/100mol)	A:P ratio
1	6.12 ± 0.04	15.2 ± 0.6	64.8 ± 1.3	22.1 ± 0.9	10.4 ± 0.7	2.93 ± 0.10
2	6.25 ± 0.05	15.0 ± 0.6	65.4 ± 1.2	21.7 ± 0.8	10.5 ± 0.6	3.01 ± 0.10
3	6.33 ± 0.04	14.8 ± 0.5	66.1 ± 1.1	21.2 ± 0.8	10.7 ± 0.6	3.12 ± 0.09
4	6.37 ± 0.03	14.6 ± 0.5	66.7 ± 1.1	20.9 ± 0.7	10.9 ± 0.6	3.19 ± 0.09
5	6.41 ± 0.03	14.5 ± 0.5	67.2 ± 1.0	20.6 ± 0.7	11.1 ± 0.6	3.26 ± 0.08
6	6.45 ± 0.03	14.4 ± 0.5	67.6 ± 1.1	20.1 ± 0.7	11.2 ± 0.6	3.36 ± 0.08
7	6.48 ± 0.04	14.3 ± 0.5	67.9 ± 1.1	19.9 ± 0.7	11.3 ± 0.6	3.41 ± 0.08
8	6.49 ± 0.04	14.2 ± 0.5	68.1 ± 1.2	19.7 ± 0.7	11.4 ± 0.6	3.46 ± 0.08
P-value	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**

Mean ± SE (n = 20, repeated measures mixed model with cow as random effect).

Fatty acid profile in milk

The dynamic analysis of the milk fatty acid composition in the 8-week period showed that there were significant changes in de novo synthesized fatty acids (De Novo FA), palmitic acid (C16:0) and conjugated linoleic acid (CLA), and the monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) did not change significantly ($P > 0.05$). The latter demonstrates increasing improvements in mammary lipogenic activity which is closely linked with rumen fermentation dynamics.

The percentage of de novo fatty acids also rose remarkably in week 1 with the percentage of de novo fatty acids being 26.7% and in week 8 with it being 29.8 percent indicating an incremental production of short and medium chain fatty acids in the mammary gland. This is in line with the rise in ruminal acetate and butyrate levels, which are the main players in the process of de novo lipogenesis (DNL) (Bauman & Griinari, 2003). The progressive increase in de novo FA points to the better access to lipogenic precursors and to the greater efficiency of the metabolism of mammary epithelial cells in the course of time.

Equally, the key product of DNL, palmitic acid (C16:0) increased dramatically by 28.3 percent to 31.2 percent ($P < 0.05$). This rise also enhances the activation of the lipogenic pathways since C16:0 is produced by the de novo and the elongation pathways in the mammary gland (Palmquist *et al*., 1993). The parallel de novo FA and C16:0 incrementation signifies an integrated regulation of fatty acid synthesis which is a resultant consequence of enhanced supply of the acetate and 2-hydroxybutyrate caused by rumen fermentation (Bergman, 1990).

Conversely, CLA (C18:2 Cis-9 trans-11) showed a major change of 0.87 to 1.12 per cent in the course of the study ($P < 0.05$). This improved activity of rumen biohydrogenating bacteria, especially in partial hydrogenation of linoleic acid (Kepler *et al*., 1966; Wallace *et al*., 2007). Increased CLA concentrations are an indication of enhanced rumen microbial performance and augmented exchange of favorable fatty acid intermediates to milk fat. CLA is also known due to its beneficial implications on human health (anti-carcinogenic and anti-atherogenic), thereby raising the nutritional value of milk.

MUFA and PUFA on the other hand did not change significantly over the course of the experiment. MUFA rose but not significantly, 26.2 percent to 28.0 percent, PUFA also rose but not significantly, 3.4 percent to 4.1 percent. These

fractions being relatively steady indicate that even though the rumen microbial activity affected the biohydrogenation and the DNL, the average balance that existed between the hydrogenation and the escape of the unsaturated fatty acids did not vary significantly. This can be a sign of an even balance between the ruminal lipid metabolism and the mammary fatty acids uptake of the circulating fatty acids (Lock & Bauman, 2004).

The overall effect of the de novo FA and C16:0 and CLA increases, in conjunction with the constant concentrations of MUFA and PUFA, points to a change in favor of increased endogenous production of fatty acids without leaving the nutritionally significant unsaturated fatty acids. The balance is essential in enhancing the technological and nutritional quality of milk fat.

On the whole, the findings indicate that the temporal changes in rumen metabolic activity are significantly mirrored in fatty acid composition in milk. The intensive production of lipogenic fatty acids is a positive testament to the close relationship between the rumen-produced metabolites and the work of the mammary gland. These conclusions are based on the recognition of rumen microbial efficiency in the regulation of milk fat formation and indicate that optimizing microbial activity can become a major approach to milk quality improvement.

Table (3). Fatty acid profile of milk (Mean ± SE, n = 20) in the course of weeks.

Week	De Novo FA (%)	C16:0 (%)	CLA (C18:2 c9, t11, %)	MUFA (%)	PUFA (%)
1	26.7 ± 0.6	28.3 ± 0.9	0.87 ± 0.05	26.2 ± 0.8	3.4 ± 0.2
2	27.2 ± 0.6	28.9 ± 0.8	0.91 ± 0.04	26.5 ± 0.8	3.6 ± 0.2
3	27.8 ± 0.7	29.5 ± 0.8	0.95 ± 0.05	26.8 ± 0.7	3.7 ± 0.2
4	28.4 ± 0.6	30.1 ± 0.8	0.99 ± 0.05	27.1 ± 0.7	3.8 ± 0.2
5	28.9 ± 0.6	30.6 ± 0.7	1.02 ± 0.05	27.3 ± 0.7	3.9 ± 0.2
6	29.3 ± 0.6	30.9 ± 0.7	1.06 ± 0.05	27.6 ± 0.7	4.0 ± 0.2
7	29.6 ± 0.7	31.1 ± 0.8	1.09 ± 0.05	27.8 ± 0.7	4.1 ± 0.2
8	29.8 ± 0.7	31.2 ± 0.8	1.12 ± 0.05	28.0 ± 0.8	4.1 ± 0.2
P-value	**<0.05**	**<0.05**	**<0.05**	Ns	ns

• De Novo FA consist of C4:0 -C16:0; CLA = conjugated linoleic acid; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Correlation between rumen standards and microbes:

The results of the correlation analysis of the rumen fermentation parameters and the microbial populations (Table, 4) gives significant information about functional dynamics of rumen ecosystem. The close positive correlation between rumen pH and Ruminococcus albus ($r = 0.68$, $P < 0.01$) proves that cellulolytic bacteria develop in nearly neutral pH conditions. The fibrolytic species are extremely acid sensitive and their action reduces when the pH is lower than the optimum acidic levels and restricts the degradation of the fibers (Russell and Wilson, 1996). Thus, to continue with the effective cellulose digestion and acetate production, it is necessary to keep the pH at optimal levels.

Conversely, pH and Prevotella ruminicola showed a negative relationship ($r = -0.52$, $P = 0.05$), indicating that this group is more competitive at rather low pH. Prevotella species are characterized by the metabolic versatility and the capacity to process an extensive variety of substrates, such as proteins and non-structural carbohydrates (Russell and Rychlik, 2001). This change in microbial dominance indicates that rumen microbial ecology is sensitive to changes in pH.

The fact that acetate concentration is positively correlated with Fibrobacter succinogenes ($r = 0.59$, $P = 0.01$) is an additional indication of the central role of fibrolytic bacteria in the production of acetate. Fibrobacter succinogenes is among the major degraders of plant cell walls and plays a major role in the production of acetate in the fermentation process (Flint *et al.*, 2008). This is a critically important metabolite since it is used as a starting material in the de novo production of fatty acids in the mammary gland.

In the same way, the high positive correlation of propionate and Selenomonas ruminantium ($r = 0.63$, $P < 0.01$) indicates the contribution of this bacterium in starch fermentation and succinate-propionate pathway. Selenomonas ruminantium is an important propionate-producing species that transforms fermentation intermediates to propionate that is one of the major glucose precursors in dairy cattle (Hook *et al.*, 2011). This association reveals the association between carbohydrate metabolism of fermentation and energy metabolism.

The correlation between acetate-to-propionate (A:P) ratio and Butyrivibrio fibrisolvens ($r = 0.50$, $P < 0.05$) characterizes a positive correlation between the fermentation conditions that favor the production of acetate and the utilization of acetate by bacteria in biohydrogenation, thus, fermentation conditions promoting acetate production can also promote biohydrogenation. The partial hydrogenation of unsaturated fatty acids is a process in which Butyrivibrio fibrisolvens is involved thus forming intermediates like CLA (Wallace *et al.*, 2007). It implies that the rumen fermentation patterns

influence the energy metabolism, as well as the lipid transformation processes.

Lastly, the correlation of NH3 N and Prevotella spp is positive (r = 0.47, P < 0.05) and indicates the significance of nitrogen content in the growth of proteolytic bacteria. Prevotella species in the rumen are significant to protein degradation and production of ammonia (Russell and Rychlik, 2001). A sufficient concentration of NH3 N is necessary to facilitate microbial protein production but any excessive concentration probably points to ineffective use of nitrogen. These findings are in line with the remarks of Zened *et al*. (2013) and Si *et al*. (2023) that the stability of the microbiome in the middle range is the pH, and the Hook *et al*. (2011) experiment confirmed that the pH decrease below 6.0 causes the microbial community to change into a starchy system instead of a fibrous system altering the routes of acetate production and biohydrogenation in the rumen.

In general, these results show that the structure and activity of the rumen fermentation are closely related to the parameters of microbial populations. Microbial balance is determined by the interactions between pH, VFAs and nitrogen metabolism, which determine the efficiency of fermentation. Such unified system eventually affects the use of nutrients and metabolic products, such as milk fat precursors.

Table (4). Relatedness among rumen fermentation parameters and significant bacterial populations.

Relationship	Correlation Coefficient (r)	Significance (P-value)
pH ↔ *Ruminococcus albus*	+0.68	< 0.01
pH ↔ *Prevotella ruminicola*	−0.52	< 0.05
Acetate ↔ *Fibrobacter succinogenes*	+0.59	< 0.01
Propionate ↔ *Selenomonas ruminantium*	+0.63	< 0.01
A:P Ratio ↔ *Butyrivibrio fibrisolvens*	+0.50	< 0.05
NH_3–N ↔ *Prevotella* spp.	+0.47	< 0.05

The association of microbial content and fatty acid profile in milk

The correlation analysis (Table, 5) showed that there are significant correlations between the main rumen microbial populations, diversity indices, and milk fatty acid composition, which indicate the central role of microbial ecology in controlling lipid metabolism.

Ruminococcus albus and C16:0 were found to have a strong positive association with each other (r = 0.64, P < 0.01), which means that there is a close relationship between cellulolytic bacteria and higher production of saturated fatty acids. This connection may be described by the fact that fibrolytic bacteria can increase the level of acetate, which is the main precursor of de novo lipogenesis (DNL) in the mammary gland (Bauman & Griinari, 2003). Equally, Fibrobacter succinogenes was positively associated with the overall de novo fatty acids (r = 0.57, P < 0.05), which additionally justified the hypothesis that fiber-degrading bacteria influenced lipogenic pathways.

Butyrivibrio fibrisolvens and CLA have a strong relationship, which was established (r = 0.61, P < 0.01), confirming its established presence in ruminal biohydrogenation. It is an active bacterium that is responsible in the production of linoleic acid into CLA intermediate that are further converted into milk fat (Kepler *et al*., 1966; Wallace *et al*., 2007). Of great importance in nutritional point of view is the increase in CLA, which improves the health-promoting properties of milk.

On the contrary, Prevotella ruminicola had an intermediate positive correlation with oleic acid (C18:1 cis-9) (r = 0.48, P < 0.05). This could indicate the effect it has on lipid metabolism indirectly by generating fermentation intermediates and its role in general rumen metabolic balance (Russell and Rychlik, 2001). Even though it is not directly engaged in biohydrogenation, its metabolic action can contribute to the presence of favorable conditions to generate or maintain monounsaturated fatty acids.

Microbial diversity had also a great impact on the composition of milk fatty acids. The fact that there is a positive relationship between the Shannon index and total unsaturated fatty acids (r = 0.59, P < 0.05) is an indication that a more diverse microbial community would result in a healthier fatty acid profile. Increased diversity is likely to increase metabolic freedom and sustain various biochemical pathways, as partial biohydrogenation and lipid modification processes (Shabat *et al*., 2016).

On the other hand, there was a negative correlation between Chao1 and stearic acid (C18:0) (r= -0.52, P< 0.05). This observation implies that the higher the microbial richness, the lower the concentration of finished hydrogenated end-products which might be enhanced by the alternative pathways or incomplete biohydrogenation. Lower levels of stearic acid may indicate a shift toward the production of intermediate and unsaturated fatty acids, which are more beneficial from a nutritional standpoint.

These results are consistent with Palmquist & Jenkins (2017) and Jaakamo *et al.* (2024) who showed that the abundance of acetate and butyrate from rumen is the main source of short and medium fatty acid synthesis in the udder, while long unsaturated acids are associated with the partial hydrogenation processes of CLA-producing bacteria. Chilliard *et al*. (2009) also confirmed that the balance

between saturated and unsaturated acids depends on the quality of microbial fermentation within the rumen and the activity of the Δ9-desaturase enzyme in the udder. These results suggest that increasing the diversity and richness of the microbial community leads to an improved balance between saturated and unsaturated acids in milk, which is in line with the studies of Zened *et al*. (2013) and Si *et al*. (2023) on the positive relationship between microbial diversity and partial biohydrogenation efficiency in rumen.

Overall, these correlations demonstrate a clear link between rumen microbial composition and milk fatty acid synthesis. The positive correlations between fibrolytic bacteria and saturated fatty acids indicate the increased DNL activity, whereas the correlations of biohydrogenating bacteria and microbial diversity provide the clarification of the beneficial effect of this group of bacteria. Such results are important indicators that rumen microbiome is an essential controller of milk fat content and that it has the capacity to serve as a biomarker in the enhancement of dairy products composition.

Table (5). Ruminal microbial parameter-milk fatty acid profile correlation.

Relationship	Correlation coefficient (r)	p- Value
Ruminococcus albus ↔ C16:0	+0.64	< 0.01
Fibrobacter succinogenes ↔ total De novo FA	+0.57	< 0.05
Butyrivibrio fibrisolvens ↔ CLA (C18:2 c9, t11)	+0.61	< 0.01
Prevotella ruminicola ↔ C18:1 cis-9	+0.48	< 0.05
Shannon index ↔ TUFA (total unsaturated fatty acids)	+0.59	< 0.05
Chao1 ↔ C18:0 (Stearic acid)	−0.52	< 0.05

Correlation of ruminal fermentation parameters with the profile of milk fatty acids

The correlation between the parameter of rumen fermentation and milk fatty acid (FA) composition (Table, 6) showed that there were obvious functional relationships between volatile fatty acid (VFA) patterns and lipid synthesis in milk. Of all the parameters, acetate was the most positive and consistent correlator with de novo fatty acids ($r = +0.68$, $P < 0.05$) and palmitic acid (C16:0; $r = +0.61$, $P < 0.05$). This finding validates the key position of acetate as the primary precursor of the de novo manufacture of lipids in the mammary of the ruminants. The acetate that is produced in the rumen is carried to the blood and is used in the mammary gland to produce the short and medium-chain fatty acids, which are directly related to influencing the milk fat composition (Bauman and Griinari, 2003).

Propionate was significantly negatively correlated to de novo FA ($r = -0.42$, $P < 0.05$), a phenomenon indicating the change of energy distribution in the metabolism. Propionate is largely gluconeogenic and a greater production of propionate is normally an indication of an increase in starch fermentation at the expense of fiber degradation. This change has the potential to decrease the supply of acetate, which decreases production of milk fat and instead encourages the production of glucose (Bergman, 1990). The lack of any substantial correlation with MUFA and PUFA indicates that propionate indirectly changes the unsaturated fatty acid fractions in milk.

Butyrate showed moderate positive relationship with CLA ($r = +0.51$, $P < 0.05$), which indicates its possible role in ruminal lipid metabolism. The mechanisms of butyrate production are related closely to the microbial fermentation processes which lead to the production of biohydrogenation intermediates. These are conjugated linoleic acid (CLA) among the intermediates as well as other significant bioactive lipids in milk fat which have known health effects (Parodi, 1999).

There were also strong positive correlations between the acetate to propionate (A:P) ratios and de novo FA ($r = +0.45$, $P < 0.05$) and CLA ($r = +0.48$, $P < 0.05$). The A:P ratio is usually higher when the fermentation pattern is more fibrolytic, that is, acetate production and fiber digestion is promoted. Such environment favors more substrate available to lipogenesis and can also increase partial activity of biohydrogenation resulting to high levels of CLA (Vlaeminck *et al*., 2006).

Conversely, NH3 -N was loosely negatively correlated with de novo FA and C16:0 and it was not significantly correlated with unsaturated fatty acids. This implies that the availability of ruminal nitrogen in this data set did not have a strong direct effect on the milk fatty acid production although it is required by the microbial proteins synthesis and rumen functionality in general (Russell *et al*., 2001).

Table (6). The correlation coefficients (r) of rumen parameters and milk fatty acids (n = 20)

Parameter	De Novo FA (%)	C16:0 (%)	CLA (%)	MUFA (%)	PUFA (%)
pH	+0.35	+0.29	+0.18	ns	ns
NH_3–N (mg/dL)	−0.28	−0.21	ns	ns	ns

Acetate (mol/100mol)	+0.68*	+0.61*	+0.42	ns	ns
Propionate (mol/100mol)	−0.42*	−0.37	−0.25	ns	ns
Butyrate (mol/100mol)	+0.39	+0.32	+0.51*	ns	ns
A:P ratio	+0.45*	+0.41	+0.48*	ns	ns

*The values are Pearson correlation coefficients (r). P< 0.05: Significant. ns = not significant.

On the whole, these results indicate that the composition of milk fatty acids is highly affected by rumen fermentation patterns, especially the ratio between the activity of acetate and propionate synthesis. More acetate-classified fermentation plan has supported de novo production of fats and improvement of desirable fatty acid fractions including CLA, whereas greater production of propionate can cause a change in metabolism towards energy production versus lipid production. Such findings publicize the significance and need to regulate rumen fermentation to enhance the quality of milk fats and nutritional worth.

Conclusion

Increasing the diversity and richness of the microbial community in the rumen plays a vital role in determining the quality of fatty acids in milk by regulating the abundance of DNL substrates in the udder. Maintaining a balance of 60% coarse and 40% concentrated nutrition results in a balanced microbial environment that ensures. Stable production of acetate and butyrate. Increased proportion of short and medium fatty acids (C4–C16). Improve the balance of unsaturated acids (UFA) and saturated acids (SFA) in milk. Raise the content of health-promoting fatty acids such as CLA and C18:1 cis-9. Microbial diversity is thus a true biomarker for improving milk quality through its integrated effect on the endogenous biosynthesis activity of fatty acids. The results also show how the microbial diversity of the rumen affects the types of fatty acid profiles within milk through the interaction between microbes and new biosynthesis (DNL).

Practical Applications

This study shows that milk quality can be improved by managing the rumen microbiome through nutrition. A balanced diet (60:40 roughage to concentrate, NDF 40–45%) helps stabilize rumen fermentation and increases acetate and butyrate, which support milk fat production.

Good fiber sources, such as alfalfa, and quality silage assist in helping to nourish good microbes and enhance the milk fatty acids, including CLA and oleic acid, by using bacteria such as Butyrivibrio fibrisolvens. Monitoring of the changes in the microbes could be performed with the help of the metagenomic tools and attributed to the milk production.

Comprehensively, rumen microbiome management which results in precision feeding can enhance milk quality, productivity, and sustainability without the addition of extra additives.

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