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Early lactational response to rumen-protected methionıne supplementation in dairy buffalo fed forage-based diets

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Abstract

This study determined the effects of rumen-protected methionine (RPMet) supplementation on milk yield and milk composition in early lactation dairy buffalo fed a forage-based diet. A total of 20 lactating, multiparous Anatolia water buffalo were used, with a mean initial milk yield of 4.63 ± 0.52 kg/d, 42.75 ± 6.21 days in milk, and a body weight of 452.8 ± 28.92 kg. The buffalo were separated into two groups, each containing 10 animals of similar body weight, parity, days in milk, and milk yield. The first group (CON) received a ration without feed additives as a control, whereas the second group (RPMet) was fed the control diet supplemented with 10 g/head/day of RPMet. The buffalo were housed in two separate free-stall barns. The experiment lasted for nine weeks, including one week for adaptation and eight weeks for the trial period. Experimental animals were fed a total mixed ration with a forage to concentrate ratio (R:C ratio) of 70:30 containing 15% crude protein and 1.72 Mcal/kg NE_L. Daily milk yield was recorded for individual animals and milk samples were collected at 2-w intervals. The supplementation with 10 g/d of RPMet considerably increased milk yield (+ 0.16 kg/d), FCM (+ 0.66 kg/d), energy-corrected milk (+ 0.74 kg/d), milk protein yield (+ 0.02 kg/d), and milk fat yield (+ 0.05 kg/d), but did not affect milk lactose and SNF yield. A higher milk protein percentage was obtained with RPMet compared to the CON (0.23% vs.0.21); fat, lactose, and solid non-fat were similar. Early lactation RPMet supplementation beneficially affects milk yield, milk protein content and yield, and milk fat yield. Further studies are needed to assess the efficiency of RPMet under different dietary conditions in lactating buffalo.

Key Words: Anatolian water buffalo, milk performance, rumen-protect methionine
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Introductıon

Buffalo possess remarkable adaptability and resilience, allowing them to thrive in diverse environments characterized by varying topography, climates, vegetation, and disease challenges. This exceptional trait makes them invaluable contributors to numerous rural economies. Despite their widespread presence worldwide, only 0.2% of water buffalo can be found in Europe, with 93% concentrated in Italy (Minervino *et al.,* 2020). The significance of buffalo in Italy is further highlighted by their substantial contribution to the country's milk production. Italy ranks sixth globally in milk production, with an impressive output of 257,460 tons (FAOSTAT, 2021). One of the prime examples of this milk's economic importance is the production of high-quality mozzarella, which is an economically-important commodity (Borghese, 2017). As per TUIK data (2021), the Anatolian buffalo population in Turkey decreased to 85,000 head in 2020. However, through the implementation of the National Anatolian Water Buffalo Project by the Ministry of Agriculture and Forestry starting in 2011, the number saw a

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significant increase, reaching 185,000 head by 2021. Within the scope of the project, it was aimed to improve on 1200 kg of milk in 250 days of lactation (Ozturk *et al.,* 2022). As a result of the National Anatolian Water Buffalo Project, there has been a trend in dairy buffalo breeding to use diets consisting of corn silage, legume forages, and small amounts of concentrates throughout the year instead of diets based on poor-quality roughages to maintain production over the entire lactation (Muruz & Selçuk, 2019). Although these diets have positive impacts on feed costs and reduce environmental impacts associated with dairy farming (Kim & Lee, 2021), they can contribute to lower levels of economic efficiency and profitability (Ozturk *et al.,* 2022). Such diets can cause production losses due to insufficient amino acids (AA), especially methionine (Met), the first limiting essential AA, thus limiting milk protein synthesis and milk production (NRC, 2001). The requirements of the lactating dairy buffalo must be met without excessive protein feeding (Kim & Lee, 2021). The addition of a rumen-protected Met (RPMet) form of this AA to the diet may be a useful strategy to improve lactation performance (Awawdeh, 2016). As observed in previous studies with dairy cattle (Broderic *et al.,* 2008; Patton, 2010; Zanton *et al.,* 2014), the addition of rumen-protected Met (RPMet) to dairy diets has been associated with enhanced milk production, increased milk protein content, and higher milk protein yield. Consequently, incorporating RPMet supplementation into the feeding regimen enables the use of diets with reduced crude protein (CP) content without compromising milk and protein yields (Broderic *et al.,* 2008). While positive effects of feeding RPMet to lactating dairy cows have been observed, it is unclear if similar outputs can be achieved in buffalo, particularly in early lactation.

Limited research has focused on feeding RPMet, specifically during the early lactation period, to assess milk performance in dairy buffalo (Rathwa *et al.,* 2022). The objective of this study was to determine the effects of protected Met supplementation on early lactation performance in buffalo maintained on forage-based diets. This study tested the hypothesis that feeding RPMet would enhance milk performance in early-lactation dairy buffalo.

Material and Methods

Ondokuz Mayıs University Local Animal Ethics Committee approved all experimental procedures for the present study (Protocol No. 2023/41). The study was conducted at a commercial dairy buffalo farm in the Tokat district of Turkey. This investigation used 20 early lactating Anatolian water buffalo with body weights (BW) of 452.8 ± 28.92 kg, initial milk yield of 4.63 ± 0.52 kg/day and parities of 2.85 ± 0.74. All the experimental buffalo were selected at 42.75 ± 6.21 days in milk. Buffalo in the experiment were separated into two equal groups, each containing 10 animals with similar body weight, parity, days in milk, and milk yield. The first group (CON) was fed a ration without feed additives as a control; group two (RPMet) was fed the control ration supplemented with 10 g/head/day of the RPMet. The basal mixed ration was offered *ad libitum* twice daily (at 07:00 and 18:00) with free access to fresh drinking water. The experiment lasted for 9 w, consisting of 1 w of adaptation and * w of data collection. Buffalo used in this study were kept in one of two identical free-stall pens. Stalls were bedded with rubber alley mats. In this study, individual feeders were not used, instead, animals were fed in their respective experimental groups. Individual headlocks were used to guarantee RPMet consumption at feeding time. For this reason, buffalo were adapted to the headlocks using self-locking feed stanchions during the adaptation period.

The basal diet consisted of a 70:30 forage:concentrate (Table 1). The basal diet was formulated to meet or exceed the nutrient requirements for lactating buffalo of 463 kg of body weight, 5.0 kg/d of milk yield, and 8.80% milk fat and 4.5% milk protein (NRC, 2001). The source of RPMet used was Smartamine M (75% DL-Met and 80% bioavailability; Adisseo Inc., Antony, France). The dose of RPMet (10 g/animal/day) was mixed into 40 g of wheat bran per buffalo daily as a carrier. The buffalo were administered 50 g of wheat bran per buffalo daily as a placebo in the control group. Both RPMet and placebo were individually top-dressed on the fresh, basal mixed ration. To ensure that buffalo could not consume RPMet from adjacent feed bunks due to group feeding, they were locked to the headlocks of the feed bunk for an average of 30 min at feeding time. Thus, the animals were monitored to consume RPMet individually. If the buffalo did not voluntarily enter the feed bunk headlocks, the researchers gently guided them. All buffalo had no *ad libitum* access to water during this time.

Feed allowance and refusal amounts were recorded daily to calculate the group feed intakes. Basal mixed ration samples were collected for two consecutive days weekly to determine dry matter (DM) content. Feed samples were taken and dried at 60 °C for 48 h. At the end of the experimental period, feed samples were pooled, and representative samples were taken for further chemical analysis. Samples were ground through a 1-mm screen in a Wiley mill and subjected to chemical analysis using appropriate methods specified by AOAC (2005) and Van Soest *et al.* (1991).

The dairy buffalo were milked twice daily at 06:30 and 18:30. Milk yields were recorded at each milking. Milk samples were collected individually every 2 w from two milkings conducted on the same day. Raw milk samples were immediately chilled to 4 °C and then analyzed to determine milk protein, fat, lactose, and solid no-fat (SNF) contents (MilkoScan FT120 instrument, Foss Electric, Denmark) on the same day, preserving cold chain. Milk component yields of dairy buffalo were calculated by multiplying the percentages of components sampled every two weeks by the amount of milk produced at milking. Fat-corrected milk (FCM) standardized to 4% fat and energy-corrected milk (ECM) calculations were conducted using the following equations recommended by the NRC (2001).

$$FCM = [0.4 \times milk\ yield\ (kg)] + [12.86 \times milk\ fat\ (kg)] \quad (1)$$

$$ECM = [0.3246 \times milk\ yield\ (kg)] + [12.86 \times fat\ yield\ (kg)] + [7.04 \times protein\ yield\ (kg)] \quad (2)$$

Table 1 Ingredients and nutrient compositions of the experiment basal diet

Ingredient, g/kg of dry matter	**Quantity**
Corn silage	244.74
Alfalfa hay	194.48
Wheat straw	265.13
Corn grain	127.76
Wheat bran	19.01
Soybean meal	135.77
Limestone	7.28
Salt	3.46
Vitamin and trace minerals[1]	2.19
Nutritional composition[2], g/kg of dry matter	
CP[2]	142.75
ADF[2]	274.45
NDF[2]	449.22
Ash[2]	70.70
Starch[3]	168.11
NE_L[3], Mcal/kg	1.38

CP: Crude protein; RDP: Rumen degradable protein; RUP: Rumen undegradable protein; ADF: Acid-detergent fibre; NDF: Neutral detergent fibre; MP: Metabolizable protein; NE_L: Net energy lactation
[1]Cu 0,14 mg; Mn 12 mg; Zn 45 mg; Se 0,15 mg; I 0,72 mg; Co 0,14 mg; Vit A 9.000 IU; Vit D3 1800 IU
[2]Laboratory analysis
[3]Calculated from NRC (2001)

All statistical analyses were performed using IBM SPSS Statistic 25. The means between different groups were determined using independent *t*-tests. Differences were considered significant at $P < 0.05$

Result and Discussion

In this study, RPM was supplemented to a forage-based diet of dairy buffalo. The objective was to determine the impact on milk production performance. In the present experiment, the DMI average of the group-fed cows in each treatment was not affected ($P > 0.05$) by treatment (14.47 kg/d for CON vs. 14.80 kg/d for RPMet; Table 2). Whereas some studies have not observed any significant impact on DMI (Chen *et al.,* 2011; Lee *et al.,* 2015), others have reported an increase in DMI (Zhou *et al.,* 2016; Batistel *et al.,* 2017) due to supplementation of protected Met. In these particular studies, the observed increases in DMI were partially attributed to a reduction in inflammation and oxidative stress, leading to improvements in immunometabolic status and liver function (Osorio *et al.,* 2014; Zhou *et al.,* 2016). Additionally, Patton (2010) suggests that various factors such as the level of RPMet supplementation, the presence of potentially co-limiting AAs, the duration of the feeding period, and the stage of lactation could all potentially influence feed intake and, consequently, may confound the results related to DMI.

The data related to milk yield and milk composition are presented in Table 2. The supplementation with 10 g/d of RPMet considerably increased milk yield (+ 0.16 kg/d), FCM (+ 0.66 kg/d), and ECM (+ 0.74 kg/d). Figure 1 illustrates the lactation curves, depicting the average weekly milk yields in relation to the treatments administered over the 8-w experimental duration. Particularly noteworthy is the rapid and pronounced response of the cows to Met supplementation, which was similar to the result reported by Junior *et al.* (2021), where there was a progressive response with advancing weeks. This observation suggests that Met served as the primary limiting AA. Many researchers reported inconsistent results concerning the effect of RPMet supplementation on milk yield.

Table 2 Effect of RPMet supplementation on feed intake, milk yield, and composition in dairy buffalo

Item	**Treatment**[1]		***P-value***
	CON	**RPMet**	
Dry matter intake[2], kg/d	14.47 ± 1.60	14.80 ± 1.77	0.272
Milk production, kg/d			
Milk yield	4.92 ± 0.37	5.08 ± 0.45	<0.001
FCM[3] yield	7.33 ± 1.00	7.99 ± 0.99	0.004
ECM[4] yield	8.51 ± 1.07	9.25 ± 1.04	0.002
Milk composition, %			
Fat	8.57 ± 1.22	8.83 ± 1.19	0.331
Protein	4.50 ± 0.04	4.54 ± 0.05	<0.001
Lactose	4.85 ± 0.76	4.85 ± 0.42	0.970
SNF[5]	10.07 ± 0.90	10.14 ± 1.48	0.785
Milk component yield, kg/d			
Fat	0.41 ± 0.07	0.46 ± 0.07	0.012
Protein	0.21 ± 0.01	0.23 ± 0.01	0.001
Lactos	0.23 ± 0.04	0.25 ± 0.02	0.057
SNF[5]	0.49 ± 0.08	0.51 ± 0.05	0.368

[1]CON = Control diet; RPMet = Control diet and administration of RPMet (10 g/head/d)
[2]Statistics were not possible due to group feeding
[3]Fat-corrected milk (4%)
[4]Energy-corrected milk (kg/d)
[5]Solid non-fat
P <0.05 is considered statistically different

Results of milk production were in line with the findings of Lee *et al.* (2012) who concluded that the supplementation of Met to the diet of lactating cows had a marked effect on milk production as a consequence of increased DMI. Moreover, Wang *et al.* (2010) reported improved milk protein yields and nitrogen utilization efficiency by improving AA balance in metabolizable proteins and reducing the deamination of absorbed AAs. Dairy cow diets supplemented with Met can substantially improve milk production by improving DMI and liver function (Batistel *et al.* 2017). In contrast to with our results, no substantial impact on milk yield was observed by Guo *et al.* (2023), who found that buffalo receiving RPMet consumed less DMI when compared to buffalo receiving a basal diet. Yang *et al.* (2010) reported that as the diet went from a deficit (control) to adequate (42 g/d of RPMet) and excessive (56 and 70 g/d of RPMet) Met levels, AA imbalance led to a negative effect on milk yield.

It is generally accepted that milk volume is regulated by lactose synthesis; as lactose concentration is relatively constant, increased lactose synthesis will increase milk yield (Sadovnikova *et al.,* 2021). Milk lactose concentration was not affected by RPMet supplementation in this trial. Swanepoel *et al.* (2020) suggested that metabolizable protein deficit limited milk production due to lower availability of microbial CP. Therefore, in our study, there are several potential reasons for the increased milk yield with the supplementation of RPMet. One possibility would be that milk yield was increased as a consequence of increased effective utilization of metabolizable protein (NRC, 2001). Another option would be that Met supplementation helped to optimize the utilization of energy available (Cardoso *et al.,* 2021). A third explanation would be that Met can activate protein translation by binding to mTOR (Appuhamy *et al.,* 2012). Thus, the supplementation of Met may enhance the overall efficiency of the utilization of AAs for protein synthesis, potentially contributing to increased milk yield (Junior *et al.,* 2021). Differences in milk yield among studies might be attributed to the degree to which Met was limiting in the diets fed, an excess of AAs, an imbalance in AAs, increased intake of compounds used to protect the AAs from ruminal degradation, or other factors (Lara *et al.,* 2006).

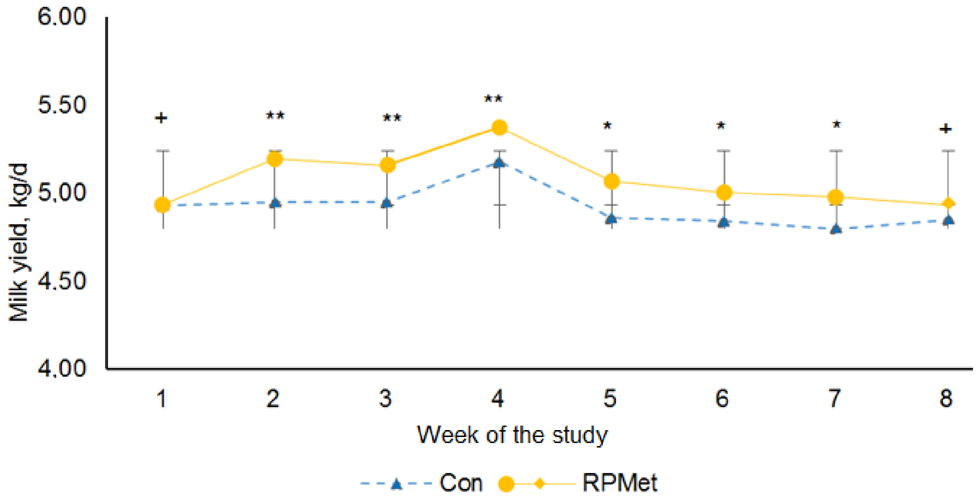

Figure 1 Effect of rumen-protected methionine (RPMet) supplementation on milk yield of Anatolian dairy buffalo during the experimental period. Values are expressed in average weekly milk yield. ** P <0.01; * P <0.05; + not significant

Supplementing the diet of dairy cows with RPMet is typically linked to an enhancement in milk protein concentration, yield, or both (Patton, 2010; Zanton *et al.,* 2014), the primary variables examined in this study. The results of the present study confirmed our hypothesis, as Met supply led to an increase in milk protein percentage (4.50 vs. 4.54%, P <0.001) and yield (0.21 vs. 0.23 kg/d, P <0.01, Table 2). In the study of Tauqir *et al.* (2022), the response of buffalo to milk protein percentage was observed immediately after the first week of RPMet supplementation. Some research studies have reported positive effects of Met supplementation on milk production parameters. For example, a study by Yoder *et al.* (2020) observed that supplementing Met improved milk protein yield and milk protein percentage in dairy cows. Our findings are in line with earlier studies (Patton, 2010; Ardalan *et al.,* 2021; Junior *et al.,* 2021). These studies confirmed the benefits of dietary RPMet supply on the milk protein content of dairy cows.

Younge *et al.* (2001) reported that RPMet supplementation increased milk protein content since cows fed diets deficient in Met were digested in the small intestine. In the present study, RPMet increased milk protein content, which may indicate that our CON diet was deficient in Met. Besides serving as direct precursors for protein synthesis, AAs also function as regulators of protein synthesis. In the current study, the increases in milk protein concentration in response to RPMet supply might be associated with the activation of mTOR, a serine/threonine kinase that plays a central role in regulating protein synthesis and cell growth (Appuhamy *et al*., 2012). Because there was an adequate intake of Met in the diet, Strezetelski *et al.* (2009) observed no substantial variations in the monitoring of milk protein content across different treatments. Several studies have reported no effect on milk protein yield but observed changes in milk protein percentage (Trinacty *et al.,* 2009; Chen *et al.,* 2011; Quintero & Olivera-Angel, 2019; Toledo *et al.,* 2021; Tauqir *et al.,* 2022). Our study found that milk protein yield and percentage increased substantially by +0.02 kg/d and +0.04%, respectively. The differences observed in the results between these studies may be attributed to variations in the balance of essential AAs in the diet and the specific bioavailability of RPMet.

In the present study, buffalo fed the RPMet diet had a higher milk fat yield (+0.05 kg/d), likely due to higher milk yield and numerically higher milk fat percentage than cows fed the CON diet (Table 2). Similarly, a study by Junior *et al.* (2021) demonstrated that Met supplementation increased milk fat yield in dairy cows, but there was no effect on milk fat percentage. Previous studies, including those conducted by Patton *et al.* (2015), Lee *et al.* (2012), Zang *et al.* (2017), Yoder *et al.* (2020), and Li *et al.* (2022), have indicated that Met supplementation generally has a limited impact on milk fat production. Several studies investigating the supplementation of RPMet to lactating cows have demonstrated a consistent improvement in milk fat percentage (Toledo *et al.,* 2021) and yield (Zhou *et al.,* 2016; Batistel *et al.,* 2017; Swanepoel *et al.,* 2020; Cardoso *et al.,* 2021). According to the findings of Bionaz and Loor (2008) and Martinov *et al.* (2010), the influence of Met on milk fat synthesis can be attributed to its ability to enhance the activity of lipogenic gene networks and alter the expression of critical miRNA molecules involved in regulating the balance of lipid production. However, the present study demonstrated that RPMet does not substantially affect the milk fat percentage. The lack of change in milk fat percentage with Met supplementation may be related to the metabolic pathways of Met and its methylated compounds (Benefield *et al.,* 2009). In addition to its role as a precursor for protein synthesis, Met may serve as a methyl donor in the synthesis of choline, a crucial component for generating phospholipids necessary in the formation of chylomicrons and very low-density lipoproteins (Benefield *et al.,* 2009). Secondly, Met may contribute to the *de novo* production of short and medium-chain fatty acids within the mammary gland, thus increasing the fat content in milk (Wei *et al.,* 2022). In the current study, perhaps most of the absorbed Met was used for protein synthesis rather than choline/lipid synthesis and thus did not benefit milk fat concentration. Guretzky *et al.* (2006) reported that the inhibition of choline synthesis from Met reduced milk and milk fat yields.

The present study found no difference between the milk lactose and NNF yields and percentages between the CON and RMet groups (Table 2). Lactose is very stable in milk ingredients and is not sensitive to other factors (Wei *et al.* 2022). In the current study, RPMet did not affect the lactose yield and percentage in milk, which is consistent with the results of Yoder *et al*. (2020). However, other studies have shown that RPMet supplementation and a reduction in dietary CP can increase milk lactose content (Broderick *et al.,* 2008; Nursoy *et al.,* 2018). The higher milk lactose percentage observed by Rathwa *et al.* (2022) was attributed to higher blood glucose levels in RPMet which helps in mammary lactose biosynthesis. Most of the glucose required for lactose synthesis in the mammary gland is derived from gluconeogenesis (Aschenbach *et al.,* 2010) and a glycogenic AA such as Met is not prioritized for glucose production, and the quantitative contribution of EAA to glycogenic carbon is minimal (Larsen & Kristensen, 2013). This may explain why Met did not affect milk lactose production in our study.

Under the conditions of this experiment, the lack of a significant difference in SNF percentage relative to those fed the CON diet may be because of the observed similar lactose percentage obtained with the RPMet diet (Junior *et al.,* 2021). In the experiment of Rathwa *et al.* (2022), with buffalo fed concentrate and green and dry fodder-based total mixed diet, supplementation of RPMet increased milk SNF percentage due to higher milk protein and lactose percentage. Discrepancies in the influence of additional Met on the production of milk lactose and SNF can be ascribed to factors such as fluctuations in nutritional circumstances, dietary composition, and the distinct physiological traits of the animals.

Conclusions

The results showed that feeding RPMet increased milk yield, protein percentage, and protein yield. However, adding RPMet did not substantially affect the percentages and yields of fat, lactose, and SNF during early lactation. Based on these findings, it can be concluded that early lactation RPMet supplementation has a beneficial effect on milk yield, milk protein content and yield, and milk fat yield. However, further studies are needed to assess the efficiency of RPMet under different dietary conditions in lactating buffalo.

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Author Contributions

HM (ORCID: 0000-0002-1975-4545) conceptualization, data collection, methodology, analysis, writing, and original draft preparation; SÇ (ORCID: 0000-0002-9784-5469) data collection, methodology, and review; ZS (ORCID:0000-0002-6060-4514) Laboratory analysis, writing, and editing; BB (ORCID: 0000-0002-0732-0192) laboratory analysis; SE (ORCID: 0000-0003-3392-2792) laboratory analysis.

Conflicts of Interest

The Authors declare that there is no conflict of interest

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