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Abstract. In the Basque Country of Northern Spain dairy sheep (Latxa breed) production systems are based on part-time grazing starting in late winter or early spring. Pasture feeding is supplemented with indoor forage (alfalfa and pasture hay) and concentrate to meet production requirements. The objective of this study was to evaluate the effect of actual grazing time on the antioxidant capacity of the resulting milk. The experiment was conducted during 4 weeks in spring. Sheep were separated into 4 homogeneous groups of 12 sheep each, and randomly assigned to 3 different alfalfa hay supplements: 300 g/day (Group 1), 600 g/day (Group 2), and 900 g/day (Group 3). The control group (Group 0) received 600 g alfalfa hay/day and was not allowed to graze outdoors. All animals received 500 g concentrate/day at milking. Groups 1-3 were given access to pasture during 4 hours a day. Grazing time was visually monitored. Group 3 spent the least time grazing ($P<0.05$). Water-soluble total antioxidant capacity (TAC) was determined by the spectrophotometric method, based on the scavenging of the radical cation $ABTS^{-+}$ (2,2'-azinobis[3-ethylenbenzothiazoline-6-sulphonic acid]) by Trolox® as a reference antioxidant, monitored at 730 nm. Results are given as the equivalent amount of Trolox® normalized according to the protein content of each milk sample (mmol Trolox® equivalents/g protein). TAC values were significantly ($P<0.05$) higher in whole milk samples from Groups 1 and 2 than in milk samples from Group 3. Likewise, milk samples from Group 2 exhibited significantly ($P<0.05$) higher antioxidant capacity than those from Group 0. TAC values of whole milk samples were 3.5 higher than those of whey samples. TAC values for whey samples were significantly ($P<0.05$) higher in samples from Group 2 than in those of Group 0. We conclude that increased pasture grazing increases the total antioxidant capacity of the resulting milk.

Keywords. Antioxidant capacity – Milk – Pasture – Trolox®.

Développement de la capacité anti-oxydante totale soluble à l´eau du lait de brebis comme résultant de l'augmentation du temps de pâturage

Résumé. *Au nord de l´Espagne dans le Pays Basque, les systèmes de production du lait de brebis (race Latxa) sont basés sur le pâturage partiel, qui commence à la fin de l´hiver ou au début du printemps. L'alimentation avec du pâturage est supplémentée avec du fourrage (luzerne et fourrage conservé) et du concentré quand ils sont à l´intérieur, pour arriver aux prévisions de production. L´objectif de cette étude a été l´évaluation de l´effet du temps du pâturage actuel sur la capacité anti-oxydante du lait de brebis. L´expérience a été réalisée pendant 4 semaines de printemps. Les brebis ont été séparées en 4 groupes homogènes de 12 brebis, avec une alimentation supplémentée avec 3 différentes quantités de foin de luzerne distribuées au hasard: 300 g/jour (Groupe 1), 600 g/jour (Groupe 2), 900 g/jour (Groupe 3). Le groupe témoin (Groupe 0) a reçu 600 g de foin de luzerne/jour et ne pouvait pas pâturer dehors. Tous les animaux ont reçu 500 g du concentré/jour pendant la traite. Les Groupes 1-3 ont pâture pendant 4 heures chaque jour. Le temps de pâturage a été contrôlé visuellement. Le Groupe 3 a pâturé pendant le temps le plus court ($P<0.05$). La capacité anti-oxydante totale soluble à l´eau (TAC) a été déterminée avec une méthode spectro-photométrique, sur la base de l'élimination du radical cation $ABTS^{-+}$ (2,2'-azinobis[3-éthylenbenzothiazoline-6-sulfonique acide]) avec Trolox® comme référence anti-oxydante, contrôlée à 730 nm. Les résultats sont exprimés comme la quantité de l'équivalent de Trolox® normalisé selon le contenu de protéine de chaque échantillon du lait (mmol Trolox® équivalents/g protéine). Les valeurs TAC ont été significativement plus supérieures ($P<0.05$) sur*

les échantillons du lait entier des Groupes 1 et 2 que sur des valeurs des échantillons du Groupe 3. De la même manière, les échantillons du lait du Groupe 2 ont présenté des valeurs de la capacité anti-oxydante significativement supérieures (P<0.05) à celles du Groupe 0. Les valeurs TAC du lait entier ont été 3.5 fois plus élevées que les échantillons du sérum du lait. Les valeurs TAC du sérum du lait ont été significativement supérieures (P<0.05) pour les échantillons du Groupe 2 à celles du Groupe 0. Par conséquent, on conclut que le temps plus long de pâturage accroît la capacité anti-oxydante totale du lait.

Mots-clés. *Capacité anti-oxydante – Lait – Pâturage – Trolox®.*

I – Introduction

In recent years consumers have become increasingly interested in foods that, in addition to being nutritious and good-tasting, contain compounds that provide health benefits and do not contain compounds that could have a negative health impact. Feed also has an important effect on the concentration of various compounds that exhibit antioxidant capacity (Havemose *et al*., 2004). The oxidative stability of the milk depends on the relative concentrations of pro- and anti-oxidant compounds, in addition to the fat composition and amount of protein, among other variables (Havemose *et al*., 2006). Antioxidant compounds potentially present in milk can belong to very different chemical families, such as phenols, terpenoids, water-soluble vitamins such as vitamin C, or even proteins and peptides. Here we determine the water-soluble total antioxidant capacity of the milk as an appropriate way to determine overall differences among samples from animals which received different feeds, instead of determining the concentrations of individual compounds. Water-soluble total antioxidant capacity in sheep's milk samples was measured by the spectrophotometric method of Re *et al.* (1999) as modified by Chen *et al.* (2003), which relies on the reduction of the water-soluble, coloured radical cation $ABTS^{-+}$ (2,2'-azinobis[3-ethylenbenzothiazoline-6-sulphonic acid]). The water-soluble total antioxidant capacity is quantified as the equivalent concentration of Trolox®, a water-soluble analog of vitamin E, used as reference antioxidant. Therefore, this method measures water-soluble total antioxidant capacity.

Part-time grazing with indoor supplementation is the traditional, most frequently used, sheep flock management system during much of the lactation period in the Basque Country of Northern Spain, from late winter until late spring, when pastures are available (Oregui and Falagán-Prieto, 2003). This flock management method takes advantage of locally available resources. Most of the milk produced during this time is used for Idiazabal cheesemaking. The work presented herein is part of a wider study designed to evaluate the effect of grazing time on the nutritional quality of the milk. In this manuscript we report the results of the water-soluble antioxidant capacity of the milk, as a quality parameter.

II – Materials and methods

The experiment was conducted during 4 weeks from mid-April until mid-May with an experimental flock of sheep, with one week to allow the sheep to get accustomed to their new feed. Forty-eight multiparious *Latxa* dairy ewes 2 months *post-partum* were separated into 4 homogeneous groups (according to live-weight, body condition score and milk production) of 12 sheep each. Initial mean values were 2nd or 3rd lactation, 40-60 day of lactation, milk yield of 1.40-1.50 l/d and 57-62 kg live weight. Groups were randomly assigned to one of the feeding regimes described in Table 1. The nutritional characteristics of the feeds are described in Table 2.

Table 1. Feeding regimes of the four groups of animals

	Pasture feeding	Indoor feeding program		
Group	**Access to pasture (h/d)**	**Concentrate (g/d)**	**Alfalfa hay (g/d)**	**Grass hay (g/d)**
0	0	500	600	1,000
1	4	500	300	0
2	4	500	600	0
3	4	500	900	0

Table 2. Nutritional characteristics of the feeds

Feed	Crude Protein (%)	Neutral-Detergent Fibre (%)
Concentrates	22.0 ± 1.0	–
Alfalfa hay	19.6 ± 3.2	36.4 ± 6.5
Grass hay	9.3 ± 3.1	56.1 ± 5.3
Pasture†	23.4 ± 2.0	40.0 ± 3.3

† Polyphite pasture was composed mainly of *Lolium perenne, Dactylis glomerata* and *Trifolium repens*.

As described in Table 1, Group 0 was kept indoors during the entire 5 weeks with no access to pasture, whereas the other three Groups were allowed access to pasture during 4 hours per day, after the morning milking. Concentrate was provided during the morning (250 g) and evening milking (250 g) and alfalfa hay was given after the evening milking, according to Table 1. Groups 1-3 grazed on the same pasture, although in different parts of the field, so as to facilitate monitoring. Actual time spent grazing was visually monitored.

During the 4 weeks milk production was daily recorded (l/d) and milk samples from individual sheep were taken each week to determine the percent fat and protein (Method PE/ALVO/02, 2005). Milk samples (evening and morning milkings combined) were taken once a week. Protein content of whole milk and whey was determined by the Kjeldhal method.

Total antioxidant capacity (TAC) of milk samples was determined by monitoring the decrease in absorbance at 730 nm as the coloured radical cation $ABTS^{-+}$ (2,2'-azinobis[3-ethylenbenzothiazoline-6-sulphonic acid]) is reduced. Trolox® is used as a reference antioxidant. The radical cation is produced by the overnight reaction (12-16 h) of ABTS (7mM) with potassium persulfate (2.45 mM) in 10 ml dionized water, in the dark, at room temperature. The radical cation thus prepared is stable for up to 6 days if kept in the dark at room temperature. The working solution is prepared by dilution with saline phosphate buffer (pH 6.7) until the absorbance at 730 nm is 0.7 ± 0.02. To 2.0 ml working solution various amounts of Trolox® (or milk samples) are added and the absorbance is read at 730 nm after 10 minutes at 25ºC. Under these conditions, a 25 mM Trolox® concentration provides 100% scavenging of $ABTS^{-+}$. Trolox® calibration curves were linear up to 50 mM. The total antioxidant capacity of milk (TAC) is given as the equivalent amount of Trolox® normalized according to the protein content of each milk sample (mmol Trolox® equivalents/g protein).

The pH of all milk samples was systematically adjusted to 6.7. Samples were sonicated for 10 minutes and diluted 10 to 15 times to avoid excessive turbidity. Whey was prepared from skim milk by precipitating proteins at pH 4.6 and removing the precipitate by centrifuging at 2000 g during 30 min at 4ºC. Under the conditions described, values for percent scavenging were linear with the amount of diluted sample used. Milk fractions studied were whole milk and whey.

Statistical analysis of the data (one-way ANOVA) was done using the SPSS statistical package version 16.0. Values reported are the average and standard deviation.

III – Results and discussion

The amount of milk produced during the 4 weeks by all Groups was essentially identical (P>0.05), with no statistically significant differences (P>0.05) observed in the protein and fat content (Table 3). In contrast, the actual amount of time spent grazing depended on the amount of alfalfa hay each group received. Thus, Groups 1 and 2 grazed significantly longer time (P<0.05) than Group 3, although Group 1 received half the amount of alfalfa hay than Group 2. These results indicate that it is possible to implement a flock management method based on part-time grazing using locally available resources and reducing the amount of indoors-provided supplements without compromising milk yield or its gross composition.

Table 3. Milk yield, gross milk composition and grazing time

	Group 0	Group 1	Group 2	Group 3
Milk Yield (l/d)	1.03 ± 0.22^{a}	1.32 ± 0.32^{a}	1.36 ± 0.35^{a}	1.46 ± 0.30^{a}
Protein (%)	5.05 ± 0.34^{a}	4.88 ± 0.45^{a}	5.05 ± 0.49^{a}	4.96 ± 0.47^{a}
Fat (%)	6.98 ± 0.89^{a}	6.12 ± 1.00^{a}	6.34 ± 1.12^{a}	6.30 ± 0.95^{a}
Grazing time (min/4h)	0	228 ± 8^{a}	224 ± 6^{a}	209 ± 14^{b}

^{a,b} different superscripts in the same row indicate statistically significant differences (p<0.05).

As it can be seen in Table 4, the total antioxidant capacity (TAC) values of whole milk samples were directly correlated with the actual amount of time that sheep were grazing. TAC values for milk samples from Groups 1 and 2 were significantly higher (P<0.05) than those of milk samples from Group 3. Whole milk samples from Group 2 (which received the same amount of alfalfa hay as Group 0) exhibited significantly (P<0.05) higher antioxidant capacity than those from Group 0, clearly demostrating the importance of fresh grass.

Table 4. Total antioxidant capacity values (mmol Trolox® equivalents/g protein) of whole milk and whey

Sample	Group 0	Group 1	Group 2	Group 3
Whole milk	2,762.48 ± 109,74^{a}	3,022.78 ± 234,92^{b}	2,967.93 ± 112.25^{b}	2,703.57 ± 120.41^{a}
Whey	713.20 ± 72.29^{a}	778.79 ± 54.21^{b}	835.65 ± 66.15^{b}	789.49 ± 32.20^{b}

^{a,b} different superscripts in the same row indicate statistically significant differences (p<0.05).

TAC values of whey samples were between 3.4 and 3.8 times lower than those of whole milk in all cases. Values for Groups 1-3 were not significantly different, but they were significantly higher than that of Group 0. Comparing the TAC values of whole milk with those of whey samples, it can be concluded that caseins contributed the largest amount of total antioxidant capacity, as observed by Zulueta *et al.* (2009) and Cervato *et al.* (1999). Once the effect of caseins is eliminated, the effect of grazing in Group 3 (as compared to Group 0) is clearly seen.

Other investigators (see references in Zulueta *et al*., 2006) have reported that the total antioxidant capacity of milk depends, primarily, on the protein content and the composition of the fat fraction (see references in Havemose *et al.*, 2004 and 2006). In this investigation no attempt has been made to study the antioxidant capacity of the fat fraction. However, our results clearly indicate that when animals are fed fresh grass other water-soluble components contribute significantly to the water-soluble total antioxidant capacity of the milk and of the whey. Although we have not yet iden-

tified these water-soluble components, vitamin C is likely to be one of the main compounds because it has been known for a long time that pasture increases its concentration in milk.

We conclude that only a small amount of supplement in conjunction with part-time grazing is necessary to produce milk with a significantly higher total antioxidant capacity than that obtained by intensive practices.

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