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REGULAR ARTICLES

Effect of grape seed and skin supplement on milk yield and composition of dairy ewes

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Abstract In the present study, we investigated the effect of grape seed and skin supplement (GSSS), on lactating dairy ewes' production. Ten dairy pregnant ewes from northern Tunisia were allocated to two groups: control diet (C) and supplemented with 20 % (*w*/*w*) GSSS. The experiment lasted for 8 weeks and took place after 2 months of lambing. During the experiment, daily milk yield and milk composition were determined. Supplementation of the diet with GSSS increased milk production ($P < 0.001$), calcium ($P < 0.01$), free iron ($P < 0.01$) and urea content ($P < 0.001$) but had no effect on milk fat nor protein. From these data, it is concluded that the inclusion of GSSS in sheep diets increased significantly ewes' milk yield.

Keywords Calcium · Dairy ewes · Free iron · GSSS · Milk yield

Introduction

The agro-industrial by-products are of considerable interest for animal feeding, especially in the Mediterranean area, considering the nutritional characteristics of the forage resources available, often obtained in unfavourable climatic conditions (Molina and Aguilera 1991) which penalise the exploitation of such resources in the countries.

There have been growing interests among the researchers to improve the nutritional value of ruminant-derived milk and products. One of the most important strategies is the use of tannins to protect proteins against excessive degradation in the rumen, by forming reversible complexes with proteins. These complexes are not degraded at low pH found in the rumen (Mangan 1988; Butter et al. 1999), but they disintegrated at higher pH in the abomasum and small intestine (Jones and Mangan 1977). Dietary condensed tannins enhance nutritional performance (Waghorn et al. 1999), resulting in higher milk production in dairy cows (Woodward et al. 2000), and credited with reduced methane emission for both sheep (Waghorn et al. 2002) and dairy cows (Woodward et al. 2001). Moreover, tannins may reduce volatile compounds such as skatole and indole (responsible for off-flavour) and improve antioxidant activities in dairy products. However, contrasting results have been described among studies that might be explained by various tannin origins and doses used. In the past years, the functional properties of many plant extracts have been investigated for their potential use as novel nutraceuticals. Davies et al. (2009) demonstrated that feeding grape seed supplement caused no adverse effect in terms of animal health (temperature, pulse and respiration rates) and even induced positive effects related to a presumed altered fermentation in the hindgut. Moreover, incorporation of grape supplement in chicken diets up to 2.5 g/kg had no adverse effect on growth performance or protein digestibility and reduces the free plasma minerals (Chamorro et al. 2013). Grape seed extract is beneficial as it reduces the negative effect of heat stress in rabbits (Hassan et al. 2016). Grape seed extract also contributed to the increase in milk production in cows, an effect that was assigned to ruminal metabolism

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modifications (Gessner et al. 2015). However, little is known about the effect of grape seed and skin supplement (GSSS) on yield and quality of ewe milk.

The present study aimed to determine the effect of GSSS on ewes' milk production and quality by means of some physicochemical parameters determination as protein, fat, or minerals.

Materials and methods

Ten post-pregnant ewes from a farm located in northern Tunisia were stratified according to their body weight and randomly divided into a first group that was fed a control diet (C) and a second group (GSSS) that was fed a diet supplemented with 20 % GSSS (*w*/w). GSSS was processed from a grape cultivar (carignan) of *Vitis vinifera* from northern Tunisia.

To determine the composition of this supplement diet, seeds and skins were dried and grounded separately with an electric mincer (FP3121 Moulinex) until a fine powder was obtained. Total phenolic content was determined by the colorimetric method of Folin–Ciocalteu (Singleton and Rossi 1965). Flavonoids and condensed tannins were determined according to Dewanto et al. (2002) and Sun et al. (1998), respectively (Table 1).

Ewes used in this study gave birth the same week. The experiment started 2 months after lambing and continued for 2 months of lactation. An adaptation period was carried out during 10 days before the beginning of the study, during which all ewes were on standard diet.

Determination of milk yield

Milk was collected twice daily at fixed time on the morning and the evening, and only morning milk was used to evaluate daily yield. Milk samples were aliquoted and stored at −20 °C for further composition analysis.

Fat and protein content analysis

Milk was analyzed for fat and protein by IR spectrometry (MilkoScan 133/; Foss Electric, Hillerod, Denmark) after appropriate calibration of the apparatus according to Gerber (BSI 1955) and Kjeldahl (IDF 1993) methods, respectively.

Determination of milk pH

The pH of fresh milk from control and GSSS groups was measured using an electronic pH meter (Jenway 3505 pH Meter) calibrated with pH 4 and 7 buffers.

Table 1 Phenolic levels in carignan GSSE

Phenolics	Seed	Skin
Total phenolics (mg/g extract)	67	51
Total flavonoids (mg/g extract)	16	14
Non flavonoids (mg/g extract)	51	37
Condensed tannins (mg/g extract)	1.22	3.43
Total anthocyanins (μg/g extract)	0.997	0.962

Determination of milk urea

Urea is transformed into ammonia by urease. The ammonium ions react with a phenolic derivative to form a blue-green-coloured complex whose intensity, measured at 700 nm, is proportional to the concentration of urea in the sample.

Lipoperoxidation

Thiobarbituric acid-reactive substances (TBARs) were used as a measure of the secondary lipid oxidation products using the double heating method (Draper and Hadley 1990). Briefly, after precipitation of milk proteins with trichloroacetic acid (TCA), malondialdehyde (MDA) from supernatant was allowed to react with thiobarbituric acid (TBA). Spectrophotometric measurement of the colour produced was measured at 532 nm, and MDA concentration was calculated by using the absorbance coefficient of the MDA–TBA complex: $1.56 \times 10^5\ cm^{-1}\ M^{-1}$.

Free iron measurement

Free iron level was determined according to Leardi et al. (1998) using a commercially available kit from Biomaghreb, Tunisia. At acidic pH 4.8, all Fe^{3+} released from transferrin was reduced by ascorbic acid into Fe^{2+}, which constituted with ferrozine a purple colourful complex measurable at 560 nm. Briefly, aliquots of milk were added to a reaction mixture containing ascorbic acid (5 g/L) and ferrozine (40 mM) and incubation performed at 37 °C for 10 min.

H_2O_2 content

H_2O_2 was determined enzymatically according to Kakinuma et al. (1979) using a commercially available kit from Biomaghreb. Briefly, in the presence of peroxidase, H_2O_2 reacts with 4-amino-antipyrine and phenol to give a red-coloured quinoeimine which absorbed at 505 nm. Results are expressed as millimoles of H_2O_2 per milligram of protein.

Calcium measurement

Ionizable calcium was determined according to Stern and Lewis (1957) using a commercially available kit from Biomaghreb. At basic pH, calcium constituted with cresolphthalein a purple colourful complex measurable at 570 nm. Briefly, aliquots of milk were added to a reaction mixture containing 2-amino-2-methyl 1-propanol buffer (500 mmol/L), cresolphthalein (0.62 mmol/L) and hydroxy-8 quinoleine (69 mmol/L). Incubation was carried out at room temperature for 5 min assuming the complex was stable for 1 h.

Statistical analysis

Data were analyzed by unpaired Student's *t* test or one-way analysis of variance (ANOVA) and expressed as means ± standard error of the mean (SEM.). All statistical tests were two-tailed, and $P < 0.05$ was considered significant.

Results

The daily milk production increased gradually by 42 % after 20 days and reached an optimum of 300 % ($P < 0.001$) after 2 months of supplementation with 20 % GSSS (Fig. 1). Differences in ewes' daily milk yield mean did not reflect any variation in body weight gain (data not shown).

The daily protein yield was not significantly different between the two groups ($P > 0.05$) during the entire treatment period (Fig. 2a).

Similarly, the daily fat yield did not show any significant difference upon GSSS treatment at all time points studied (Fig. 2b). There was also no difference between the two groups of ewes concerning pH (Fig. 3a). However, an increase in urea ($P < 0.01$) has been recorded during the last month in the GSSS-supplemented group when compared to control (Fig. 3b).

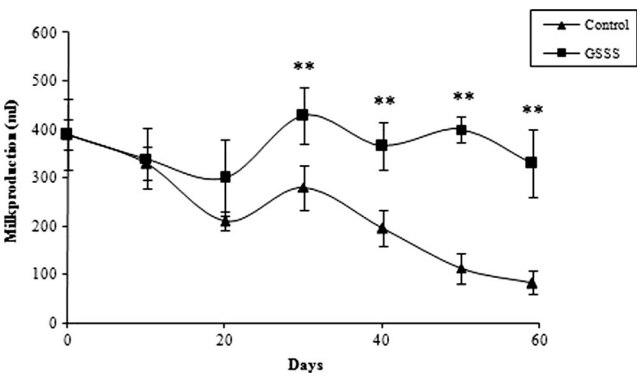

Fig. 1 Effect of GSSS on daily milk production. Ewes were ingested or not with GSSS (20 %) during 2 months. Results are expressed as mean ± SEM ($n = 5$). **$P < 0.01$ vs. control

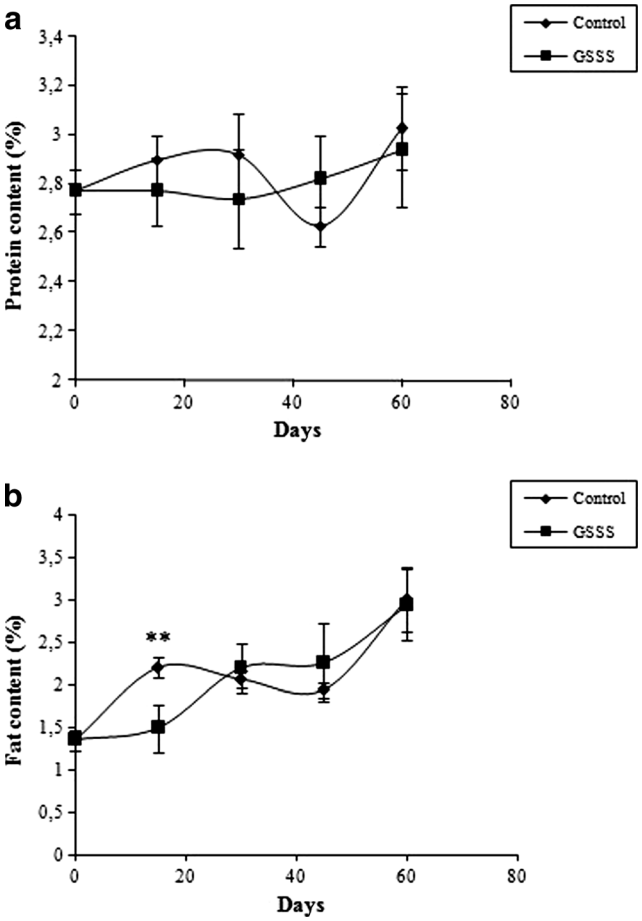

Fig. 2 Effect of GSSS on milk protein (**a**) and fat (**b**). Ewes were supplemented or not with GSSS (20 %) during 60 days and milk protein (**a**) or fat (**b**) determined. Results are expressed as mean ± SEM ($n = 5$)

The inclusion of GSSS in the animal diet provoked an increase ($P < 0.01$) in lipoperoxidation by 60 and 130 % after 30 and 45 days, respectively, versus control which disappeared after 60 days of treatment (Fig. 4a). No difference in H_2O_2 level was detected between the two groups of ewes (Fig. 4b). GSSS increased free iron level from 15 days of treatment ($P < 0.01$), reaching an optimum of 83 % after 40 days, and declined thereafter reaching 24 % at the end of the experiment (Fig. 5a). GSSS also increased significantly ($P < 0.01$) the calcium content by 30 and 70 % after 30 and 45 days, respectively, and this effect disappeared after 60 days of treatment (Fig. 5b).

Discussion

GSSS is a by-product derived from *V. vinifera*. It is obtained following wine making after drying and processing to produce a polyphenolic compound-rich extract (Lau and King 2003). Grape seed and skin extract (GSSE) is a commercially dietary supplement listed on the "Everything Added to Food in the

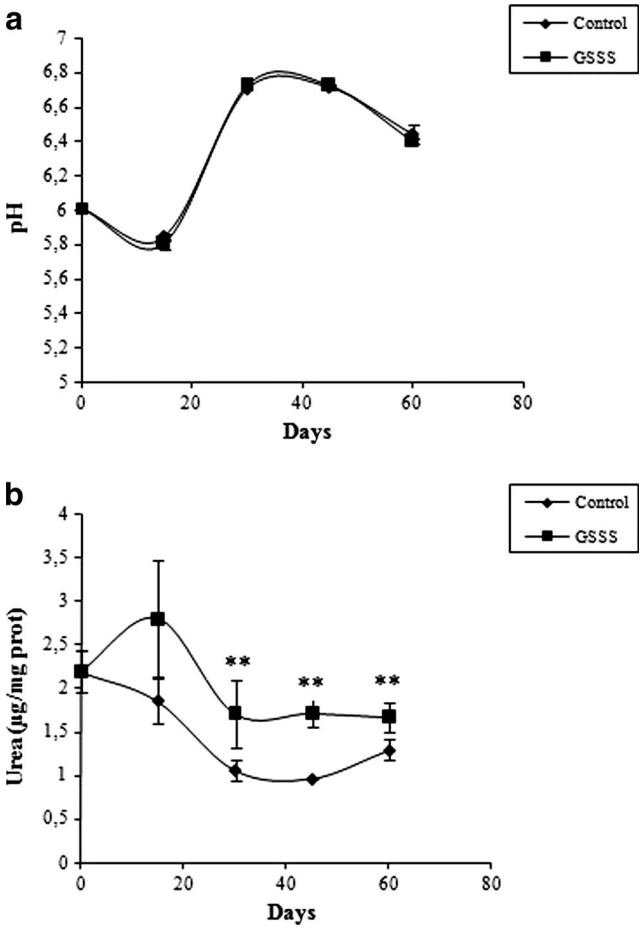

Fig. 3 Effect of GSSS on milk pH (**a**) and urea (**b**). Ewes were supplemented or not with GSSS (20 %) during 2 months. Results are expressed as mean ± SEM ($n = 5$)

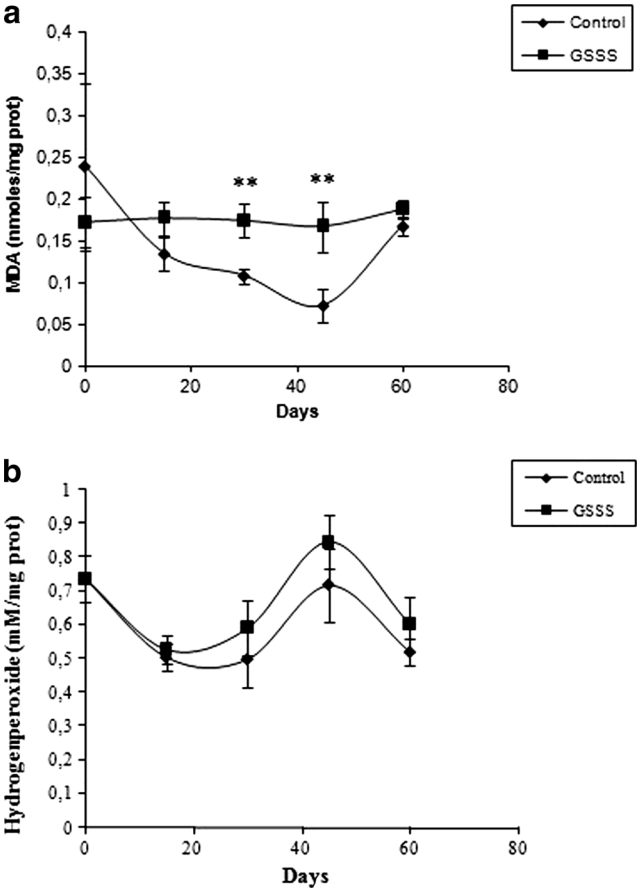

Fig. 4 Effect of GSSS on milk MDA (**a**) and hydrogen peroxide (**b**). Ewes were ingested or not with GSSS (20 %) during 2 months. Results are expressed as mean ± SEM ($n = 5$). $^{**}P < 0.01$ vs. control

United States (EAFUS)" and received the Generally Recognized as Safe (GRAS) certification from US FDA. As mentioned in Table 1, GSSE contained polyphenolic compounds, mainly flavonoids (25.42 %), non-flavonoids (74.57 %) and tannins (5.25 %). Tannins constitute a heterogeneous group of high molecular weight phenolic compounds with the capacity to form complexes with proteins (Schofield et al. 2001). The beneficial effects of tannins in ruminants depend on the ingested amount and on its metabolism in the digestive tractus (Hagerman and Butler 1991). Proteins form complexes with condensed tannins that are stable over the pH range 3.5 to 7.0. This complexation protects proteins from microbial hydrolysis and deamination in the rumen and increases the feed proteins available for digestion and absorption post-rumen (Dutta et al. 2012). It has been routinely observed that the high antioxidant activity of GSSS makes it a good candidate as a supplement into food and beverages to retard deterioration. Moreover, the high antioxidant activity of GSSS particularly in its proanthocyanidin part, as well as digestion products, has reportedly been shown to prevent and reduce the risk of several diseases (Dai and Mumper 2010).

Supplementation with 20 % GSSS significantly ($P < 0.05$) increased the daily milk yield of ewes after 20 days of treatment. There are only scarce reports referring to the effect of supplemental GSSS on milk yield and composition in dairy ewes. Thus, Buccioni et al. (2015) and Dschaak et al. (2011) found that milk yield was not affected by tannin extract respectively in dairy ewes and cows. Moate et al. (2014) described a negative effect in grape marc-fed dairy cows, and Dey and De (2014) stated about a perceptible positive impact on milk production in cows supplemented with condensed tannins.

It is clearly established that GSSS is safe and did not induce any toxic side effects when fed to rats at dietary concentrations as high as 5 % for at least 90 days (Bentivegna and Whitney 2002; Charradi et al. 2013). Increased milk production could be explained by an increase in energy supply, since a strong correlation between these two parameters was previously established (Goulas et al. 2003).

No major effect of GSSS supplementation was observed on milk protein and fat content. This data confirmed those of Jeronimo et al. (2010) who reported that the inclusion of grape seed extract did not modify meat fatty acid profile.

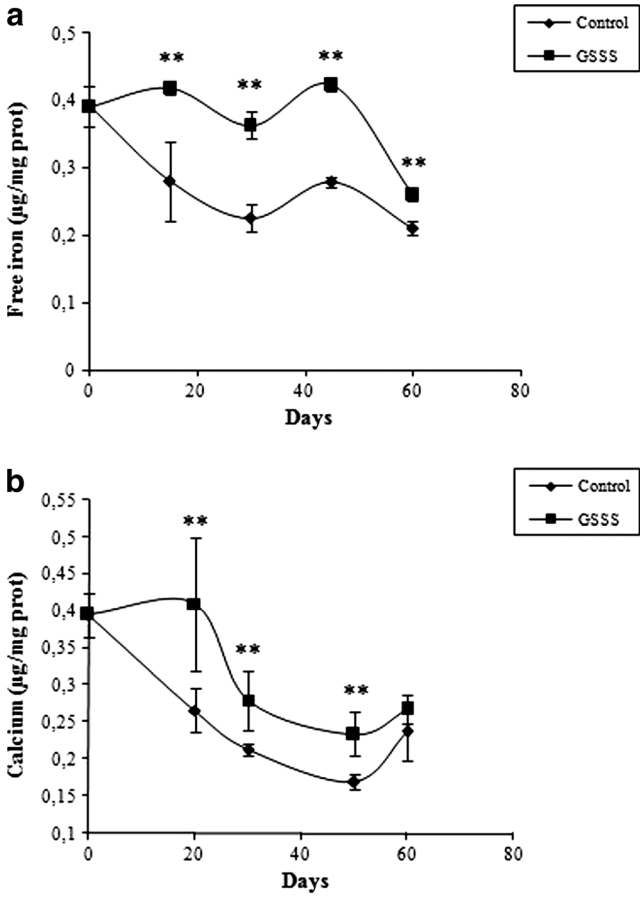

Fig. 5 Effect of GSSS on milk free iron (**a**) and calcium (**b**). Ewes were ingested or not with GSSS (20 %) during 2 months. Results are expressed as mean ± SEM ($n = 5$). $^{**}P < 0.01$ vs. control

These results also corroborate the lack of any effect of dry citrus pulp supplement on milk production and composition of dairy ewes (Fegeros et al. 1995). However, our data are in discordance with those of Anantasook et al. (2015) who described a positive effect of tannins on milk protein and fat content.

pH of ewes' milk changed consistently during lactation as it decreased slowly till the end of lactation (Sahan et al. 2005). In our study, pH slightly decreased between the 1st and 15th day of lactation and increased thereafter to reach a plateau till the end of lactation. A similar tendency was reported by Pugliese et al. (2000). On the other hand, Pavič et al. (2002) reported a gradual increase of pH during lactation and proteins are well-known contributors to milk acidity (Sahan et al. 2005). In fact, acidity changes as a result of the variations in the amount of proteins which could explain the stability of pH since our results also showed no difference in protein content during GSSS treatment. The percentage of lactic acid present in milk is a rough indication of its age and the way whereby it has been handled.

Interestingly, there was a significant ($P < 0.05$) improvement in urea content in GSSS-supplemented animals during the last month of treatment. A similar tendency and a comparable urea level were observed for goat lactation by Kuchtík and Sedláčková (2003). Milk urea nitrogen (MUN) content is related to the protein content of animal nourishment and is used to define an adequate diet.

Since oxidation of polyunsaturated fatty acids generates MDA, the measurement of this lipoperoxidation by-product could be used as an indicator of milk alteration. Lipoperoxidation was slightly ($P < 0.05$) increased in ewes supplemented with 20 % GSSS during 20 days (from the 20th day to the 40th day). At the end of the treatment, MDA levels were almost similar for the two groups. Such a result is rather conflicting as GSSS is well recognised as a powerful antioxidant in goats (Di Trana et al. 2015). Moreover, our results also indicated no difference in hydrogen peroxide levels between the two groups which is in contrast with those of Lin et al. (2001), who described the ability of *Terminalia catappa tannin* extract to prevent lipid peroxidation and modification of mitomycin C-induced clastogenicity. Such a discrepancy could be explained by differences in polyphenol origin (Bhat et al. 2013).

According to the data of calcium and free iron content in milk samples, GSSS induces a clear enrichment in these two minerals. Data on mineral content of ewes' milk during lactation are scarce (Voutsinas et al. 1988). Generally, the ewes' milk is a convenient source of calcium, phosphorus and potassium. Average calcium level in ewes' milk samples found in the present study is higher than previously reported (Yücecan 1992; Mehaia 1994) as it varied from 0.4 µg/mg of proteins to 0.26 µg/mg of proteins, depending on the period of lactation. The highest contents in Ca and free iron were found at the beginning of the treatment and declined thereafter. Fluctuation in Ca content and a markedly higher Ca level in the course of lactation were also described by Sahan et al. (2005).

Future studies should extend the present findings, in particular by evaluating the dosing effect of GSSS on lactose and casein content and on iron and calcium enrichment.

Conclusion

The addition of GSSS to lactating dairy ewes' diets clearly increased milk production. After the 8th week of lactation, there was no significant difference in milk composition (protein, fat) except for urea, free iron and calcium content. The addition of GSSS at the first stage of ewes' lactation increases milk yield, free iron and calcium and could be used to improve milk production and quality.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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