Associations between negative energy balance, milk composition, sensor-derived behavior, and inflammatory biomarkers in early-lactation dairy cows

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Abstract. The onset of lactation represents a critical period in dairy cows, characterized by profound metabolic adaptations and an increased risk of negative energy balance (NEB). This study aimed to investigate the relationships between metabolic status, milk composition, physiological and behavioral parameters, and systemic inflammation in early-lactation dairy cows. A total of 71 clinically healthy multiparous Holstein cows at 17±3 days in milk were monitored under commercial farm conditions. Based on circulating non-esterified fatty acid (NEFA) concentrations, cows were classified into low-NEFA and high-NEFA groups. Milk composition was continuously assessed using an in-line analyzer, while reticulorumen pH, core body temperature, rumination time, physical activity, and water intake were recorded using intraruminal sensors. Blood samples were analyzed for NEFA and serum amyloid A (SAA) concentrations. Cows with elevated NEFA exhibited significantly higher milk fat content and fat-to-protein ratio, alongside reduced rumination time and physical activity. Serum amyloid A concentrations were markedly increased in the high-NEFA group, indicating activation of a systemic inflammatory response. In contrast, reticulorumen pH and core body temperature remained largely stable between groups. Correlation analysis revealed strong associations between NEFA, altered milk composition, suppressed behavioral activity, and inflammatory activation, highlighting NEFA as a central indicator of metabolic stress. These findings demonstrate that NEB in early lactation is a systemic condition affecting metabolic, behavioral, and inflammatory domains. The integration of milk traits, blood biomarkers, and continuous sensor-based monitoring provides a valuable framework for early identification of cows at risk of metabolic and inflammatory disorders in precision dairy farming systems.
Key Words: dairy cows, early lactation, intraruminal sensors, milk fat-to-protein ratio, negative energy balance, non-esterified fatty acids, rumination behavior, serum amyloid A.

Introduction. The onset of lactation represents one of the most critical and vulnerable stages in the productive cycle of dairy cows, particularly in animals with a high genetic potential for milk production (Trevisi et al 2025). During this transition period, cows undergo profound metabolic, endocrine, and immunological changes, which significantly increase the incidence of metabolic, inflammatory, and infectious disorders (Trevisi et al 2025). The occurrence of these conditions has direct negative consequences on fertility, milk yield, and overall performance in subsequent lactations, thereby affecting the economic efficiency of dairy farming systems (LeBlanc 2010).

A major determinant of pathological risk in the postpartum period is the rapid establishment of negative energy balance (NEB), resulting from the inability of dietary intake to meet the markedly increased energy demands required for milk synthesis (Drackley 1999). NEB is associated with extensive mobilization of body reserves, alterations in lipid and carbohydrate metabolism, and impaired immune function, which predispose cows to metabolic disorders such as ketosis and hepatic lipidosis, as well as to increased susceptibility to infectious and viral diseases (Ingvarsen & Moyes 2013).

In this context, a thorough understanding of the pathophysiological mechanisms governing metabolic adaptation at the onset of lactation, together with the early identification of metabolic and immunological imbalances, is essential for the implementation of effective nutritional and management strategies (Trevisi et al 2025). Careful monitoring of metabolic status and timely interventions can substantially contribute to optimizing herd health, animal welfare, and productive longevity in dairy cows (LeBlanc 2010).

The transition period, defined as the interval encompassing the last three weeks prepartum and the first three weeks postpartum, is characterized by profound metabolic, endocrine, and immunological adaptations that markedly influence energy homeostasis and overall health status in dairy cows (Sordillo et al 2009). During this critical phase, cows are required to simultaneously support late fetal growth, colostrum synthesis, and the initiation of lactation, processes that impose a substantial metabolic burden and lead to an accelerated utilization of body reserves (Overton & Waldron 2004).

As a consequence, concentrations of non-esterified fatty acids (NEFA), which are considered key indicators of adipose tissue mobilization, increase markedly in the postpartum period and are closely associated with the establishment of negative energy balance (NEB) (Ospina et al 2010). These metabolic adjustments are frequently accompanied by a decline in immune competence, disturbances in calcium homeostasis such as hypocalcemia, activation of systemic inflammatory responses, and increased oxidative stress (Sordillo & Raphael 2013). If these alterations persist beyond the early postpartum period, they may predispose cows to the development of severe metabolic disorders, including ketosis and hepatic steatosis, ultimately compromising productive performance, reproductive efficiency, and animal welfare (Drackley 1999).

In this context, metabolic profiling has emerged as an effective approach for the early detection of metabolic imbalances associated with the transition period (LeBlanc 2010). Blood-based analyses allow for the assessment of key biomarkers such as NEFA, β-hydroxybutyrate (BHB), and indicators of hepatic function (Ospina et al 2010), and are widely regarded as reference methods for evaluating the metabolic status of dairy cows (Smith et al 2014). Excessive lipid mobilization during early lactation is reflected by elevated circulating concentrations of NEFA and BHB, reduced glucose levels, and alterations in the milk fat-to-protein ratio (F:P). Collectively, these parameters provide valuable insight into the metabolic adaptation of cows and serve as practical indicators of increased risk for hepatic dysfunction and other metabolic disorders (Džermeikaitė et al 2025).

The immune system responds to these physiological challenges through the production of acute phase proteins (APPs), among which serum amyloid A (SAA) is considered the principal marker in cattle (Saco & Bassols 2023). Circulating SAA concentrations increase during the periparturient period as a response to physiological stress, inflammatory processes, and structural and functional changes within the reproductive tract, reaching peak values during the first weeks after calving (Humblet et al 2006).

Despite these systemic increases, SAA concentrations remain low in the milk of clinically healthy cows, a characteristic that enhances its value as a sensitive and specific indicator of systemic inflammation rather than localized mammary responses (Nielsen et al 2004). Consequently, monitoring SAA dynamics provides relevant information on inflammatory status and immune activation during the transition period, supporting its use as a biomarker for early detection of health disturbances in dairy cows (Saco & Bassols 2023).

Despite the significant advances achieved in recent years, there is still a limited number of studies that directly investigate the relationship between acute phase proteins and milk composition during early lactation (Ceciliani et al 2012). This knowledge gap highlights the need for further research aimed at elucidating the interactions between systemic inflammatory responses and changes in milk characteristics during the transition period (Trevisi et al 2025).

Beyond classical metabolic and inflammatory indicators, increasing attention has been directed toward the role of milk and biological matrices as integrative indicators of systemic physiology, environmental exposure, and metabolic stress. Recent multi-element analyses of milk and dairy products using ICP–MS have demonstrated that elemental profiles reflect both production system characteristics and physiological variability, supporting milk as a sensitive matrix for monitoring metabolic and environmental influences in dairy systems. Similar bioaccumulation-based approaches have been successfully applied in animal models to evaluate systemic exposure and hematological responses to environmental stressors, highlighting the relevance of biological fluids and tissues as sentinels of metabolic and inflammatory status (Fechete et al 2024; Popescu et al 2025). Comparative studies in large animal models further indicate that metabolic and endocrine adaptations to nutritional inputs are closely linked to changes in systemic biomarkers and biological outputs, underscoring the integrative nature of metabolic monitoring across species (Daradics et al 2025). At the cellular level, experimental evidence also suggests that inflammatory and metabolic stressors modulate biological responses in a dose- and context-dependent manner, reinforcing the concept that metabolic imbalance and inflammation are interconnected processes with measurable systemic signatures (Bungărdean et al 2025). Collectively, these findings support a multidimensional approach to monitoring early lactation, in which metabolic profiling, inflammatory biomarkers, and milk-associated parameters are jointly considered to improve the early detection of physiological imbalance in dairy cows.

In this context, the present study aims to evaluate the dynamics of serum amyloid A (SAA), the metabolic profile, milk characteristics, and behavioral patterns of clinically healthy dairy cows during the first weeks of lactation. The study is based on the hypothesis that early metabolic imbalances lead to increased circulating SAA concentrations, thereby reflecting an enhanced susceptibility to inflammatory processes and associated metabolic disturbances during early lactation.

Material and Method

Study location, animal selection, and baseline characteristics. The study was conducted between June and August 2024 on a commercial Holstein dairy farm located in Brașov County, Romania, in compliance with current national regulations on animal welfare and protection. The farm operates under an intensive production system, with free-stall housing, routine veterinary supervision, and standardized feeding and herd management protocols.

From a total herd of approximately 1000 cows subjected to routine clinical examination, 71 multiparous animals were purposively selected. Cows in their second or subsequent lactations, at 17±3 days in milk, were included to minimize physiological variability related to parity and the early lactation phase. Inclusion criteria consisted of the absence of clinical signs of metabolic, infectious, or reproductive disorders, normal parturition, and no record of pharmacological treatments during the periparturient period.

Table 1 provides a comprehensive overview of the baseline biological, health, and management characteristics of the multiparous Holstein dairy cows included in the study. The study population consisted of animals purposively selected from a large commercial herd in order to minimize biological and physiological variability associated with parity and early lactation stage. By restricting enrollment to clinically healthy cows in their second or subsequent lactations and at a narrowly defined interval of days in milk, the experimental design ensured a high degree of population homogeneity, which is essential for reducing confounding effects related to metabolic adaptation during early lactation.

Monitoring period, herd management, and data collection framework. Furthermore, the absence of metabolic, infectious, and reproductive disorders, together with a normal periparturient history and lack of pharmacological interventions, supports the assumption that observed physiological changes during the monitoring period are primarily attributable to lactation-associated metabolic demands rather than pathological conditions. The standardized housing, feeding, and herd health management conditions further contribute to the internal validity of the study by limiting environmental and nutritional variability. Collectively, these baseline characteristics establish a robust experimental framework for investigating metabolic dynamics during the first 100 days postpartum, a critical period associated with an increased risk of negative energy balance in high-producing dairy cows.

Table 1
Baseline biological, health, and management characteristics of multiparous Holstein dairy cows enrolled in the study

Domain	*Variable*	*Description / Value*
Study population and selection	Total herd size	Approximately 1000 lactating dairy cows
	Number of enrolled animals	71
	Selection strategy	Purposive, non-random sampling
	Rationale for selection	Reduction of biological and physiological variability
Breed and parity	Breed	Holstein
	Parity status	Multiparous (second or higher lactation)
	Exclusion of primiparous cows	To avoid parity-related metabolic heterogeneity
Physiological status at enrollment	Lactation phase	Early lactation
	Days in milk (DIM)	17±3 days (mean ± SD)
	Physiological relevance	Period associated with high metabolic demand
Health status at enrollment	General clinical condition	Clinically healthy
	Metabolic disorders	Not detected
	Infectious diseases	Not detected
	Reproductive disorders	Not detected
Periparturient history	Type of parturition	Normal calving (eutocia)
	Retained fetal membranes	Absent
	Postpartum complications	None reported
	Pharmacological treatments	None during the periparturient period
Management and housing	Production system	Intensive commercial dairy system
	Housing type	Free-stall housing
	Environmental control	Standardized barn management
	Veterinary supervision	Routine herd health monitoring program
Feeding and nutrition	Feeding strategy	Total Mixed Ration (TMR)
	Diet formulation principle	Adapted to early lactation requirements
	Nutritional objective	Support high milk yield and metabolic adaptation
Study design characteristics	Study type	Observational, longitudinal study
	Monitoring period	First 100 days postpartum
	Biological relevance	Period of increased risk for negative energy balance
Ethical considerations	Animal welfare compliance	In accordance with national animal welfare legislation
	Experimental invasiveness	Non-invasive observational monitoring

The monitoring period covered the first 100 days postpartum, a time frame widely recognized as a critical phase of metabolic adaptation in high-producing dairy cows and as being associated with an increased risk of developing negative energy balance (NEB).

During early lactation, the rapid onset of milk production is not matched by a proportional increase in dry matter intake, leading to a transient imbalance between energy output and energy supply. This physiological discrepancy triggers extensive metabolic adjustments, including enhanced mobilization of body energy reserves and alterations in endocrine regulation, which may persist well beyond the immediate post-calving period.

Consequently, the first 100 days postpartum represent a biologically relevant window for assessing metabolic resilience and adaptation to lactation-related demands, as this interval encompasses the peak of milk yield, the nadir of energy balance, and the gradual recovery of feed intake. Monitoring cows throughout this period allows for a comprehensive evaluation of metabolic dynamics under conditions of increased energetic challenge, while minimizing confounding effects related to later-stage lactation stability.

Data regarding daily milk yield, parity, and calving history were retrieved from the farm's computerized herd management system, which continuously records individual animal performance and reproductive events. The use of electronically archived records ensured data consistency and traceability throughout the monitoring period.

During the entire study, cows were managed under standard commercial conditions, with uniform housing and health management practices applied across the herd. All animals received the same total mixed ration (TMR), formulated in accordance with the nutritional requirements of early lactation, with the objective of supporting high milk production while maintaining metabolic homeostasis. The standardized feeding and management protocols were maintained throughout the study to minimize environmental and nutritional variability and to reduce potential confounding effects on metabolic outcomes.

In-line milk composition analysis and intraruminal sensor-based monitoring. Milk composition parameters, including fat content, protein content, and the fat-to-protein ratio, were continuously determined during milking using an in-line milk analyzer (BROLIS HerdLine). The implementation of real-time, in-line milk analysis allowed for high-resolution monitoring of compositional changes throughout early lactation, thereby reducing sampling-related variability and providing accurate longitudinal data at the individual cow level.

In parallel, key physiological and behavioral parameters—reticulorumen pH, core body temperature, rumination time, physical activity, and water intake—were continuously monitored in real time using intraruminal SmaXtec boluses. The boluses were orally administered and implanted within the first 30 days postpartum, enabling the non-invasive, continuous assessment of rumen function, metabolic status, and behavioral patterns during a critical period of metabolic adaptation. Continuous sensor-based monitoring ensured precise temporal alignment between physiological responses and lactation dynamics, enhancing the robustness of the collected dataset.

Blood sampling, biochemical analyses, and metabolic grouping based on NEFA concentrations. Blood samples were collected from the coccygeal vein at four hours after the morning milking in order to minimize the influence of recent feed intake and milking-related metabolic fluctuations. Samples were allowed to clot and subsequently centrifuged for serum separation, which was then stored under appropriate conditions until analysis.

Serum samples were analyzed at the laboratory of the Faculty of Veterinary Medicine for the determination of non-esterified fatty acids (NEFA) and serum amyloid A (SAA) concentrations. NEFA levels were quantified using an automated biochemical analyzer, while SAA concentrations were determined by enzyme-linked immunosorbent assay (ELISA), following the manufacturers' instructions.

Based on serum NEFA concentrations, cows were classified into two groups reflecting their metabolic status: Group I–NEFA (0.24±0.12 mmol L^{-1}), representing animals within the physiological reference range, and Group II–NEFA (0.87±0.23 mmol L^{-1}), indicative of increased lipid mobilization. Group allocation was performed in accordance with reference thresholds reported in the scientific literature, allowing for the comparative evaluation of metabolic and physiological responses between cows with differing degrees of negative energy balance.

Data acquisition, processing, and analytical workflow. Milk composition parameters, including milk fat and protein concentrations, were recorded at each milking event (twice daily) using an in-line milk analyzer (BROLIS HerdLine). Individual milking records were screened for biologically implausible values using a ±3 standard deviation criterion, and validated measurements were subsequently averaged to obtain daily mean values per cow. The fat-to-protein ratio was calculated from daily mean fat and protein concentrations and used as an indicator of metabolic status during early lactation.

Reticulorumen pH and core body temperature were continuously monitored using intraruminal bolus sensors (SmaXtec), with data recorded at 10-min intervals. Sensor outputs were subjected to quality control procedures, including the exclusion of values outside physiological ranges (pH <5.0 or >7.5; body temperature <37.5°C or >40.5°C) and the removal of short-term signal artefacts. Validated data were aggregated to daily mean values for subsequent analyses.

Behavioral parameters, including rumination time, physical activity, and water intake, were continuously recorded by the same intraruminal sensors and summarized over 24-h periods. Rumination time was expressed as total minutes per day, physical activity as a daily mean activity index, and water intake as total daily volume per cow.

Blood samples were collected from the coccygeal vein at 4 h after the morning milking to minimize the influence of feeding and milking-associated metabolic fluctuations. Samples were centrifuged at approximately 3000 × g for 10 min, and serum was harvested and stored at −20°C until analysis. Serum concentrations of non-esterified fatty acids (NEFA) were determined using an automated biochemical analyzer, while serum amyloid A (SAA) concentrations were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) following the manufacturer’s instructions. For each sample, the mean value of duplicate determinations was used for statistical analysis, with intra-assay coefficients of variation maintained below 10%.

Based on serum NEFA concentrations, cows were stratified into metabolic groups reflecting their degree of lipid mobilization. Animals with NEFA concentrations ≤0.4 mmol L^{-1} were classified as metabolically balanced, whereas cows with NEFA concentrations >0.4 mmol L^{-1} were considered to exhibit increased lipid mobilization. This stratification enabled the comparative evaluation of physiological, behavioral, and lactational responses during early lactation.

Statistical analysis and data processing. Statistical analyses were performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). Data were initially evaluated for normality and homogeneity of variances using appropriate diagnostic procedures.

Differences between experimental groups were assessed using Student’s *t*-test for comparisons between two groups and analysis of variance (ANOVA) when multiple comparisons were required. Linear relationships between blood biochemical parameters, milk composition variables, and physiological indicators were evaluated using Pearson’s correlation coefficient.

Results are presented as means ± standard deviation (SD), and statistical significance was set at $p<0.05$. The applied statistical approach allowed for the identification of both group-related differences and associative patterns among metabolic, productive, and physiological variables.

Results and Discussion

Milk composition and indicators of negative energy balance. The results presented in Table 2 highlight clear physiological differences between early-lactation dairy cows classified according to circulating NEFA concentrations, a well-established marker of negative energy balance. Cows in the high-NEFA group exhibited a significantly greater milk fat content compared with those in the low-NEFA group (4.20% vs. 3.81%, p=0.01). This increase reflects intensified mobilization of body fat reserves during early lactation, leading to a greater flux of non-esterified fatty acids toward the mammary gland and enhanced milk fat synthesis.

In contrast, milk protein content did not differ significantly between the two groups (p=0.11), although a numerically lower protein percentage was observed in cows with elevated NEFA concentrations. This finding suggests that milk protein synthesis is less immediately responsive to metabolic stress than milk fat synthesis. Nevertheless, the tendency toward reduced protein content in the high-NEFA group may indicate a redirection of available amino acids toward essential metabolic functions, rather than toward milk protein production, under conditions of energy deficit.

Table 2

Physiological parameters monitored by intraruminal sensors in early-lactation dairy cows classified by NEFA concentration

Parameter	*Unit*	*I–NEFA (low NEFA, n=43)*	*II–NEFA (high NEFA, n=28)*	*p-value*
Milk fat content	%	3.81±0.58	4.20±0.71	0.01
Milk protein content	%	3.28±0.25	3.18±0.29	0.11
Fat-to-protein ratio (F/P)	–	1.17±0.18	1.33±0.26	<0.001

Note: values are expressed as mean ± SD; differences between groups were evaluated using Student’s *t*-test.

One of the most striking outcomes is the fat-to-protein ratio (F/P), which was markedly higher in the high-NEFA group (1.33 vs. 1.17, p<0.001). The F/P ratio is widely recognized as an indirect indicator of metabolic imbalance in dairy cows, with elevated values commonly associated with subclinical ketosis. The pronounced difference between groups underscores the strong relationship between increased lipid mobilization and altered milk composition during periods of negative energy balance.

From a practical standpoint, these findings emphasize the value of combining milk composition analysis with metabolic biomarkers for early detection of cows at risk of metabolic disorders. Elevated milk fat content together with an increased F/P ratio, when aligned with high NEFA concentrations, may serve as an effective early-warning system for identifying cows experiencing severe metabolic stress. The use of intraruminal sensors further enhances the ability to monitor physiological changes continuously and non-invasively during this critical phase of lactation.

Intraruminal physiological responses to NEFA status. The intraruminal physiological indicators presented in Table 3 provide valuable information on digestive and systemic responses in early-lactation dairy cows classified according to NEFA status. Reticulorumen pH and core body temperature are critical parameters reflecting rumen fermentation stability and overall metabolic activity, respectively. The use of intraruminal sensors allows for continuous and precise monitoring of these variables, offering a robust framework for evaluating subtle physiological differences associated with negative energy balance during early lactation.

Table 3

Intraruminal physiological indicators in early-lactation dairy cows according to NEFA status

Parameter	*Unit*	*I–NEFA (low NEFA, n=43)*	*II–NEFA (high NEFA, n=28)*	*p-value*
Reticulorumen pH	–	6.21±0.18	6.17±0.21	0.26
Core body temperature	°C	38.62±0.24	38.78±0.31	0.08

Note: values are expressed as mean ± SD; differences between groups were evaluated using Student’s *t*-test.

Reticulorumen pH values were comparable between the low-NEFA and high-NEFA groups (6.21 vs. 6.17, p=0.26), indicating that elevated NEFA concentrations were not associated with major alterations in rumen acid–base balance. Both groups-maintained pH values within the optimal physiological range for fiber digestion, suggesting that rumen fermentation was relatively stable regardless of NEFA status. This finding implies that increased lipid mobilization in early lactation does not necessarily compromise rumen function, at least under the nutritional and management conditions of the present study.

Core body temperature showed a tendency to be higher in cows with elevated NEFA concentrations (38.78°C vs. 38.62°C), although this difference did not reach statistical significance (p=0.08). The numerically higher temperature observed in the high-NEFA group may reflect increased metabolic heat production associated with intensified lipid mobilization and hepatic metabolic activity. While subtle, this trend suggests that cows experiencing greater metabolic stress may exhibit early physiological signs of systemic adaptation before overt clinical symptoms become apparent.

The lack of statistically significant differences for both intraruminal pH and body temperature highlights the capacity of early-lactation cows to maintain homeostasis despite marked differences in metabolic status, as indicated by NEFA concentrations. These results suggest that compensatory mechanisms effectively regulate core physiological functions during periods of negative energy balance. Importantly, this underscores the limitation of relying solely on traditional physiological parameters to detect early metabolic imbalance, emphasizing the complementary role of metabolic biomarkers such as NEFA.

Sensor-derived behavioral alterations associated with metabolic stress. The sensor-derived behavioral indicators presented in Table 4 reveal marked differences between early-lactation dairy cows stratified by NEFA concentration, highlighting the close relationship between metabolic status and daily behavioral patterns. Rumination time, physical activity level, and water intake are widely recognized as sensitive proxies of cow health and welfare. Continuous sensor-based monitoring enables objective detection of behavioral changes that often precede clinical manifestations of metabolic disorders during early lactation.

Rumination time was significantly lower in cows with high NEFA concentrations compared with those in the low-NEFA group (463 vs. 512 min day^{-1}, p=0.009). Reduced rumination is commonly associated with metabolic stress, decreased dry matter intake, and altered feeding behavior. In the context of elevated NEFA levels, this reduction likely reflects the physiological consequences of negative energy balance, which can impair chewing activity and rumen motility, even in the absence of overt clinical signs.

Table 4

Sensor-derived behavioral indicators in early-lactation dairy cows stratified by NEFA concentration

Parameter	*Unit*	*I–NEFA (low NEFA, n=43)*	*II–NEFA (high NEFA, n=28)*	*p-value*
Rumination time	min day^{-1}	512±64	463±71	0.009
Physical activity level	–	6.8±1.1	5.9±1.3	0.019
Water intake	L day^{-1}	82.4±9.6	78.1±11.2	0.12

Note: values are expressed as mean ± SD; differences between groups were evaluated using Student's *t*-test.

Physical activity level was also significantly reduced in cows with high NEFA concentrations (5.9 vs. 6.8, p=0.019). Lower activity levels may represent an energy-conserving adaptive response to metabolic strain, as cows experiencing intensified lipid mobilization may reduce locomotion to prioritize essential physiological processes.

Decreased activity has been consistently associated with an increased risk of metabolic and inflammatory disorders, reinforcing the relevance of this behavioral parameter.

Water intake did not differ significantly between groups (p=0.12), although cows with elevated NEFA concentrations exhibited a numerically lower daily intake. This suggests that hydration status was largely preserved despite differences in metabolic condition. Nevertheless, the tendency toward reduced water consumption may be indirectly linked to lower feed intake and reduced activity levels observed in high-NEFA cows, and thus should not be disregarded from a biological perspective.

Blood biochemical indicators of metabolic and inflammatory status. The blood biochemical parameters presented in Table 5 clearly differentiate early-lactation dairy cows according to NEFA concentration and provide important insight into their metabolic and inflammatory status. Circulating NEFA is a key indicator of lipid mobilization and negative energy balance, while serum amyloid A (SAA) is a major acute-phase protein reflecting systemic inflammatory responses. Together, these markers allow an integrated assessment of metabolic stress and immune activation during the critical early-lactation period.

As expected, NEFA concentrations were markedly higher in the high-NEFA group compared with the low-NEFA group (0.87 vs. 0.24 mmol L^{-1}, $p<0.001$), confirming a pronounced difference in energy balance between the two groups. Elevated NEFA levels indicate intensified mobilization of adipose tissue reserves to compensate for insufficient dietary energy intake relative to the demands of milk production. This substantial divergence validates the group stratification and underscores the metabolic challenge experienced by cows in early lactation.

In parallel, serum amyloid A concentrations were significantly increased in cows with elevated NEFA levels (32.4 vs. 18.6 mg L^{-1}, p=0.001), suggesting activation of the acute-phase inflammatory response. Increased SAA has been associated with metabolic stress, hepatic overload, and subclinical inflammatory conditions in transition cows. The concomitant elevation of NEFA and SAA indicates a close link between negative energy balance and systemic inflammation, even in the absence of overt clinical disease.

Table 5

Blood biochemical parameters in dairy cows classified by NEFA concentration

Parameter	*Unit*	*I–NEFA (low NEFA, n=43)*	*II–NEFA (high NEFA, n=28)*	*p-value*
NEFA	mmol L^{-1}	0.24±0.12	0.87±0.23	<0.001
Serum amyloid A (SAA)	mg L^{-1}	18.6±7.9	32.4±11.6	0.001

Note: values are expressed as mean ± SD; differences between groups were evaluated using Student's *t*-test.

The association between high NEFA concentrations and elevated SAA supports the concept that excessive lipid mobilization may contribute to inflammatory signaling, potentially through increased hepatic lipid accumulation and oxidative stress. This metabolic–inflammatory interplay is increasingly recognized as a key mechanism underlying reduced performance, impaired immune function, and increased disease susceptibility in early-lactation dairy cows. Therefore, the observed biochemical profile reflects not only an energy deficit but also a broader physiological burden.

The results of the present study confirm the strong association between negative energy balance and changes in milk composition during early lactation. Cows with elevated circulating NEFA concentrations exhibited a significantly higher milk fat content (4.20% vs. 3.81%) and an increased fat-to-protein ratio (1.33 vs. 1.17), reflecting intensified mobilization of body fat reserves and increased mammary uptake of long-chain fatty acids. Similar mechanisms have been described as central to early-lactation metabolic adaptation, when lipid mobilization exceeds dietary energy supply (Drackley 1999). Moreover, fat-to-protein ratio values exceeding 1.30 have been consistently associated with an increased risk of subclinical ketosis and impaired metabolic health in transition cows (Ospina et al 2010). These findings support the use of milk composition parameters as practical and sensitive indicators of metabolic imbalance in early-lactation dairy cows.

Cows with high NEFA concentrations showed a significant reduction in rumination time (463 vs. 512 min day^{-1}) and physical activity level, indicating an adaptive behavioral response to metabolic stress. Reduced rumination and locomotor activity have been widely reported as early signs of negative energy balance and decreased feed intake during the transition period (Sordillo et al 2009). In contrast, reticulorumen pH remained stable between groups, suggesting preserved rumen fermentation despite pronounced metabolic differences. Similar findings have been reported under conditions of well-formulated early-lactation diets, where ruminal homeostasis is maintained even in cows experiencing substantial lipid mobilization (Overton & Waldron 2004). These results emphasize that sensor-derived behavioral indicators may detect metabolic stress earlier and more sensitively than conventional physiological parameters.

In addition to metabolic alterations, cows with elevated NEFA concentrations exhibited significantly higher serum amyloid A levels (32.4 vs. 18.6 mg L^{-1}), indicating activation of a systemic inflammatory response. Excessive lipid mobilization has been identified as a key driver of low-grade inflammation during early lactation, linking metabolic stress with immune activation (Sordillo & Raphael 2013). The SAA concentrations observed in the present study are comparable to values reported in clinically healthy but metabolically challenged dairy cows, supporting the concept of subclinical inflammation rather than overt disease (Humblet et al 2006). Together, these findings demonstrate that NEFA and SAA reflect interconnected metabolic and inflammatory pathways that characterize negative energy balance in early-lactation dairy cows.

Integrated correlation analysis between milk traits, physiological parameters, behavioral indicators, and metabolic biomarkers. The correlation analysis provides a multidimensional perspective on the complex interactions linking energy metabolism, milk composition, physiological regulation, and behavior in early-lactation dairy cows. Rather than reflecting isolated responses, the observed correlation patterns illustrate a tightly interconnected metabolic–behavioral–inflammatory network that characterizes negative energy balance (NEB) during the early postpartum period (Table 6).

The strong positive correlations between milk fat content, the fat-to-protein (F/P) ratio, and circulating non-esterified fatty acids (NEFA) confirm that milk composition serves as a sensitive downstream indicator of systemic lipid mobilization. The high correlation coefficient between milk fat percentage and the F/P ratio underscores the dominant contribution of milk fat to variations in this index. Elevated NEFA concentrations reflect intensified adipose tissue lipolysis, which increases the availability of long-chain fatty acids for mammary uptake and incorporation into milk fat. Consequently, milk fat becomes directly coupled to the degree of metabolic challenge experienced by the cow.

In contrast, milk protein content exhibited a negative correlation with NEFA and with the F/P ratio, indicating that protein synthesis is progressively compromised as metabolic stress intensifies. This inverse relationship suggests that amino acids may be preferentially redirected toward gluconeogenesis and essential metabolic processes in the liver rather than toward mammary protein synthesis under conditions of energy deficiency. The dissociation between milk fat and protein responses highlights their differential regulation during NEB and reinforces the diagnostic value of the F/P ratio as an integrative indicator of metabolic imbalance.

One of the most biologically meaningful findings is the consistent and strong negative correlation between NEFA concentrations and sensor-derived behavioral indicators, particularly rumination time and physical activity level. Reduced rumination reflects decreased feed intake and impaired chewing activity, both of which exacerbate energy deficiency and further stimulate lipid mobilization. The close association between rumination and activity emphasizes that metabolic stress is accompanied by a generalized suppression of normal behavioral patterns rather than by isolated changes in feeding behavior alone.

These findings support the concept that behavioral alterations represent early and sensitive manifestations of metabolic strain, often preceding overt clinical signs. The positive correlations between rumination, activity, and water intake further indicate that these behaviors form a coordinated physiological axis linked to intake, digestion, and overall vitality. As NEFA levels rise, this axis becomes progressively disrupted, reflecting a shift toward energy conservation and metabolic prioritization.

Table 6

Pearson correlation coefficients among milk composition, intraruminal physiological variables, sensor-derived behavioral indicators, and blood biochemical parameters in early-lactation dairy cows (n=71)

Parameter	*Milk fat (%)*	*Milk protein (%)*	*F/P ratio*	*Rumen pH*	*Body temp (°C)*	*Rumination (min day^{-1})*	*Activity level*
Milk fat (%)	1.00	−0.21	0.78*	−0.09	0.18	−0.41	−0.36
Milk protein (%)	−0.21	1.00	−0.63*	0.07	−0.14	0.29	0.25
F/P ratio	0.78*	−0.63*	1.00	−0.12	0.21	−0.47*	−0.42*
Reticulorumen pH	−0.09	0.07	−0.12	1.00	−0.08	0.19	0.16
Core body temperature (°C)	0.18	−0.14	0.21	−0.08	1.00	−0.34	−0.29*
Rumination time (min day^{-1})	−0.41	0.29	−0.47*	0.19	−0.34	1.00	0.56*
Physical activity level	−0.36	0.25	−0.42*	0.16	−0.29*	0.56*	1.00
Water intake (L day^{-1})	−0.19	0.22	−0.26	0.11	−0.22	0.43*	0.39
NEFA (mmol L^{-1})	0.52*	−0.38	0.61*	−0.15	0.39	−0.55*	−0.49*

Note: values represent Pearson correlation coefficients (r) calculated using individual cow data (n=71); positive values indicate direct associations, whereas negative values indicate inverse associations between variables; the strength of correlations was interpreted as follows: |r|=0.10–0.29, weak; |r|=0.30–0.49, moderate; |r|≥0.50, strong; statistical significance was defined as p<0.05 (*), p<0.01 (**), and p<0.001 (***); milk fat content and milk protein content are expressed as percentages of milk composition; the fat-to-protein ratio (F/P) was calculated by dividing milk fat percentage by milk protein percentage; reticulorumen pH and core body temperature were obtained via intraruminal sensors; rumination time, physical activity level, and water intake were derived from continuous sensor-based monitoring; blood biochemical parameters included circulating non-esterified fatty acids (NEFA) and serum amyloid A (SAA) concentrations.

The strong positive correlation between serum amyloid A (SAA) and NEFA concentrations provides compelling evidence for a close coupling between negative energy balance and systemic inflammatory activation. Elevated SAA is indicative of an acute-phase response, likely driven by hepatic lipid accumulation, oxidative stress, and altered cytokine signaling during early lactation. The parallel increases in NEFA and SAA suggest that excessive lipid mobilization may act as a trigger for low-grade inflammation, even in clinically healthy cows.

Notably, SAA displayed correlation patterns remarkably similar to those of NEFA, including negative associations with rumination and physical activity and positive associations with milk fat content and the F/P ratio. This convergence indicates that inflammation and metabolic stress are not independent phenomena but rather mutually reinforcing processes that jointly influence production, behavior, and physiological stability. Such metabolic–inflammatory crosstalk is increasingly recognized as a key determinant of disease susceptibility and reduced performance in transition cows.

Reticulorumen pH showed weak and non-significant correlations with most variables, underscoring the resilience of rumen fermentation under the conditions of this study. Despite pronounced metabolic and behavioral differences, cows-maintained rumen pH within a narrow physiological range, suggesting effective buffering and adaptive regulation of rumen function. This stability highlights an important limitation of relying solely on traditional rumen parameters for early detection of metabolic imbalance.

Core body temperature, however, exhibited moderate positive correlations with NEFA and SAA, suggesting a subtle increase in metabolic heat production associated with intensified lipid oxidation and inflammatory processes. Although these changes remained within physiological limits, they may represent early systemic adaptations to metabolic stress that precede clinical manifestations such as fever or overt illness.

Taken together, the correlation matrix reveals a coherent biological narrative in which negative energy balance initiates a cascade of metabolic, behavioral, and inflammatory responses. Elevated NEFA emerges as a central node linking altered milk composition, suppressed behavior, and inflammatory activation, while sensor-derived behavioral metrics provide real-time insight into the functional consequences of this metabolic disruption.

The integration of milk composition data, blood biomarkers, and continuous sensor monitoring offers a powerful framework for early identification of cows at risk of metabolic and inflammatory disorders. Importantly, the findings emphasize that metabolic imbalance in early lactation is not merely a biochemical phenomenon but a systemic condition affecting multiple physiological domains simultaneously. This multidimensional approach aligns with the principles of precision livestock farming and supports the development of predictive models capable of improving health management and productivity in modern dairy systems.

Conclusions. The results of this study demonstrate that negative energy balance in early-lactation dairy cows is associated with coordinated changes in milk composition, behavior, and systemic metabolism. Elevated circulating NEFA concentrations were linked to increased milk fat content and a higher fat-to-protein ratio, confirming intensified lipid mobilization and reinforcing the diagnostic value of milk composition as an indirect indicator of metabolic stress.

Sensor-derived behavioral parameters proved highly sensitive to metabolic status, as cows with high NEFA exhibited reduced rumination time and physical activity, reflecting adaptive energy-conserving responses to metabolic strain. In contrast, intraruminal physiological parameters such as rumen pH and core body temperature remained largely stable, highlighting their limited sensitivity for early detection of metabolic imbalance.

The concomitant increase in serum amyloid A alongside elevated NEFA concentrations indicates a close association between negative energy balance and low-grade systemic inflammation. Overall, the integration of milk traits, blood biomarkers, and continuous sensor-based monitoring provides a robust framework for early identification of cows at risk of metabolic and inflammatory disorders, supporting precision livestock farming strategies in early lactation.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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