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Effect of a high forage : concentrate ratio on milk yield, blood parameters and oxidative status in lactating cows

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Abstract. A feeding strategy that requires a forage : concentrate ratio equal to 70 : 30, with at least five different herbs in the forage and the use of silages prohibited, has recently been introduced in Italy. Despite the benefits in terms of human health (lower ω6 : ω3 ratio, higher conjugated linoleic acid level) of the obtained milk, little information regarding the possible effects on cows' health is available. The aim of this study was to evaluate the effects of such a feeding strategy in dairy cows (90 days in milk at the beginning of the trial) on milk yield and composition, and blood metabolic profile, including the evaluation of oxidative stress. The proposed feeding strategy, compared with a semi-intensive strategy, resulted in an improvement of animal oxidative status (lower levels of reactive oxygen metabolites, higher levels of antioxidant potential and anti-reactive oxygen metabolites) and a significant increase of milk urea only in the first part of the trial. No differences in milk yield and composition were detected throughout the trial.

Additional keywords: animal health, dairy cows, dairy milk yield.

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Introduction

A feeding strategy for lactating dairy cows has recently been introduced in Southern Italy and is now spreading across the whole country. Such a feeding strategy includes several rules to reach more natural feeding (Rubino 2014): the cows must have free access to outdoor paddocks; the forage : concentrate ratio must be at least 70 : 30; and the forage must contain at least five different herbs, and silages are prohibited. The aim is to obtain no negative effects (i.e. low ruminal pH, subclinical acidosis) on cow metabolic homeostasis and the improvement of milk nutritional value by lowering the ω6 : ω3 ratio and by increasing conjugated linoleic acids (CLAs; Hanuš *et al.* 2018). Due to the increasing interest of consumers for healthy foods, the fatty acid profile is considered one of the most important parameters to determine their nutritional value. Particularly, the ω3 and ω6 fatty acids, being essential for humans, must be provided through the diet, and should contain a ω6 : ω3 ratio within 2.1 and 4.1 (Simopoulos 2002). Similarly, high importance has been given to the CLAs that are reported to have immune-modulating, anti-carcinogenic and anti-atherosclerosis properties (Pastushenko *et al.* 2000; Whigham *et al.* 2000). Actually, there is no recommended human intake for CLAs; most studies indicated dosages varying from 0.7 g/day to 6.8 g/day (Benjamin and Spener 2009). Animal diet is recognised as the most important factor determining the fatty acid profile of cow milk. The ω6 : ω3 ratio in milk from cows fed a high percentage of forage, mainly due to the increase of ω3 fatty acids, is closer to the recommended ratio for human health (Ellis *et al.* 2005), whereas such a ratio is more than two times higher (Simopoulos 2002) in the milk produced in intensive dairy farms, where animals' diets have a lower forage : concentrate ratio (Harvatine *et al.* 2009). Similarly, the CLA levels in the milk of sheep (Meluchová *et al.* 2008), cows (White *et al.* 2001) and goats (Tsiplakou *et al.* 2006; D'Urso *et al.* 2008; Tudisco *et al.* 2010, 2012; 2014) are known to be significantly higher when animals are fed with fresh forage compared with the total mixed ration. Compared with the fresh forage, the hay, as well as the silage, shows a decrease of PUFA content due to the oxidative phenomena during the storage process (Chilliard *et al.* 2007). Therefore, milk nutritional value can be improved by increasing the forage : concentrate ratio. Such an

increase affects the rumen and metabolic status (i.e. increase of rumen pH) (Bjerre-Harpøth *et al*. 2012) and, consequently, the nutrient supply used for synthesis of milk components, by improving rumen microbiome activity (Aguerre *et al*. 2011). A high proportion of concentrates increases DM and digestible carbohydrate intake, but, in contrast, it reduces fibre digestibility, altering volatile fatty acid patterns (Dixon 1986). In addition, it reduces chewing activity, and hence saliva production, decreasing rumen pH (Yang and Beauchemin 2009; Lechartier and Peyraud 2010), thus increasing the risk of subclinical or clinical rumen acidosis (Wang *et al*. 2013). From the above, it is clear that, when increasing the forage : concentrate ratio, several factors, that range from animal welfare to milk production and quality should be considered. It is known that blood metabolites reflect the animal nutritional status, as well as its physiological condition (Da Chuan *et al*. 2015). Within these parameters, a growing importance has been recently given to the assessment of oxidative stress. A high level of reactive oxygen species, due to an increased production of pro-oxidant species and/or a decreased efficacy of the anti-oxidant system, can lead to oxidative stress, an emerging health risk factor involved in many diseases, including inflammatory, infectious and degenerative disorders, both in humans and animals (Halliwell and Cross 1994; Bildik *et al*. 2004; Kiral *et al*. 2005; Kumaraguruparan *et al*. 2005; Vajdovich *et al*. 2005). Several authors underlined the importance of nutrition in modulating oxidative stress. By comparing the relationship between body condition score and oxidative status, Bernabucci *et al*. (2005) suggested that nutrition is involved in free radical-mediated lipid peroxidation, which is critical in high-yielding cows that are naturally prone to oxidative stress.

Despite the benefits of the milk obtained by the already mentioned feeding strategy in terms of human health that have recently been reported (Cavaliere *et al*. 2018), little information regarding the possible effects on cows' welfare is available. The aim of this study was to evaluate the effects of the feeding strategy suggested by Rubino (2014), described above, in dairy cows on milk yield and chemical composition, and blood metabolic profile, including the evaluation of oxidative stress.

Materials and methods

Animals and diets

The study was carried out during spring/summer from March to July 2016 on Italian Friesian cows, in a farm located in a hilly area of Centre Italy (Segni, Rome, Italy; longitude 13°0′E, latitude 41°41′N, altitude 668 m above sea level). The farm produces two types of commercial milk (lower forage milk and higher forage milk) from animals fed as semi-intensive (forage : concentrate 55 : 45) or the feeding strategy described above respectively. The ingredients and forage : concentrate ratio of the two diets are reported in Table 1. To assess the metabolic changes due to the introduction of the new feeding strategy, 30 animals, selected from those fed as semi-intensive were included in this study and divided into two groups of 15 animals (control group, low forage concentration (LFC), and new feeding strategy group, high forage concentration (HFC)), homogenous for parities (3.35 ± 0.99 and 3.33 ± 0.90 respectively), days in milk (90 ± 62.8 and 94 ± 63.5 days respectively) and average daily milk production (27.0 ± 2.4 and 26.8 ± 3.0 kg/day respectively). Diets were offered (3.25% of liveweight) as total mixed ration. Refusals were weighed daily, and feed intake was calculated as the difference between offered and residual feed. During the trial, animals were housed and fed in groups in two adjacent pens; the HFC and LFC groups had free access to outdoor shaded paddocks without pasture and with five automatic water bowls, of 200 m^2 and 100 m^2 respectively. After acclimation of 30 days, the experimental period started and lasted 4 months, during which samples of milk and blood were collected monthly (sampling I, II, III, IV).

Table 1. Diet ingredients, forage : concentrate ratio and DM intake
LFC, low forage concentration; HFC, high forage concentration

	Group LFC	Group HFC
Diet ingredients (kg as fed)		
Corn silage	24	–
Mixed hay[A]	–	7.6
Alfalfa hay	5.0	9.8
Wheat bran	1.3	1.2
Cornmeal	4.0	3.1
Triticale	1.9	1.0
Faba bean	–	2.0
Sunflower cake	1.7	–
Soybean meal	2.0	–
Forage : concentrate ratio (DM basis)	55 : 35	70 : 30
Intake, kg DM	20.9	21.2

[A] *Vicia sativa*, *Avena sativa*, *Lolium multiflorum*, *Trifolium alexandrinum* and *Trifolium squarrosum*.

Feed analysis

Samples of both diets were collected monthly to determine DM, crude protein (CP), ether extract (EE) and ash as described by AOAC (2005) procedures (ID number: 2001.12, 978.04 and 920.39 respectively). Structural carbohydrate fractions were also determined as described by Van Soest *et al*. (1991). Starch content was analysed with polarimetric detection (Polax L; Atago, Tokyo, Japan) as indicated by Martillotti *et al*. (1987). The net energy for lactation was calculated as follows: NEl, Mcal/kg = (0.703 × ME − 0.19) + {[(0.097 × ME + 0.19) / 0.97] × (EE − 3)} (NRC 2001).

Milk determinations

Milk yield was recorded daily during automatic milking (Parallel parlour 8 + 8; Tecnozoo s.r.l. Torreselle di Piombino Dese, Padua, Italy). At each sampling day, representative individual milk samples (300 mL, obtained pondering milk yield at the two daily milkings, at 0500 hours and 1600 hours) were collected by a milk recording device and stored at 4°C. The milk parlour was very close to the pen. Two hours after sampling, fat, protein, lactose and

milk urea nitrogen (MUN) concentrations were measured by the infrared method using a MilkoScan FT 6000 (Foss Electric A/S, Hillerød, Denmark).

Blood sampling and chemistry

Starting the first Monday of April, at 0700 hours, after milking and before feeding, blood sampling was carried out monthly every first Monday always by the same practitioner following the rules of good veterinary practice under farm conditions (FVE 2005). Animals were fasted 8 h before the blood withdrawal. Blood samples were taken from the jugular vein in 8-mL Vacucheck tubes with gel separator and clot activator that promote blood clotting with glass or silica particles, stored at 4°C and immediately transported to the laboratory. Serum was obtained by centrifugation at 450 *g* for 15 min; then, serum samples were frozen in small aliquots at –80°C. Blood chemistry analyses were performed by an automatic biochemical analyser AUTOLAB PM4000 (AMS, Rome, Italy) using reagents from Spinreact (Santa Coloma, Spain) to determine: total proteins, albumin, blood urea nitrogen (BUN), creatinine, glucose, aspartate amino transferase, alanine aminotransferase, gamma-glutamyltransferase, lactic dehydrogenase, creatine kinase, alkaline phosphatase, cholesterol and triglycerides. Other reagents were from Catachem (Bridgeport, CT, USA) to determine β-hydroxybutyric acid, Randox (Dungloe, Ireland) for non-esterified fatty acids (NEFA) and Diacron International s.r.l. (Grosseto, Italy) to assess derivatives of reactive oxygen metabolites (d-ROMs), biological anti-oxidant potential, OXY-adsorbent and anti-reactive oxygen metabolites (anti-ROMs) tests.

Statistical analyses

Milk and blood data were analysed by one-way ANOVA (JMP software version 11; SAS Institute, Cary, NC, USA) according to the following model:

$$y_{ij} = \mu + G_i + S_j + G \times S_{ij} + \varepsilon_{ij},$$

where y is the dependent variable, μ is the mean, G is the group effect (i is LFC, HFC), S is the sampling effect (j is I, II, III, IV), $G \times S$ is the first order interaction and ε is the error. The means were compared using the *t*-test. Results were considered significant for $P < 0.05$ and $P < 0.01$

Results

Feed analysis

Table 2 shows the diets' chemical composition, feed intake and net energy for lactation. Different ingredients determined differences in diet protein level (154.0 vs 136.0 g/kg DM), structural carbohydrates (neutral detergent fibre 370.0 vs 485.0 g/kg DM), starch content (135.0 vs 97.6 g/kg DM) and net energy for lactation (6.4 vs 5.8 MJ/kg DM).

Milk

Table 3 shows milk yield and composition. No differences were detected for milk yield between groups, but the yield was significantly different among the sampling time ($P < 0.0001$). Regarding milk composition, only urea was significantly higher ($P < 0.0001$) in the HFC group, but the difference disappeared after sampling II.

Blood metabolic profile

Tables 4 and 5 show the serum lipid- and protein metabolism-related parameters respectively.

Table 2. DM (%), chemical composition (g/kg DM) and net energy for lactation (NEl; MJ/kg DM)

CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NEl, net energy for the lactation; LFC, low forage concentration; HFC, high forage concentration

	Group LFC	Group HFC
DM (%)	52.4	47.0
CP (g/kg DM)	154.0	136.0
EE (g/kg DM)	32.2	17.8
NDF (g/kg DM)	370.0	485.0
ADF (g/kg DM)	244.0	393.0
ADL (g/kg DM)	76.4	110.0
Starch (g/kg DM)	135.0	97.6
Ash (g/kg DM)	60.3	82.9
NEl (MJ/kg DM)	6.4	5.8

Table 3. Milk yield and composition

LFC, low forage concentration; HFC, high forage concentration; G, group effect; S, sampling effect; G × S, interaction between group and sampling time; RMSE, root mean square error

	Yield kg/day		Fat %		Lactose %		Protein %		Urea mg/dL	
Sampling	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC
I	22.5	18.1	3.5	3.6	4.8	4.9	3.3	3.3	24.3	30.8
II	17.1	15.3	3.5	3.6	4.8	4.8	3.3	3.3	22.9	28.6
III	12.0	11.3	3.5	3.7	4.7	4.6	3.3	3.4	24.8	24.0
IV	9.0	7.8	3.6	3.7	4.7	4.6	3.3	3.3	20.1	20.6
Mean	15.5	13.1	3.5	3.6	4.8	4.7	3.3	3.3	23.0	26.0
G	0.095		0.687		0.172		0.432		<0.0001	
S	<0.0001		0.547		0.061		0.312		<0.0001	
G × S	0.542		0.764		0.056		0.533		<0.0001	
RMSE	3.63	3.98	0.24	0.32	0.23	0.30	0.28	0.33	5.13	7.94

Table 4. Lipid metabolism indicators

LFC, low forage concentration; HFC, high forage concentration; GLU, glucose; NEFA, non-esterified fatty acids; BHBA, beta-hydroxybutyrate; CHO, cholesterol; TRI, triglycerides; G, group effect; S, sampling effect; G × S, interaction between group and sampling time; RMSE, root mean square error

	GLU mg/dL		NEFA µmol/L		BHBA mg/dL		CHO mg/dL		TRI mg/dL	
Sampling	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC
I	62.9	52.4	55.1	64.7	4.5	6.1	138.9	144.6	8.9	9.2
II	61.3	52.5	51.0	63.5	5.0	5.3	139.0	143.4	8.6	9.6
III	58.3	54.6	52.5	59.2	7.2	6.2	144.4	146.4	9.5	10.7
IV	65.9	56.7	55.8	54.2	8.5	6.0	138.1	142.7	10.4	10.4
Mean	62.1	54.1	53.6	60.4	6.3	5.9	140.1	144.3	9.4	10.0
G	0.008		0.052		0.409		0.296		0.596	
S	<0.0001		0.237		0.234		0.875		0.079	
G × S	0.235		0.430		0.074		0.699		0.321	
RMSE	8.13	7.05	28.62	27.86	2.16	1.51	42.38	30.44	1.26	2.06

Table 5. Protein metabolism indicators

LFC, low forage concentration; HFC, high forage concentration; TP, total proteins; ALB, albumin; BUN, blood urea nitrogen; CREA, creatinine; G, group effect; S, sampling effect; G × S, interaction between group and sampling time; RMSE, root mean square error

	TP g/dL		ALB g/dL		BUN mg/dL		CREA mg/dL	
Sampling	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC
I	9.2	9.5	3.1	3.3	12.2	19.7	0.84	0.87
II	9.2	9.4	3.2	3.2	11.9	21.3	0.85	0.89
III	9.1	9.0	3.3	3.3	16.3	16.2	0.85	0.92
IV	9.0	8.9	3.2	3.3	14.8	14.1	0.88	0.92
Mean	9.1	9.2	3.2	3.3	13.9	17.9	0.85	0.89
G	0.872		0.294		<0.0001		0.558	
S	0.074		0.064		0.040		0.597	
G × S	0.826		0.437		<0.0001		0.933	
RMSE	0.736	0.747	0.30	0.28	2.90	3.70	0.094	0.111

Glucose was higher in the LFC group and showed significant ($P < 0.008$) differences at all sample days, but no differences were detected for interaction between group and sampling. An opposite trend was observed for triglycerides and NEFA whose values were higher in the HFC group, but no significant differences were observed. Also, no significant differences were detected for cholesterol and beta-hydroxybutyrate (BHBA).

Regarding protein metabolism, a significant difference was detected only for BUN, showing higher values ($P < 0.0001$) in the HFC group up to sampling II. Furthermore, a significant difference was also seen by sampling day and interaction. No significant difference was detected for total proteins, albumin and creatinine.

Table 6 shows the activity of enzymes mainly related to liver and muscle function. Aspartate amino transferase and creatine kinase were significantly higher in the HFC group only at the first sampling ($P = 0.003$ and $P = 0.009$ for aspartate amino transferase and creatine kinase respectively). For the other parameters, no significant differences were found.

Oxidative status

Table 7 shows levels of oxidative status markers. No differences were found for OXY-adsorbent tests and anti-ROMs1. In the HFC group, d-ROMs were significantly ($P = 0.008$) lower at the second sampling until the end of the experiment, whereas the biological anti-oxidant potential levels showed a sharp increase after the third sampling ($P = 0.0001$) in the HFC group. Furthermore, a significant interaction (group × sampling) was also recorded for both parameters. At the last sampling, anti-ROMs2 values were also significantly ($P = 0.028$) higher in the HFC group.

Discussion

High-producing dairy cows need to provide enough glucose precursors to meet the requirements of the mammary gland for milk production. Several studies have shown that the glucose requirement of the mammary gland accounts for up to 85% of the glucose required by lactating ruminants, and lactose synthesis accounts for up to 85% of the glucose taken by

Table 6. Liver- and muscle-specific enzymes

LFC, low forage concentration; HFC, high forage concentration; ALT, alanine amino transferase; AST, aspartate amino transferase; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; LDH, lactic dehydrogenase; CK, creatine kinase; G, group effect; S, sampling effect; G × S, interaction between group and sampling time; RMSE, root mean square error

	ALT		AST		GGT		ALP		LDH		CK	
	mg/dL		μmol/L		g/dL		mg/dL		mg/dL		U/L	
Sampling	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC
I	27.0	31.7	75.3	86.0	22.6	25.0	69.8	64.2	1029.6	1121.3	135.7	212.2
II	29.2	29.4	74.3	79.3	24.8	23.8	76.7	56.0	1050.8	976.6	172.8	143.4
III	26.0	31.6	71.0	73.7	26.7	23.7	56.0	55.8	968.4	965.9	153.9	139.1
IV	24.6	26.3	75.1	87.7	25.1	22.9	63.3	54.9	1004.1	1004.1	169.4	214.2
Mean	26.8	29.7	74.0	81.8	24.7	23.8	67.0	57.8	1015.6	1012.9	156.7	178.2
G	0.875		0.075		0.120		0.459		0.540		0.102	
S	0.641		0.003		0.094		0.098		0.556		0.009	
G × S	0.074		0.081		0.067		0.141		0.097		0.086	
RMSE	1.9	2.3	11.14	17.6	2.1	2.7	7.56	12.1	306	276	33.9	40.3

Table 7. Oxidative status indicators

LFC, low forage concentration; HFC; high forage concentration; d-ROMs, reactive oxygen metabolites; BAP, biological antioxidant potential; OXY, OXY-adsorbent; Anti-ROMs1, anti-reactive oxygen metabolites 1; Anti-ROMs 2, anti-reactive oxygen metabolites 2; UCARR, Unit Caratelli; G, group effect; S, sampling effect; G × S, interaction between group and sampling time; RMSE, root mean square error

	d-ROMs		BAP		OXY		Anti-ROMs1		Anti-ROMs2	
	UCARR		μmol/L		mmol/L		μeq/L		μeq/L	
Sampling	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC	LFC	HFC
I	68.7	68.0	1359	1708	475	439	52.1	50.4	275.3	218.0
II	60.2	53.8	1548	1259	405	409	52.3	43.8	261.6	292.67
III	78.8	74.8	1326	2329	410	383	52.8	46.8	236.9	371.4
IV	65.0	46.2	1329	2401	394	362	53.6	47.6	206.5	297.8
Mean	68.2	60.7	1384	1924	411	398	55.1	47.1	245.0	295.0
G	0.008		0.003		0.407		0.858		0.018	
S	<0.0001		0.0001		0.086		0.970		0.028	
G × S	0.003		<0.0001		0.921		0.966		0.082	
RMSE	8.97	6.13	764.5	877.5	95.9	87.63	19.56	22.62	96.00	93.00

the mammary gland. Thus, limited glucose precursors availability may reduce milk yield in cows (Amaral-Phillips *et al*. 1993). In any event, a non-significant decrease in milk yield was registered in this trial. Also, milk composition did not differ between the groups. Milk urea was higher in the HFC group at the first and second sampling, thus reflecting serum urea levels and confirming the transitory change of protein metabolism. Some authors suggested that milk urea can be used to monitor nutritional status of lactating dairy cows (Baker *et al.* 1995). Previous studies showed the relationship of milk urea to dietary protein and energy. Variation in milk urea has been hypothesised to be related to the protein : energy ratio of the diet (Roseler *et al*. 1993). In contrast, according to Westwood *et al*. (1998), due to the high aptitude of cows in adapting to high-protein diets, milk urea determination may have limited usefulness as a marker of nutritional status.

As the urea increase was seen up to 60 days from the beginning of the trial, such a result suggests that the acclimation time to the proposed feeding strategy should be prolonged and carefully monitored (Huntington and Archibeque 1999).

The increase of the forage : concentrate ratio resulted in less available energy and, as a result, body reserves were used as alternative energy sources. Serum glucose was significantly lower in the HFC group, but despite being essential to milk volume (Amaral-Phillips *et al*.1993), the potential energy impairment did not result in a lower milk yield and lactose. In contrast, Cao *et al*. (2010) found a statistically significant association between MUN and lactose, which may be an indirect result of milk yield, explained by the role of lactose synthesis in the regulation of milk secretion. This condition is common in early lactation, especially in the first 3–5 weeks and up to 2 or 3 months after parturition, and mainly in high-yielding dairy cows, when feed intake is insufficient to cover the requirements for energy (Aeberhard *et al*. 2001).

In this study, even at a later lactation stage, the lower glucose in the HFC group was a consequence of the diet characteristics, but the negative energy balance due to the

reduced energy intake was compensated by the mobilisation of NEFA from adipose tissues (Baird 1982). The increase of NEFA detected throughout the trial, although non-significant, seemed to be sufficient to provide enough energy to animals in the HFC group. In fact, in the case of excessive fat mobilisation, associated with marked formation of acetyl-coenzyme A, the tricarboxylic acid cycle cannot fully metabolise fatty acids (Brumby *et al*. 1975). As a result, acetyl-coenzyme A is converted to acetoacetate, which is then reduced to BHBA by BHBA dehydrogenase or spontaneously decarboxylated to acetone (Brumby *et al*. 1975). Hence, the presence of ketone bodies in body fluid is normal to a certain degree, whereas high concentrations of ketone bodies indicate that adaptability of metabolism is exceeded; that is whole-body homeostasis cannot be maintained (Brumby *et al*. 1975).

In our experiment, BHBA concentration was not higher in the HFC group, showing that NEFA did not provide the substrate for BHBA synthesis. Furthermore, both BHBA and NEFA fall in the physiological range for mid-late lactation (Djokovic *et al*. 2016). Therefore, the reduced glucose was compensated by a slight NEFA mobilisation with no increase of BHBA and, as a consequence, no metabolic risk for animal health.

Even with only one sample per month collection, the absence of significant differences between the two groups suggests that animals from the HFC group were able to maintain body homeostasis by changing metabolism in a non-dangerous manner, despite the low-energy diet. A higher level of urea was detected, both in serum and milk, in the first two HFC samplings. Remarkably, the difference disappeared at sampling III and IV, suggesting just a transitory use of protein as energy substrate because of the lower energy diet. Such transitory use may be responsible for the significant difference seen by sampling day and interaction.

The higher concentration of MUN and BUN in the HFC group may also be caused by the higher protein fraction degradable in the rumen in this diet due to the presence of Leguminosae plants (*Vicia sativa*, *Trifolium alexandrinum*, *Trifolium squarrosum*) in the mixed hay. Indeed, in lactating dairy cows, an imbalance of degradable and undegradable intake protein increased BUN and MUN (Roseler *et al*. 1993). The presence of rumen degradable protein can cause asynchrony between amino acids and energy availability favouring a higher NH_3 production at the rumen level and, consequently, increasing the concentration of blood urea (Chumpawadee *et al*. 2006). It is conceivable that the diet affected rumen bacteria activity, but further studies including rumen ecology should be performed to address such hypothesis. According to Adduci *et al*. (2015), an incorrect feeding management of dairy cattle requires a fast correction, being detrimental on animal productivity, health and fertility, thus negatively affecting the overall farm profitability. Moreover, the higher losses of nitrogen through urine and faeces as a consequence of unbalanced nitrogen energy in the diet are known to have potential negative environmental effects (Dijkstra *et al*. 2013).

Aspartate amino transferase and creatine kinase values showed significant differences at the first sampling, but levels were within the physiological range (Cozzi *et al*. 2011). Both enzymes are considered to be markers of muscle integrity being largely present in the skeletal muscle of cows (Sattler and Fürll 2004), and their increase can be associated with exercise. Therefore, such difference was probably due to the muscle adaptation that the HFC group underwent by using a larger outdoor paddock (Radwowska and Herbut 2014). All other parameters did not show any difference, confirming that body tissue function was not affected by the diet.

The results regarding the oxidative status may suggest a probable beneficial effect of a HFC diet. The d-ROMs were lower in the HFC group, showing a general improvement of oxidative status, as this test provides a measure of the whole oxidant capacity of plasma, and its decrease is considered a measure of cellular health (Cesarone *et al*. 1999). Even if OXY-adsorbent tests were not statistically different, the lower d-ROMs level in the HFC group was probably due to an improvement of the biological anti-oxidant potential, as shown by the dramatic increase of biological anti-oxidant potential levels after the second sampling. Such a biological anti-oxidant potential is attributed to the major component of the plasma barrier to oxidation (vitamin C, vitamin E, uric acid, bilirubin; Benzie and Strain 1996; Dohi *et al*. 2005). The higher levels of anti-ROMs2 in the HFC group suggest a prevalence of "slow" anti-oxidants, such as uric acid and certain thiols (i.e. cysteine), which play a role in the defence against free radicals (Maruoka *et al*. 2013; Pizza *et al*. 2013). In our study, the diet forage : concentrate ratio was higher in the HFC group, thus providing higher intake of omega 3 than omega 6. These two classes of essential fatty acids have different physiological functions, such as pro- and anti-inflammatory activity for omega 6 and omega 3 respectively. It is important to underline that the metabolic changes induced by inflammation include alterations in mitochondrial function that may lead to higher free radicals production (Chan 2006).

Conclusions

The results of this study showed that the proposed feeding strategy did not decrease milk yield. Animals fed a higher forage : concentrate diet were able to maintain body homeostasis, probably increasing NEFA without increasing BHBA, despite the low-energy diet. Moreover, the cows in the HFC group showed a general improvement of oxidative status, probably due to an improvement of the biological anti-oxidant potential. This last result is also interesting in terms of prolongation of productive life, but further studies are required to address such a hypothesis.

Conflicts of interest

The authors declare no conflicts of interest.

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