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Organic Acid Supplementation in Holstein Cows: Blood Biochemistry, Colostrum Quality, Cryptosporidium Detection, and Milk Composition

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ABSTRACT: This study evaluated the effects of dietary supplementation with an organic acid blend during the transition period on the biochemical profile, colostrum quality, neonatal calf health, milk composition, and mammary health of Holstein dairy cows.

In the first protocol, 20 cows were assigned to a control group ($n = 8$) or a supplemented group ($n = 12$) receiving 40 g/day beginning 60 days before calving. A second protocol included 36 cows (18 per group) and assessed milk composition at 3 and 6 weeks postpartum and mammary health by the California Mastitis Test (CMT). Prepartum biochemical parameters, maternal serum and colostrum Brix values, and microscopic Cryptosporidium detection in neonatal calves were also evaluated.

Supplementation did not significantly affect the biochemical parameters evaluated before calving ($P > 0.05$). Maternal serum Brix values were comparable between groups, whereas colostrum Brix values were significantly lower in supplemented cows. Cryptosporidium detection at three days of age was uncommon in both groups, with no apparent reduction in calves born to supplemented cows. At the first milk sampling, supplemented cows had higher milk fat content ($P = 0.001$) and lower milk protein content ($P = 0.036$). Density, solids-not-fat, salts, and lactose did not differ significantly between groups. The proportion of CMT-positive cows was lower in the supplemented group ($P = 0.007$).

INTRODUCTION

Dairy production contributes to food supply and agricultural economies worldwide. Maintaining herd health and productive performance remains a central concern in intensive dairy systems (Ali et al., 2013).

The transition period involves profound metabolic, physiological, and hormonal adjustments in high-producing dairy cows (Dingwell et al., 2003). As calving approaches and lactation begins, nutritional requirements rise faster than feed intake can meet them. This imbalance affects the energy and protein available for colostrogenesis and subsequent synthesis of lactose, milk lipids, and milk proteins (Devant et al., 2007).

These changes may impair periparturient immune function and increase susceptibility to metabolic and infectious disorders, including subclinical mastitis (El-Zaiat et al., 2019). Colostrogenesis also involves selective transfer of immunoglobulins, particularly IgG1, from the circulation into the mammary gland (Kessler et al., 2021). Colostrum characteristics and the effectiveness of passive immune transfer affect neonatal calf health (Bartier et al., 2015). During this vulnerable period, calves may be exposed to enteric pathogens, including *Cryptosporidium parvum* (Ouakli et al., 2018).

Periparturient nutritional interventions may help support maternal metabolism, lactation, and calf health. Organic acids have therefore attracted interest as functional feed additives. Previous work has investigated their potential effects on fermentation, nutrient utilization, and dairy cow performance (Gheller et al., 2020).

This study examined associations between dietary supplementation with an organic acid blend and prepartum biochemical variables, maternal serum and colostrum Brix values, early neonatal *Cryptosporidium* detection, milk composition, and CMT status in Holstein dairy cows.

MATERIALS AND METHODS

Study area

The study was conducted between December 2023 and April 2024 on a commercial dairy farm in the Wilaya of Tizi Ouzou, Algeria. The region has a Mediterranean climate, with hot, dry summers and cool, rainy winters; mountainous areas experience colder and wetter conditions.



Location of the municipality within the wilaya of Tizi-Ouzou

Ethical approval

Additive composition

RUMITOX is a powdered feed additive for ruminants (cattle, sheep, and goats). It contains a blend of organic acids and their salts, including sodium salts, malic acid, calcium propionate, calcium formate, and calcium citrate.

Blood sampling and biochemical analysis

Blood was collected from the coccygeal vein into Vacuette® heparinized vacuum tubes and centrifuged at 3,000 rpm for 10 minutes. The recovered plasma was aliquoted and stored at -20°C until analysis at the Medical Biochemistry Laboratory of the Higher National Veterinary School, Algiers, Algeria.

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, creatinine, and total proteins were measured using commercial diagnostic kits according to the manufacturers' instructions and routine laboratory procedures. Results were expressed in the conventional units used for each parameter.

Serum and colostrum refractometric analysis

At parturition, blood samples were collected from each cow by jugular venipuncture to obtain serum for refractometric evaluation. Colostrum samples were collected from the first milking after calving. Both serum and colostrum samples were analyzed immediately after collection using a digital refractometer.

Serum Brix values were determined as an indirect indicator of circulating protein concentration, whereas colostrum Brix values were used to estimate colostrum quality. All measurements were expressed as Brix percentage (%). The refractometer was calibrated according to the manufacturer's recommendations before analysis to ensure measurement accuracy and repeatability.

Milk sampling and analysis

Individual milk samples were collected into sterile containers and analyzed immediately using a LACTOSCAN SP Ultrasonic Milk Analyzer.

Mammary health was screened using the California Mastitis Test (CMT). Cows were classified as CMT-positive or CMT-negative according to the study criterion; CMT positivity was not taken as bacteriological confirmation of intramammary infection.

Experimental design

All c healthy and had not received medical treatment before the start of the experiment.

Two experimental protocols examined different outcomes associated with organic acid supplementation: the first focused on prepartum biochemical variables, colostrum and neonatal health, and the second on postpartum milk composition and CMT status.

2.1. Experimental design of Protocol 1: Metabolic, immunological, and calf health assessment

Twenty clinically healthy Holstein dairy cows were randomly allocated to a control group ($n = 8$) or a supplemented group ($n = 12$). Housing, feeding, and routine management were the same for both groups.

Supplemented cows received 40 g/day of the organic acid blend in their diet, beginning 60 days before the expected calving date (time 1). Control cows received the same basal diet without the additive.

Blood samples were collected from all animals at two time points during the prepartum period: 60 days before calving (time 1), immediately before the start of supplementation, and 30 days before calving (time 2). Serum samples were analyzed for creatinine (CREAT), total cholesterol (CHOL), triglycerides (TRIG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total proteins (TP).

The measured parameters were used to characterize biochemical status, including markers associated with hepatic, lipid, protein, and renal metabolism during the prepartum period.

At calving, maternal blood samples were collected and evaluated using a digital refractometer. Colostrum samples obtained from the first milking after parturition were also analyzed by refractometry to estimate colostrum quality.

Fecal samples were collected from 20 calves at three days of age: eight from the control group and 12 from the supplemented group. Modified Ziehl-Neelsen staining was used for microscopic detection of *Cryptosporidium*-like oocysts, and calves were classified according to whether oocysts were observed.

2.2. Experimental design of Protocol 2: Milk composition and mammary health assessment

The second protocol involved 36 lactating Holstein cows: 18 controls and 18 cows receiving 40 g/cow/day of the organic acid blend. Housing, feeding, watering, and milking conditions were comparable between groups.

Supplemented cows received the organic acid blend with their daily ration, whereas controls received the same basal diet without the additive.

Milk composition was evaluated at 3 and 6 weeks postpartum (days 21 and 42 after calving), with three weeks between sampling points. At each time point, individual milk samples were analyzed for fat, protein, lactose, solids-not-fat (SNF), density, and mineral salts using the Lactoscan SP ultrasonic milk analyser.

Mammary health was assessed using the California Mastitis Test (CMT). Each cow was classified as CMT-positive or CMT-negative according to the predefined study criterion, and the proportion of CMT-positive cows was compared between the control and supplemented groups.

The second protocol assessed associations between supplementation, milk composition, and mammary health in lactating Holstein cows.

2.3. Statistical analysis

Data were entered and analyzed using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). Data distribution was assessed before inferential analysis. Because of the relatively small group sizes, between-group comparisons of continuous variables at each sampling time were evaluated using the Mann-Whitney U test when normality assumptions were not met; normally distributed variables were compared using the independent-samples t-test. Changes across sampling times were evaluated using the corresponding repeated-measures procedure according to data distribution. Categorical outcomes, including CMT status and *Cryptosporidium* detection, were compared between groups using Pearson's chi-square test, with Fisher's exact test used when expected cell counts were small. Results are presented as mean $\pm$ standard error of the mean (SEM), and $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Effects of organic acid supplementation on serum biochemical parameters

Biochemical results measured at time 1 and time 2 are summarized in Table 1.

Serum aspartate aminotransferase (AST) concentrations were numerically higher in supplemented cows than in control cows at time 1 (68.17 ± 5.59 vs. 61.29 ± 8.43 U/L), whereas lower values were recorded in the supplemented group at time 2 (38.26 ± 0.93 vs. 42.73 ± 3.78 U/L). However, these differences were not statistically significant ($P > 0.05$).

Similarly, alanine aminotransferase (ALT) activity tended to be lower in supplemented cows at both sampling times. At time 1, ALT values were 20.00 ± 1.84 U/L and 27.29 ± 7.55 U/L in the supplemented and control groups, respectively. At time 2, the corresponding values were 11.43 ± 0.49 U/L and 12.65 ± 0.56 U/L. No significant differences were detected between groups ($P > 0.05$).

Plasma cholesterol concentrations increased as calving approached in both groups. Although cholesterol levels were lower in supplemented cows at time 2 (1.05 ± 0.16 g/L) than in control cows (1.39 ± 0.23 g/L), the difference was not significant ($P = 0.237$).

Creatinine concentrations decreased from time 1 to time 2 in both groups. No significant treatment effect was observed at either sampling time ($P > 0.05$).

Total protein concentrations increased markedly between the two sampling periods in both groups. Nevertheless, no significant differences were detected between control and supplemented cows ($P > 0.05$).

Triglyceride concentrations were numerically higher in supplemented cows at both sampling times. However, the observed differences did not reach statistical significance ($P > 0.05$).

No statistically significant between-group differences were detected for the biochemical parameters measured during the prepartum period.

Table 1. Effects of supplementation with a mixture of OAs on blood metabolites in dairy cows

Parameters	Groups		P value
	Control	Supplemented	
AST (U/L)			
Time 1	61.29±8.43	68.17±5.59a	0.489
Time 2	42.73±3.78	38.26±0.93a	0.297
ALT (U/L)			
Time 1	27.29±7.55	20.00±1.84a	0.381
Time 2	12.65±0.56	11.43±0.49a	0.135
Cholesterol (g/L)			
Time 1	0.68±0.18 ^a	0.72±0.07	0.803
Time 2	1.39±0.23 ^a	1.05±0.16	0.237
Creatinine (mg/L)			
Time 1	8.71±0.78	9.92±0.56a	0.218
Time 2	6.69±1.25	6.63±0.97a	0.970
Total protein (g/L)			
Time 1	59.14±8.24a	62.58±3.07a	0.706
Time 2	132.58±18.00 ^a	132.52±6.98a	0.997
Triglycerides (mg/dL)			
Time 1	9.29±1.21 ^a	13.25±2.32a	0.149
Time 2	24.68±4.48 ^a	29.37±2.03a	0.294

* Control: without additive; Supplemented: 40 g/cow/day of additive.

AST = aspartate aminotransferase; ALT = alanine aminotransferase; time 1 = first sampling; time 2 = second sampling.

Values are expressed as mean ± standard error.

P < 0.05 was considered statistically significant.

3.2. Effects of organic acid supplementation on maternal immune status and colostrum quality

Maternal serum Brix values at calving were similar between groups: 10.43% in controls and 10.54% in supplemented cows.

Colostrum Brix values were higher in controls than in supplemented cows. Values ranged from 27.6% to 32.7% in controls and from 7.8% to 31.4% in supplemented cows, indicating greater variability in the latter group.

The supplementation regimen was therefore not associated with improved colostrum Brix values in this study.

Table 2. Colostrum Brix values measured by refractometry

	<i>Control group</i>	<i>Supplemented group</i>	<i>P-value</i>
<i>Serum Brix (%)</i>	10.43 ± 0.29	10.54 ± 0.55	0.546
<i>Colostrum Brix (%)</i>	30.24 ± 2.23	19.87 ± 6.88	0.001

Values are expressed as mean ± standard error.

P < 0.05 was considered statistically significant.

3.3. Effects of organic acid supplementation on neonatal calf health

Early *Cryptosporidium* detection was assessed by modified Ziehl-Neelsen staining of calf fecal samples collected at three days of age.

Microscopic examination detected oocysts in one of eight control calves (12.5%) and two of twelve calves (16.7%) from the supplemented group.

Detection was uncommon in both groups, and no apparent reduction was observed among calves from supplemented cows.

Table 3. Prevalence of *Cryptosporidium* infection

<i>Group</i>	<i>Prevalence (%)</i>
<i>Control</i>	12.5
<i>Supplemented</i>	16.7

3.4. Effects of organic acid supplementation on milk composition and mammary health

Milk composition results are presented in Table 4.

At the first milk sampling, fat content was significantly higher in supplemented cows than in control cows (3.90 ± 0.62 vs. $1.42 \pm 0.19\%$; P = 0.001). By contrast, protein concentration was significantly lower in supplemented cows (3.33 ± 0.10 vs. $3.47 \pm 0.02\%$; P = 0.036).

No statistically significant differences were detected between groups for milk density, solids-not-fat content, salt concentration, or lactose content (P > 0.05).

At the second sampling, no measured milk-composition parameter differed significantly between groups (P > 0.05). The between-group differences at the first sampling were therefore not sustained at the later assessment.

Mammary health was assessed using the California Mastitis Test (CMT). A significant difference in CMT status was observed between groups (P = 0.007). In the control group, eight cows were CMT-positive and ten were CMT-negative. In the supplemented group, one cow was CMT-positive and seventeen were CMT-negative.

Supplementation was associated with fewer CMT-positive cows during the study period. As CMT is an indirect screening test for mammary inflammation, this finding does not establish a reduction in bacteriologically confirmed intramammary infection.

Table 4. Effects of supplementation with a mixture of OAs on milk components in dairy cows

Parameters	Groups		P value
	Control	Supplemented	
Fat			
Time 1	1.42 ± 0.19	3.90 ± 0.62	0.001
Time 2	2.36 ± 0.58	2.36 ± 1.15	0.129
Density			
Time 1	34.50 ± 0.26 ^a	31.67 ± 1.52	0.114
Time 2	31.74 ± 1.33 ^a	31.15 ± 2.04	0.349
Solids-not-fat			
Time 1	9.48 ± 0.06 ^a	9.24 ± 0.27	0.368
Time 2	8.92 ± 0.23 ^a	8.64 ± 0.35	0.463
Protein			
Time 1	3.47 ± 0.02 ^a	3.33 ± 0.10	0.036
Time 2	3.27 ± 0.09 ^a	3.15 ± 0.14	0.494
Salts			
Time 1	0.77 ± 0.01 ^a	0.76 ± 0.02	0.510
Time 2	0.72 ± 0.02 ^a	0.70 ± 0.03	0.498
Lactose			
Time 1	4.82 ± 0.11	4.77 ± 0.18	0.285
Time 2	4.93 ± 0.15	4.99 ± 0.17	0.821

Time 1 = first sampling; time 2 = second sampling.

* Control: without additive; Supplemented: 40 g/cow/day of additive.

Values are expressed as mean ± standard error.

P < 0.05 was considered statistically significant.

Table 5. Results of the California Mastitis Test (CMT)

	Control	Supplemented	P value
Negative	10	17	0.007
Positive	8	1	

P < 0.05 was considered statistically significant.

DISCUSSION

The transition period requires substantial adaptations in energy, protein, and lipid metabolism as dairy cows approach lactation (Timsit et al., 2025). Increased nutritional demands may outpace dry matter intake, contributing to negative energy balance and metabolic disturbances (Stojević et al., 2005). The present study examined a blend of calcium propionate, calcium formate, calcium citrate, malic acid, and sodium salts in relation to maternal biochemical variables, colostrum Brix values, early neonatal *Cryptosporidium* detection, milk composition, and CMT status.

The blend contains compounds with different proposed physiological roles. Propionate is a glucogenic precursor in ruminants, and malic acid has been investigated for its effects on ruminal fermentation. Calcium formate and calcium citrate may affect the formulation's acid and mineral properties. The study, however, cannot separate the effects of individual ingredients (Liu et al., 2010).

According to the literature, the assessment of key milk components, particularly fat, protein, and lactose, provides a non-invasive and informative approach to monitoring the health status and productivity of dairy herds (Antanaitis et al., 2023; Atasever and Stádník, 2015; Rocchetti and O'Callaghan, 2021). These milk parameters can also serve as useful indicators of the energy balance of dairy cows. A decrease in milk protein and lactose concentrations, combined with an elevated fat-to-protein ratio, may indicate a negative energy balance or energy deficit (Sakowski et al., 2012). Under normal conditions, milk fat content generally ranges from 3.5 to 5%, whereas protein content typically varies between 3.0 and 3.5% (Cauty and Perreau, 2009).

Furthermore, the milk fat-to-protein ratio (F:P) is a useful indicator of the health and metabolic status of dairy cows and can help identify animals experiencing an energy deficit (Antanaitis et al., 2023; Bunevski et al., 2020; Buttchereit et al., 2010; Jenkins et al., 2015). An optimal F:P ratio is generally considered to range between 1.2 and 1.4 (Čejna and Chládek, 2005). However, a ratio above 1.5 has been associated with an increased risk of metabolic and health disorders, including ketosis, displaced abomasum, lameness, and mastitis (Heuer et al., 1999; Klein et al., 2019). Conversely, an F:P ratio below 1.1 may indicate subacute ruminal acidosis (Đuričić et al., 2020).

No significant between-group differences were observed for AST, ALT, total proteins, triglycerides, cholesterol, or creatinine. Timsit et al. (2025) similarly reported no significant changes in AST or creatinine when evaluating an additive containing organic acids and a mycotoxin scavenger in early-lactation cows. Differences in formulation, sampling schedules, and physiological stage limit direct comparisons.

The absence of significant changes observed in this study is also consistent with the findings of Patel et al. (2024), who investigated dietary malic acid supplementation in lactating Kankrej cows. Supplementation with 30, 60, or 90 g/day of malic acid did not significantly affect serum total protein, creatinine, cholesterol, ALT, or AST. These results are particularly relevant because malic acid was one of the components of the organic acid mixture used in the present experiment. The authors similarly concluded that malic acid supplementation did not adversely affect the biochemical status of the cows. Kankrej cows. Supplementation with 30, 60, or 90 g/day of malic acid did not significantly affect serum total protein, creatinine, cholesterol, triglycerides, ALT, or AST. These results are particularly relevant because malic acid was one of the components of the organic acid mixture used in the present experiment. The authors similarly concluded that malic acid supplementation did not adversely affect the biochemical status of the cows.

Taken together, the biochemical findings indicate no statistically detectable difference between groups for the parameters measured in this study. They do not, however, establish biological equivalence or exclude effects on metabolic pathways that were not measured.

Maternal serum Brix values were similar between the control and supplemented groups ($10.43 \pm 0.29\%$ and $10.54 \pm 0.55\%$, respectively; $P = 0.546$), indicating that supplementation did not significantly modify this refractometric parameter under the conditions of the present study. By contrast, colostrum Brix values were significantly lower in supplemented cows than in control cows ($19.87 \pm 6.88\%$ vs. $30.24 \pm 2.23\%$; $P = 0.001$). This unexpected finding should be interpreted specifically as a difference in Brix-based estimation of colostrum quality and not as direct evidence of lower colostral immunoglobulin concentration (Kessler et al., 2021).

Brix refractometry is an indirect indicator of colostrum quality and is often used to estimate IgG concentration (Bartier et al., 2015). It does not directly quantify immunoglobulins. Accordingly, lower Brix values should not be interpreted as a demonstrated reduction in colostral IgG (Devant et al., 2007).

Fecal examination of newborn calves at 3 days of age detected *Cryptosporidium* in 1/8 calves (12.5%) in the control group and 2/12 calves (16.7%) in the organic-acid-supplemented group. Thus, no apparent reduction in *Cryptosporidium* detection was observed following maternal supplementation. These prevalence values were relatively low compared with those reported in several epidemiological studies of dairy calves. A worldwide meta-analysis estimated an overall prevalence of *C. parvum* of 21.9% in dairy calves, increasing to 24.9% in pre-weaned calves and 33.6% in calves with diarrhea (Chen et al., 2023). Studies conducted in Algeria have also reported substantially higher prevalence values. For example, a study of bovine cryptosporidiosis in three regions of Algeria reported an overall prevalence of 52.2% (Ouakli et al., 2018). The lower colostral Brix observed in supplemented cows also cannot be directly linked to the slightly higher proportion of positive calves in this group. Brix provides an indirect estimate of colostral IgG concentration and was not used here as a direct measurement of passive immunity (Gheller et al., 2020).

The study found a significant effect of organic acid supplementation on milk fat and protein concentrations at the first sampling. Milk fat content was significantly higher in supplemented cows than in control cows ($3.90 \pm 0.62\%$ vs. $1.42 \pm 0.19\%$; $P = 0.001$), whereas milk protein concentration was significantly lower in supplemented cows ($3.33 \pm 0.10\%$ vs. $3.47 \pm 0.02\%$; $P = 0.036$). No statistically significant differences were detected for lactose, solids-not-fat, density, or salt content. These findings suggest that the response to organic acid supplementation was selective rather than associated with a generalized change in milk composition (Orellana Rivas et al., 2021).

The fat-to-protein ratio (F:P) provides additional information for interpreting changes in milk composition and can be used as an indirect indicator of the metabolic status of dairy cows. In this study, the F:P ratio differed markedly between groups at the first sampling, reaching approximately 0.41 in control cows and 1.17 in supplemented cows. Previous studies have proposed an F:P ratio of approximately 1.2-1.4 as a desirable range, although interpretation depends on lactation stage and production conditions (Čejna and Chládek, 2005). Ratios above approximately 1.5 have been associated with an increased risk of negative energy balance and metabolic disorders such as ketosis, whereas very low ratios may be associated with milk fat depression and altered ruminal fermentation (Heuer et al., 1999; Butchereit et al., 2010; Antanaitis et al., 2023). According to these reference ranges, the F:P ratio of the supplemented group (1.17) was close to the commonly reported optimal range, whereas the control group showed a considerably lower ratio (0.41) (Antanaitis et al., 2023).

At the second sampling, no significant between-group differences in milk composition were detected. The difference in F:P ratio observed at the first sampling may therefore have been transient. Changes in feed intake, energy partitioning, lipid mobilization, and mammary activity during early lactation provide relevant physiological context (Antanaitis et al., 2023).

CMT status differed between groups: eight of 18 control cows and one of 18 supplemented cows tested positive ($P = 0.007$). This association may be relevant to mammary health, but the CMT cannot establish infection status without complementary somatic cell counts and bacteriological testing (Dingwell et al., 2003).

The results are also broadly consistent with studies investigating propionate-based supplementation. Zhang et al. (2022) evaluated calcium propionate supplementation in early-lactation Holstein cows and reported changes in milk-related and metabolic outcomes. Together with previous work on organic-acid-based products, these observations support the possibility that propionate-containing supplements may influence metabolic responses. However, differences in formulation, dose, physiological stage, and study design limit direct comparisons among studies (Ali et al., 2013).

Several limitations restrict interpretation: modest group sizes, no direct measurement of colostral IgG, a single early *Cryptosporidium* sampling time, and CMT assessment without somatic cell counting or bacteriology. Differences in some milk variables at the first sampling were not maintained at the second. Larger studies with more specific metabolic, immunological, and mammary-health measures are needed.

CONCLUSION

Supplementation with the calcium propionate–calcium formate–calcium citrate–malic acid blend was not associated with significant differences in the measured prepartum biochemical parameters or reduced early *Cryptosporidium* detection in

calves. Lower colostrum Brix values, early differences in milk fat and protein composition, and a lower proportion of CMT-positive cows were observed in supplemented animals. These outcome-specific findings require confirmation in larger studies with direct measures of colostrum immunoglobulins and intramammary infection.

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