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Effect of the inclusion of fresh lemon pulp in the diet of lactating ewes on the properties of milk and cheese

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Abbreviations: BPF, citrus by-product feedstuffs; FLP, fresh lemon pulp; GAE, gallic acid equivalent; TEAC, Trolox equivalent antioxidant capacity; FA, fatty acids; FAME, fatty acid methyl esters; CLA, conjugated linoleic acid; VA, vaccenic acid; RA, rumenic acid; SCC, somatic cell count

Highlights

- Fresh lemon pulp (FLP) was confirmed to be a high-energy feed for dairy ewes
- Milk fatty acids from FLP-fed ewes were richer in vaccenic and rumenic acids
- Total phenolic content and antioxidant activity were higher in FLP cheeses
- FLP can be used as a natural antioxidant to improve sheep health status and the health properties of dairy products

Abstract

This study investigated the effects of fresh lemon pulp (FLP), as a natural antioxidant in the diet, on the intake of feed and the production of milk and cheese of Valle del Belice lactating ewes during the hot summer in Sicily. A total of 15 second-lambing ewes, kept individually in 3 × 3 m pens, were divided into 3 homogeneous groups fed with 3 diets in a 3 × 3 Latin square design, with 3 experimental phases of 21 days each. The diets were: mixed hay ad libitum plus 600 g/day of concentrate (FLP0); mixed hay ad libitum plus 400 g/day of concentrate and 1 kg/day of FLP (FLP1); and mixed hay ad libitum plus 200 g/day of concentrate and 2 kg/day of FLP (FLP2). Nine experimental Pecorino cheeses were manufactured with bulk milk collected at the end of each phase from each group. The ewes of each group showed the same dry matter (DM) intake (2 kg/day/head), but the FLP2 group received lower (P=0.001) net energy for lactation (NE_L) than other groups (2.13, 2.36, and 2.31 Mcal/day per head for FLP2, FLP0, and FLP1, respectively). The FLP constituted 9% and 16% of the total DM intake in the FLP1 and FLP2 groups, respectively. In general, the daily milk yield was low, reflecting the effect of the high environmental temperatures, and was lower (P=0.001) in the FLP2 group than in the other groups (323, 355, and 369 g/day for FLP2, FLP1, and FLP0, respectively), probably due to the lower daily energy intake. Milk protein (P=0.046) and casein (P=0.033) percentages were higher in the FLP2 group than in the FLP1 group;

the FLP-fed groups had higher levels of (P=0.011) milk urea than the FLP0 group, due to a higher (P=0.001) CP/NE_L ratio in the ingested diet (96.4, 95.8, and 95.3 g/Mcal for FLP2, FLP1, and FLP0, respectively). The fatty acid composition of milk from FLP2-fed ewes was higher in vaccenic (10.6 vs. 7.96 mg/g fat; P=0.031) and rumenic acids (6.21 vs. 5.30 mg/g fat; P=0.048) than that in milk from FLP0 ewes. The characteristics of the cheeses were not influenced by the diet, with the exception of the total content of phenolic compounds (P=0.011) and antioxidant activity (P=0.051), both of which were higher in cheeses made with milk from FLP-fed ewes.

Key words: fresh lemon pulp; lactating ewes; milk fatty acids; cheese

1. Introduction

Citrus fruits are consumed by humans principally as fresh fruit or processed juice, either fresh or reconstituted from concentrate. The residues that remain after the juice is extracted from the fruit comprise peel (flavedo and albedo), pulp (juice sac residue), rag (membranes and cores), and seeds. These components, either individually or in various combinations, are the source materials from which citrus by-product feedstuffs (BPF) are produced. Citrus BPF are suitable energy sources for ruminant feed in many areas of the world (Bampidis and Robinson, 2006) because ruminants are able to ferment highly fibrous feeds in the rumen. An important benefit of citrus BPF–based feeding is its relatively low cost. Reduction of feed costs, while maintaining high productivity, is a primary strategy to achieve economic efficiency in ruminant production.

Citrus BPF contain a variety of energy substrates, including soluble carbohydrates and rapidly degradable NDF made up of cellulose and pectin. Because of these nutritive characteristics, which have fewer negative effects on the rumen ecosystem than supplementation with starch or sugar-rich feeds, citrus BPF can be used as a high-energy feed to support growth and lactation in ruminants (Bambidis and Robinson, 2006).

Citrus pulp is usually fed dehydrated; however, it can also be fed fresh or as silage, although fresh citrus pulp is generally transported only for short distances because of its high moisture content and high transportation costs (Bambidis and Robinson, 2006). Fresh citrus pulp can meet some of the water requirements of ruminants, which can be important in areas characterised by hot, dry summers. The use of dried and ensiled citrus pulp for ruminant nutrition has been studied extensively (Bambidis and Robinson, 2006; Volanis et al., 2006), whereas studies on fresh citrus pulp fed to sheep appear to be limited. Among citrus BPF, lemon pulp is less readily available than orange pulp, but it shows particular characteristics that make it more suitable for fresh consumption; in fact, the lower level of residual sugars, which often supports secondary fermentation and/or mould growth, and the lower pH contribute to its preservation (Bambidis and Robinson, 2006). Both lemon pulp and orange pulp are generally very rapidly accepted by ruminants, but pulp and peels from lemons are somewhat more acceptable than those from oranges and grapefruit (Bath et al., 1980). The higher antioxidant properties of lemons (total polyphenols, essential phenols, ascorbic acid) compared to other citrus fruits, such as oranges and grapefruits (Gorinstein et al., 2001), also make them preferable for the prevention of cancer and degenerative diseases in human nutrition (Tripoli et al., 2007).

Few papers have reported on the utilisation of fresh citrus pulp in the diet of dairy sheep. Studies have examined the effects of whole citrus fruits (Clementines) on lactating ewes and found analogous effects on ruminal parameters between whole citrus fruits and fresh citrus pulp (Piquer et al., 2009) and no negative effects on milk (Jaramillo et al., 2009; Piquer et al., 2011) and cheese production (Jaramillo et al., 2009). A dietary integration of 2 kg/day per head or ad libitum administration of fresh lemon pulp (FLP) has been reported for dairy ewes in Sicily, and their effects on milk yield and composition (Scatassa et al., 2006; Todaro et al., 2006) and cheese characteristics (Chiofalo et al., 2004; Todaro and Scatassa, 2011) have been evaluated. These studies have seen no negative effects on milk production, instead highlighting increased milk yield in dairy ewes fed with FLP ad libitum (Todaro et al., 2006). The effects on cheese characteristics

regarded mainly the flavour, attributed to the higher presence of limonene and β-pinene in Pecorino cheeses made with milk from FLP-fed ewes (Chiofalo et al., 2004), while some problems linked to acidification of the curd were observed in the production of *pasta filata* cheese made from raw milk, as in the Vastedda della valle del Belice Protected Designation of Origin cheese (Todaro and Scatassa, 2011).

In Sicily, a greater amount of fresh lemon pulp is available in early summer, when the by-product can be properly used to compensate for the shortage of fresh forage in the diet of dairy ewes, which are in an advanced stage of lactation at that time. Accordingly, the objective of the present study was to evaluate the effects of different levels of FLP fed to late-lactation ewes in summer, on the basis of their feed intake, in terms of milk and cheese yield and quality, with particular regard to the fatty acid (FA) composition of milk and the polyphenol enrichment and antioxidant capacity of cheese.

2. Material and methods

2.1. Animals, experimental design, and diets

The experiment was conducted at the farm of the Istituto Sperimentale Zootecnico per la Sicilia (Sicilian Region, Palermo, Italy) for a period lasting 10 weeks (June to August 2015). During the experimental period, minimum environmental temperatures ranged from 20°C to 27°C and maximum temperatures were between 28°C and 37°C. At the farm, 15 second-lambing Valle del Belice ewes in late lactation were selected and assigned to 3 groups that were homogeneous in terms of days in milk (155±12 days), body condition score (2.36±0.18) and daily milk yield (372±119 g/day). The ewes were housed in individual 3 × 3 m pens during the experimental period. After a 2-week period of adaptation to these housing conditions, the 3 groups of ewes were fed 3 different diets in succession, according to a 3 × 3 Latin square design, with each phase composed of 14 days of adaptation to the diets and 7 days for data and sample collection (sampling week).

The 3 experimental diets were based on the same vetch-oats hay offered ad libitum, integrated with 600 g/day of commercial concentrate only (FLP0), 400 g/day of commercial concentrate plus 1 kg/day of fresh lemon pulp (FLP1), or 200 g/d of commercial concentrate plus 2 kg/d of fresh lemon pulp (FLP2). In practice, therefore, 1 kg of FLP replaced 200 g of the commercial concentrate.

2.2. Sampling and analysis

At the beginning and end of each experimental phase, all ewes were weighed and monitored for their body condition score (BCS) according to Russel et al. (1969). During each sampling week, all offered and refused feeds of each ewe were weighed and sampled daily to estimate the amount and quality of feed intake.

The samples of mixed hay, commercial concentrate, and FLP were pooled for each sampling week and analysed following the procedures of the AOAC (2005) to determine the concentrations of dry matter (DM, 934.01), ash (942.05), crude protein (CP, 2001.11) and ether extract (EE, 920.39). Concentrations of NDF (aNDFom, 2002.04), ADF (ADFom, 973.18) and ADL (973.18) were determined in accordance with AOAC (2005) and Van Soest et al. (1991), using heat-stable amylase and expressed exclusive of residual ash. Non-fibre carbohydrate (NFC) content was calculated as (1000 − [CP + EE + ash + aNDF]). The energy value, expressed in Mcal of net energy for lactation (NE_L), was estimated using National Research Council equations (NRC, 2001). The samples of FLP were analysed to determine the total pectin content according to Tateo (1969).

The FA compositions of lyophilised samples of hay, FLP and concentrate (50 mg) were determined using the one-step extraction and transesterification procedure of Sukhija and Palmquist (1988), with C19:0 as the internal standard (Sigma-Aldrich, Milano, Italy). Identification of feed FA was performed using the same procedure described below for milk FA.

All feed extracts were also prepared so that their antioxidant status could be measured based on their total content of phenolic compounds and Trolox equivalent antioxidant capacity (TEAC

assay). The extraction of phenolic compounds from feeds was performed as described by López-Andrés et al. (2013). Feeds (2.5 g) were finely chopped (<1 mm) and weighed in 50-ml centrifuge tubes. Samples were homogenised with 15 ml of acetone/water (70/30, v/v) for 60 s at 4000 rpm using a high-speed homogeniser (Art-Miccra D-8, Moderne Labortechnik, Müllheim, Germany). Samples were then sonicated for 6 min (with a break of 2 min after the first 3 min of sonication) in an ultrasonic bath (LBS1 sonicator, Falc Instruments, Treviglio, Italy). Samples were kept in a water/ice bath during the homogenisation and sonication procedures. The sonicated homogenates were centrifuged at 3000 rpm for 15 min at 4°C. The supernatants were filtered through Whatman No. 541 filter paper.

The total concentration of polyphenols in the feed sample extracts was measured using the Folin–Ciocalteau colorimetric method, as described by López-Andrés et al. (2014). Briefly, 100 μl of the sample extract was transferred into a 15-ml centrifuge tube; then, 900 μl of distilled water and 500 μl of the Folin–Ciocalteau reagent, diluted to a concentration of 1 N with distilled water, were added. After 1 min, 2.5 ml of 20% (w/v) sodium carbonate was added; the mixture was vortex mixed for 30 s and incubated for 40 min in the dark at room temperature. The absorbance of the samples was read at 725 nm using an HACH DR/4000U spectrophotometer (HACH, Loveland, CO, USA) against a blank containing all of the reagents except the sample extract. Gallic acid aqueous solutions of different concentrations (0 to 1 mg/ml) were used for the calibration curve ($R^2 = 0.99$). The results were expressed as grams of gallic acid equivalent (GAE) per kilogram of sample DM.

The antioxidant activity of the feed sample extracts was analysed by the TEAC assay, as described by Re et al. (1999). This is a decolourisation assay that measures the radical-scavenging ability of samples using the radical cation ABTS•+ and Trolox as standard. The radical cation ABTS•+ was produced by reacting a 14-mM ABTS (2,2´-azinobis-(3-ethylbenzthiazoline-6-sulfonate)) aqueous solution with an equal volume of 4.9 mM potassium persulfate and incubating the mixture in the dark at room temperature for 16 h before use to allow the complete radicalisation of the ABTS. The

ABTS•+ radical solution was diluted with 5 mM phosphate-buffered saline (pH 7.4) and equilibrated at 30°C to reach an absorbance of 0.750 ± 0.020 at 734 nm. A blank reading (B_0) at 734 nm was taken after mixing 4.0 ml of the diluted ABTS•+ solution with 40 μl of distilled water; this was repeated (B_6) after 6 min of incubation at 30°C to observe any decrease in absorbance caused by spontaneous decolourisation of the radical solution during the assay, and the percentage of spontaneous inhibition (SP %) was calculated as ($[B_0-B_6]/B_0 \times 100$). For each sample, 40 μl of its extract was mixed with 4.0 ml of the diluted ABTS•+ solution, and the absorbance (S_6) at 734 nm was read after 6 min of incubation at 30°C. The percentage decrease of the absorbance in the sample mixture was calculated as ($[B_0-S_6]/B_0 \times 100$), and then the SP % was subtracted. The antioxidant capacity of samples was calculated by relating the percentage of inhibition to that measured under the same conditions (after 6 min and at 734 nm) exerted by solutions of known concentrations (ranging from 0 to 2.5 mM) of Trolox in PBS obtained from a stock solution of 2.5 mM in PBS; these results were used to perform a calibration curve ($R^2 = 0.99$). The results were expressed as mM of Trolox equivalent (TE) per 100 g of sample DM.

2.3. Milk yield and composition

During the sampling week of each experimental phase, individual milk yields were recorded daily at morning milking (7:00 am) and sampled three times on days 3, 5, and 7 of the week. Individual milk samples were analysed for lactose, fat, protein, casein, and somatic cell count (SCC) by the infrared method (Combi-foss 6000, Foss Electric, Hillerød, Denmark); pH was measured with a HI 9025 pH-meter (Hanna Instruments, Ann Arbor, Michigan, USA); titratable acidity was assessed by the Soxhlet-Henkel method (°SH/50 ml); and urea was calculated by the enzymatic method using the difference in pH (CL-10 Plus, Eurochem, Italy).

2.4. Milk fatty acid composition

Milk FAs were determined using individual milk samples collected at the end of each sampling week; these were stored at −20°C and then freeze-dried. FAs in freeze-dried milk samples (100 mg) were directly methylated in 1 ml of hexane with 2 ml of 0.5 M $NaOCH_3$ at 50°C for 15 min, followed by 1 ml 5% HCl in methanol at 50°C for 15 min, based on the bimethylation procedure described by Lee and Tweed (2008). Fatty acid methyl esters (FAME) were recovered in hexane (1.5 ml). One microliter of each sample was injected by autosampler into an HP 6890 gas chromatography system equipped with a flame-ionisation detector (Agilent Technologies, Santa Clara, CA, USA). FAME from all samples were separated using a capillary column 100 m in length with an internal diameter of 0.25 mm and film thickness of 0.25 μm (CP-Sil 88, Chrompack, Middelburg, The Netherlands). The injector temperature was kept at 255°C and the detector temperature was kept at 250°C, with an H_2 flow of 40 ml/min, an air flow of 400 ml/min, and a constant helium flow of 45 ml/min. The initial oven temperature was held at 70°C for 1 min, increased by 5°C/min to 100°C, held for 2 min, increased by 10°C/min to 175°C, held for 40 min, then finally increased by 5°C/min to a final temperature of 225°C and held for 45 min. Helium, with a head pressure of 23 psi and a flow rate of 0.7 ml/min (linear velocity of 14 cm/s), was used as the carrier gas. A FAME hexane mix solution (Nu-Check-Prep, Elysian, MN, USA) was used to identify each FA. Individual standards (Larodan Fine Chemicals AB, Malmö, Sweden) were used to identify C15:0 *iso*, C15:0 *anteiso*, C17:0 *iso*, and C17:0 *anteiso*. A standard mixture of methyl esters of C18:2 *c9 t11* and C18:2 *c10 t12* (Sigma, Milano, Italy) with published isomeric profiles (Kramer et al., 2004; Luna et al., 2005) was used to help identify the conjugated linoleic acid (CLA) isomers. Total FAs were quantified using C23:0 (Sigma) as an internal standard (4 mg/g freeze-dried sample). An index of the desaturation of C18:1 *t11* (vaccenic acid [VA]) to CLA C18:2 *c9 t11* (rumenic acid [RA]) was calculated as the ratio (RA/[RA + VA]) to express the efficiency of Δ^9-desaturase activity in the mammary gland.

2.5. Cheese making and analysis

At the end of each sampling week of the experimental phase, 1.5 L of bulk milk from each group was collected, and experimental micro–cheese-making processes were developed to manufacture Pecorino cheeses. Briefly, the raw milk from each batch was heated to 38°C, and liquid calf rennet was added to it. After the milk had clotted (after approximately 20 min), the curd was broken down until it reached the size of small maize grains. The curd was cooked under hot whey at 70°C for 20 min and, after removal from the vat, was pressed into cylindrical, perforated moulds to drain the whey. The cheeses (3 for each experimental group) were transferred to a cellar under a temperature of 16°C and a relative humidity of 80% for 7 days.

Bulk milk samples were analysed for fat and protein by the infrared method (Combi-foss 6000, Foss Electric, Hillerød, Denmark).

Cheeses aged 7 days were weighed and analysed using standard methods of FIL-IDF for DM (4A:1982; IDF,1982), protein (25:1964; IDF, 1964), and fat (5B:1986; IDF, 1986) content.

Cheese extracts were prepared, according to the method of Rashidinejad et al. (2013) with slight modifications, to measure cheese antioxidant status by the determination of total phenolic compound content and the TEAC assay with the same methods already described for feeds (López-Andrés et al., 2014; Re et al. 1999). To prepare the cheese sample extracts, milled and freeze-dried cheese samples (0.5 g) were homogenised for 30 s and then extracted for 30 min with 25 ml of methanol (95% aqueous solution) containing 1% HCl at 50°C on an orbital shaker at 200 rpm. The mixture was cooled and filtered with cheesecloth, and the residues were washed with 1 ml of the same solvent (95% methanol aqueous solution with 1% HCl) and then centrifuged at 7000 rpm for 10 min at 9°C.

2.6. Statistical analyses

Statistical analysis of each ewe's individual data was carried out using the MIXED procedure in SAS 9.3 software (2011). In the mixed model, which was used for live weight, BCS, and milk FA composition, the experimental phase (3 levels) and diets (FLP0, FLP1, FLP2) were fixed factors,

and the ewe was considered a random factor and used as an error term. For data on the ewes' feed intake and milk production, the effects of experimental phase, day within sampling week (three levels), and diet were tested by means of a repeated-measures mixed model, with the experimental day being used as the unit for repeated measures and the ewe being the repeated subject, treated as a random factor. Before analysis, SCC values were transformed into logarithmic form (Log_{10}). Statistical analyses of cheeses and bulk milk traits were performed using the GLM procedure in SAS 9.3 (2011) with experimental phase and diet as fixed factors. When a statistically significant effect ($P \leq 0.05$) of the diet was detected, means were compared using *p*-values adjusted according to the Tukey-Kramer multiple comparisons test.

3. Results

3.1. Feed characteristics

The average chemical compositions of mixed hay, concentrate, and FLP are reported in Table 1. Compared to the other dietary components, FLP is richer in water and shows a balance between NFC and NDF, with a low content of non-digestible fibre (ADL). With respect to phenolic compounds, FLP shows a value that is 4-5 times higher than hay and concentrate; consequently, the TEAC level was 5 times higher than that of the other types of feed.

3.2. Feed intake

The effects of different amounts of FLP in the diet on ewes' live weight, BCS, and DM and nutrient intake are reported in Table 2. No significant effect of diet was found for the final body weight and BCS of ewes.

Equal amounts of mixed hay were ingested by the ewes in the different experimental groups, despite the different intakes of concentrate and FLP. All concentrate administered was eaten, while the FLP2 ewes ingested only 315 g/day of lemon pulp DM, corresponding to 1.64 kg/day of the 2 kg/day administered per head. Overall, FLP constituted 9% and 16% of the total DM intake in the

FLP1 and FLP2 groups, respectively. With respect to nutrient intake, the FLP2 ewes ingested significantly lower CP and NE_L than the other groups. Both FLP groups showed a significantly higher dietary CP/NE_L ratio and phenol intake compared to the FLP0 group.

3.3. Milk yield and composition

The effects of FLP diets on milk yield and the composition of individual milk samples are reported in Table 3. Lower milk yield of the ewes was associated with a higher level of FLP in the diet. The levels of the main components showed an increase in the milk of FLP2 ewes. Whereas the increase in milk fat percentage did not reach statistical significance, milk protein and casein percentages were significantly higher in the milk of ewes fed the FLP2 diet than in that from FLP1 ewes. The milk urea content of the FLP groups was significantly higher than in the FLP0 group. The other milk parameters were not influenced by the diet.

3.4. Milk fatty acid composition

The effects of FLP diets on milk FA composition are reported in Table 4. The diet significantly affected only a few FAs, such as C15:0 *anteiso*, C15:0, VA (C18:1-*t*11), and RA (CLA C18:2 *c9 t119*). The C15:0 *anteiso* levels were significantly higher in FLP2 milk than in FLP0 milk (P=0.008), and the same trend was observed for other *iso* and *anteiso* FAs, although the differences were not significant; as a consequence, the level of branched-chain FAs tended to be higher in FLP2 milk. The C15:0 levels were higher in milk from ewes fed both FLP diets. Levels of VA (P=0.031) and RA (P=0.048) were significantly higher in FLP2 milk than in FLP0 milk. Also, CLA isomers tended to be higher in FLP2 milk. On the whole, the milk from FLP2 ewes showed higher total FA content, mainly attributable to the higher level of saturated FAs, while the levels of monounsaturated FAs and polyunsaturated fatty acids (PUFA) did not differ among groups. The desaturation ratio of RA to the sum of RA+VA tended to be higher in FLP0 milk.

3.5. Bulk milk and cheese characteristics

The effects of FLP diets on bulk milk composition and cheese characteristics are reported in Table 5. No significant effects of the diet were found for bulk milk composition and cheese characteristics, with the exception of total phenolic compounds, the levels of which were statistically significantly higher in cheeses produced with milk from FLP groups; the phenolic enrichment was directly proportional to the amount of the by-product ingested by ewes. In the same way, TEAC values of cheeses made with milk of FLP-fed ewes were statistically significantly higher than those of cheeses made from FLP0 milk. On the basis of phenols ingested by ewes and those recorded in the cheeses, phenol recovery was significantly higher in the FLP groups than in the control group.

4. Discussion

4.1. Feed characteristics

The composition of citrus fruit is affected by factors such as growing conditions, maturity, rootstock, variety, and climate (Kale and Adsule, 1995); therefore, the FLP utilised in this study shows chemical characteristics that could be different to other FLP (Bampidis and Robinson, 2006). Unfortunately, there is little available literature on the use of FLP in animal feed. The FLP used in this study, consisting of peel, pulp, and seeds, was obtained after the cold extraction of lemon juice and immediately transferred to the farm before the start of the experiment. This by-product is thus richer in water, which is especially advantageous during the hot summer months. The FLP shows a CP percentage similar to that of previous studies in Sicily (Scatassa et al., 2006; Todaro et al., 2006) but higher than others (Bampidis and Robinson, 2006). The Van Soest fibre fractions are similar to values in the literature, whereas the pectin content characteristic of this type of by-product shows intermediate values compared to other studies (Bampidis and Robinson, 2006; Wang et al., 2008). A high value of pectin produces favourable effects on NDF digestibility (Ben-Ghedalia et al., 1989); in fact, pectin is degraded very rapidly and extensively in the rumen but, unlike starch, it

yields little butyrate and lactate, causing a smaller decrease in rumen pH (Strobel and Russell, 1986; Barrios-Urdaneta et al., 2003).

The most interesting finding is the FLP's high phenol content: it was 8 to 10 times higher than other data reported in the literature (Ramful et al., 2011; Santos et al., 2014). Most of the polyphenols in citrus fruits, as well as in FLP, consist of flavonoids, which are potent antioxidant compounds with anticancer, antiatherogenic, antimicrobial and anti-inflammatory properties (Tripoli et al., 2007); thus, FLP is also a potentially healthy food. Moreover, the antioxidant activity of FLP (8.06 mmol trolox/100 g DM, equivalent to 15.5 μmol trolox/g fresh matter), as assessed using the TEAC assay, was high, in line with the content of antioxidant polyphenolics. In fact, levels were much higher than the highest value (9.92 μmol trolox/g fresh weight) reported by Ramful et al. (2011) for pulp extracts of several varieties of citrus fruits. This indicates that peel extracts have higher antioxidant activity, as suggested by Ramful et al. (2011).

4.2. Feed intake

Heat stress due to high ambient temperatures such as those which occurred during this experiment, commonly results in decreased feed intake in sheep (Marai et al., 2007). In this regard, the experimental groups responded similarly to the heat stress, since they had the same total DM and NDF intake. Nevertheless, the intake of FLP in the FLP2 group was lower than the amount offered, equal to 2 kg of fresh matter, presumably due to the high hydration of the FLP, as also discussed by Santos-Silva et al. (2016), that would have increased the volume of diet in respect to the voluntary feed intake of ewes, already reduced as a consequence of the high summer temperatures.

Since the diets were computed to be isoenergetic and isoproteic, the lower FLP intake of the FLP2 ewes resulted in lower CP and NE_L intake, with negative effects on milk yield. In addition, FLP diets resulted in a higher CP/energy ratio. This fact became more pronounced with the higher level of dietary FLP inclusion and can be considered responsible for the increase in milk urea content, as

will be discussed later. The higher phenol content of FLP compared to the other feeds resulted in a significantly and progressively higher intake of phenolic compounds as the intake of FLP increased.

4.3. Milk yield and composition

The late stage of lactation of the ewes and the hot summer season, which easily exceeds 30°C in Sicily, led to a low daily milk yield, so that the ewes were milked only once a day. In these environmental conditions, the low daily milk yield is justified for ewes of the Valle del Belice breed, which are characterised by a high level of milk production (Todaro et al., 2014). Analogous low levels of milk yield, ranging from 400 to 350 g/day, have been reported for ewes reared in Sicily during late lactation in summer (Bonanno et al., 2013). In these difficult conditions, the lower CP and NE_L intake of the FLP2 ewes further lowered their daily milk yield compared to the other ewes. Other authors, utilising several types of citrus pulp (dehydrated or ensiled) in diets for lactating ewes, obtained conflicting results: some authors did not observe any effect (Fegeros et al., 1995; Volanis et al., 2006), while others found a positive effect on daily milk yield (Piquer et al., 2011). Todaro et al. (2006), utilizing the FLP as supplement to the normal diet of lactating ewes in summer pasture, obtained an increase in daily milk yield only when the administration was made *ad libitum* (Todaro et al., 2006).

Often the changes in the percentage of milk constituents, such as fat and protein, are linked to milk yield, with negative correlation coefficients due to a dilution effect. In the present study, this relationship supports mainly the increase in protein and casein percentages in the FLP2 milk, whereas there was a small, insignificant increase in the content of milk fat. In this regard, the specific literature reports contrasting results, due to the different experimental conditions, especially with regard to the characteristics and the inclusion level of the citrus by-product used. Some authors (Volanis et al., 2006) who found a significant increase of milk fat percentage in ewes fed with ensiled citrus pulp attributed their results to the easily digestible fibre in citrus pulp silage, which creates a favourable condition in the rumen. The improved microbial activity, which leads to fibre

degradation and subsequent higher production of acetic acid than propionic acid (Piquer et al., 2009), promotes milk fat synthesis in the mammary glands. Opposite results were seen by Piquer et al. (2011), who studied the effect of adding whole citrus fruits (WCF) to the ration of dairy ewes on the composition of milk and analysed a large number of samples during the lactation period. The results obtained by these authors showed that the use of 30% of WCF in the diet led to a reduction in milk fat content, whereas greater protein content corresponded to the milk from the ewes with 10% of citrus in the ration. Moreover, Jaramillo et al. (2009) found that milk from ewes fed a diet that included 30% fresh citrus fruit (*Citrus clementina*) cultivated in the east of Spain showed 15% lower fat percentages compared to control milk. In contrast, Fegeros et al. (1995), who evaluated the use of dry citrus pulp (11%) in the ration of dairy ewes, did not observe any significant effect of diet on milk composition.

Supplementation of ruminant diets with citrus pulp may improve N utilisation (Kim et al., 2007; Piquer et al., 2009), reducing the ruminal NH_3-N escape and, consequently, the milk urea level; several studies support this hypothesis, both in dairy cows (Santos et al., 2014) and in dairy ewes (Scatassa et al., 2006). Nevertheless, the present study found the opposite, since the milk from the FLP ewes had higher urea content than the milk from the control group. An explanation for this result can be probably found in the higher CP/NE_L ratio of FLP diets; this surplus of CP intake, in respect to dietary energy, can be considered responsible for the increase in the ruminal NH_3-N escape, with the formation of urea in the liver, that is successively moved with urine and milk (Bonanno et al, 2010). However, the higher digestibility of the FLP-based diets, particularly due to the highly degradable carbohydrates (Piquer et al., 2009), could have also contributed to reducing the nitrogen losses in the faeces, thus increasing the proportion of nitrogen excreted in urine and milk.

4.4. Milk fatty acid composition

The composition of milk FAs is affected by several factors, among which the composition of diet is predominant. Because citrus pulp contains high levels of unsaturated FAs and phenolic compounds (Bampidis and Robinson, 2006), it would be reasonable to expect a change in the ruminal biohydrogenation pathway, and then in the bioavailability of unsaturated long-chain FAs for milk fat synthesis within the mammary gland. Nevertheless, recent studies have reported that feeding lactating cows pelleted citrus pulp (Santos et al., 2014) or replacing cereals fed to dairy ewes with dried citrus pulp supplemented with vegetable oil (Santos-Silva et al., 2016) alters the long-chain FA profile very little in milk. In agreement with those results, the inclusion of FLP in the diet of ewes in the present study affected the milk FA profile, but only weakly, with few significant differences but clear trends.

On the whole, all of the *iso* and *anteiso* FA, of which C15:0 *iso*, C16:0 *iso*, and C15:0 *anteiso* are recognised for their anti-cancer activity (Wongtangtintharn et al., 2004; Parodi, 2009), increased linearly with increases of FLP in the diet, and then contributed to a tendency toward increases in the total branched-chain FA. This trend confirms that observed by Lanza et al. (2015) in the FA profile of the ruminal fluid from lambs fed citrus pulp, and that by Santos-Silva et al. (2016) in sheep milk, with regard to *iso* FAs. However, in this study, the increase recorded with inclusion of the lemon by-product in the diet reached significant levels only for C15:0 *anteiso*, as well as for C15:0, for which no support has been found in the recent literature. Both branched and linear odd-chain FAs in milk derive mainly from microbial synthesis in the rumen (Vlaeminck et al. 2006); for this reason, they are considered markers of the activity of the ruminal bacteria, and their variations reflect any changes in the rumen environment and the microbial population induced by the diet characteristics. According to Vlaeminck et al. (2006), the *iso* FAs result from the biosynthesis of cellulolytic bacteria, which is favoured by diets with a high fibre level or forage:concentrate ratio; the *anteiso* and linear odd-chain FAs would derive mainly from the activity of amylolytic bacteria, whereas the *de novo* synthesis in the mammary gland of linear odd-chain FAs can be considered limited. On this basis, the increased levels of C15:0 *anteiso* and C15:0 in milk associated with the FLP-based diets

can be interpreted as induced, on the rumen level, by the particular composition of the lemon by-product; indeed, the high levels of rapidly degradable fibre, mainly pectin, together with the high lipid levels and phenolic compound content that characterise this feeding source, could have worked together to favour the activity of bacteria strains producing C15:0 *anteiso* and C15:0.

However, the main effect attributable to the FLP on the milk FA profile concerns the RA, the most abundant among CLA isomers, and its precursor VA, which increased significantly with the highest level of FLP in the diet. These results can be related to the intake of FLP polyphenols, which would have partially inhibited the PUFA biohydrogenating activity of ruminal microorganisms, and promoted the formation of biohydrogenation intermediates such as the VA, as also suggested by Santos et al. (2014) and Santos-Silva et al. (2016). On the other hand, Lanza et al. (2015) fed lambs increasing concentrations of dried citrus pulp to raise the intake of both unsaturated FAs and phenolic compounds as a means of reducing the rate of PUFA biohydrogenation in the rumen; accordingly, they improved intramuscular FA composition, particularly due to the higher content of RA. In addition, Santos-Silva et al. (2016) found that milk from ewes that ingested a level of dried citrus pulp similar to that observed in the FLP2 group in this experiment resulted higher in VA and RA. Both VA and RA originate in the rumen due to the incomplete saturation of dietary PUFA into stearic acid; VA is the last intermediate in the biohydrogenation of linoleic and α-linolenic acids, whereas RA is formed during the first biohydrogenation step of linoleic acid (Bauman et al., 2006; Chilliard et al., 2007). However, RA is formed mainly by the conversion of VA flowing from the rumen, which occurs in the mammary gland through the activity of the Δ-9 desaturase enzyme system (Chilliard et al., 2007; Jenkins et al., 2008). In this study, the efficiency of this conversion of VA into RA in the mammary gland, expressed by the desaturation ratio, was only slightly weaker with the FLP2 diet, probably because of the higher level of VA.

Since RA is well known for its benefits on human health related to its anti-cancer and anti-atherogenic properties (Parodi, 2009; Bauman and Lock 2010), its higher content in the milk enhances the health value of the dairy products obtained from animals fed the lemon by-product.

4.5. Bulk milk and cheese characteristics

The chemical constituents of bulk milk were not significantly different between groups; therefore, the yield and composition of the resultant cheeses, with regard to fat and protein percentages, did not show significant differences. Similar results have found by other authors (Chiofalo et al., 2004; Todaro and Scatassa, 2011) who fed FLP to dairy ewes. Other researchers have found that 60-day ripened cheeses produced with milk from ewes fed increasing doses of Clementine WCF showed lower levels of solids and lower fat percentages (Jaramillo et al., 2009).

The most interesting aspect of this study is the enrichment of polyphenols in cheeses produced with milk from FLP-fed ewes, along with the remarkable improvement of their antioxidant activity, proportional to the level of by-product intake. These results suggest that natural antioxidant compounds in the FLP were transferred from the diet to the milk and consequently to the cheese. On the other hand, the recovery percentage of polyphenol intake in the cheeses from FLP-based diets was 5 times higher than in FLP0 cheeses, probably because of the higher dietary content, as well as the different nature, of the FLP polyphenols.

There is a shortage of information regarding the effect of dietary citrus pulp on milk or cheese antioxidants in dairy cattle or ewes; to date, only one paper has reported on the enrichment of polyphenols and flavonoids and the increase in the antioxidant capacity in milk of dairy cattle fed with a diet with 18% of pelleted citrus pulp (Santos et al., 2014). Moreover, a strong positive correlation between polyphenols and antioxidant capacity, expressed as TEAC ($r=0.95$) or ferric reducing antioxidant power ($r=0.73$), was reported by Ramfull et al. (2011). This correlation was higher than that seen between cheese polyphenols and TEAC in the present study ($r=0.56$; $P<0.01$). This fact supports the results obtained, according to which the antioxidant capacity, expressed as TEAC, of FLP1 and FLP2 cheeses was double that of FLP0 cheeses.

Conclusions

This study increases the knowledge about the effects of including FLP in the diet of dairy ewes, confirming the great interest in this by-product from the citrus industry for animal feeding, because of its low cost, high water content, and good nutritive value. This may make it a good substitute for other, more expensive feed. In fact, based on this study, 1 kg of FLP could replace 200 g of concentrate in the diet of dairy ewes.

The inclusion of FLP in the diets for dairy ewes increased the intake of total polyphenols; accordingly, in addition to the possible benefit to animal health, this by-product could also potentially make related dairy products more healthy. Indeed, due to their ability to inhibit the complete biohydrogenation of dietary PUFA in the rumen, the phenolic compounds improved the health properties of milk fat, mainly through an increase in RA, the main CLA isomer. In addition, the transfer of lemon pulp polyphenols into the milk led to the production of sheep cheeses naturally enriched with compounds with high antioxidant capacity, further enhancing the beneficial properties for human health.

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Table 1
Chemical composition of diet constituents, mean ± SD (g/kg of dry matter)

	Vetch-oats hay	**Concentrate**	**Fresh lemon pulp**
Dry matter	907±6.5	902±3.3	192±39.9
Ether extract (EE)	12.6±4.0	27.6±2.4	47.2±0.9
Crude protein (CP)	93.7±10.4	154±1.3	118±3.3
Ash	88.2±11.9	67.2±0.6	76.5±12.2
NFC[1]	217±37.5	504±6.6	369±38.8
aNDFom[2]	588±35.4	248±5.7	390±22.4
ADFom[3]	489±39.8	156±2.6	239±6.2
Lignin	84.8±15.1	32.8±0.1	77.1±3.8
Pectin	-	-	111±12.4
Total phenolic compounds (g GAE[4]/kg DM)	4.72±1.83	2.37±0.82	18.1±0.07
TEAC[5] (mmol Trolox/100 g DM)	1.55±0.02	1.55±0.01	8.06±0.06
FA[6] composition (g/kg of DM)			
C 10	-	-	0.09
C 12	0.05	0.06	0.20
C 14	0.25	0.07	0.29
C 16	2.73	3.05	12.8
C 18	0.61	0.82	1.83
C 18:1	0.95	7.43	11.4
C 18:2	2.75	12.2	13.2
C 18:3	4.72	0.85	4.61
C 20	0.11	0.15	0.19
Unsaturated FA[6]	8.43	20.5	29.3

[1] Non-fibre carbohydrates = 1000 – (CP + EE + ash + aNDFom); [2] NDF assayed with a heat-stable amylase and expressed exclusive of residual ash; [3] ADF expressed exclusive of residual ash; [4] gallic acid equivalent; [5] Trolox equivalent antioxidant capacity; [6] fatty acids.

Table 2
Effects of diet on live weight, BCS[1], DM[2], and nutrient intake (least square means)

	FLP0	**FLP1**	**FLP2**	**SEM**[7]	***P*-value**
Final live weight, kg	47.3	44.4	47.3	1.30	0.155
Final BCS	2.35	2.37	2.35	0.07	0.941
Intake (g/day per head)					
Mixed hay DM	1517	1554	1504	60	0.719
Concentrate DM	541^{A}	359^{B}	180^{C}	0.79	0.001
Fresh lemon pulp DM	0^{A}	173^{B}	315^{C}	15	0.003
Total DM	2058	2000	2087	67	0.372
Fresh lemon pulp DM/total DM intake (%)		9.01	15.7		
aNDFom[3]	1026	1070	1052	38	0.482
CP[4]	225^{A}	221^{A}	206^{B}	6.52	0.001
NE_L (Mcal/day per head)	2.36^{A}	2.31^{A}	2.13^{B}	0.07	0.001
CP/NE_L[5] (g/Mcal)	95.3^{A}	95.8^{B}	96.4^{C}	0.09	0.001
Phenolic compounds (g GAE[6]/day)	8.44^{A}	11.32^{B}	13.22^{C}	0.46	0.001

A, B, C, means within a row with different superscripts differ ($P \leq 0.01$); [1]body condition score; [2]dry matter; [3] NDF assayed with a heat-stable amylase and expressed exclusive of residual ash; [4]Crude protein; [5]Net energy for lactation; [6]gallic acid equivalent; [7]standard error of mean

Table 3
Effects of diet on milk yield and composition (least square means)

	FLP0	**FLP1**	**FLP2**	**SEM[1]**	***P*-value**
Daily milk yield (g/day)	369^{A}	355^{A}	323^{B}	34	0.001
Fat (g/kg)	81.4	79.0	82.3	2.5	0.191
Protein (g/kg)	66.8^{ab}	66.4^{a}	68.6^{b}	2.3	0.046
Casein (g/kg)	51.9^{ab}	51.3^{a}	53.3^{b}	2.0	0.033
Urea (mg/dl)	27.9^{Aa}	31.5^{Bb}	30.5^{ABb}	1.30	0.011
Lactose (g/kg)	41.1	40.5	40.5	0.8	0.353
Somatic cell count (log_{10} n. cells/ml)	5.41	5.62	5.46	0.21	0.538
pH	6.82	6.81	6.83	0.04	0.681
Titratable acidity (°SH/50 ml)	5.82	5.75	5.95	0.26	0.412

a, b, means within a row with different superscript differ ($P \leq 0.05$), A, B, means within a row with different superscripts differ ($P \leq 0.01$); [1]standard error of mean.

Table 4

Effects of diet on milk fatty acid composition (mg/g of fat) (least square means)

	FLP0	**FLP1**	**FLP2**	**SEM[1]**	***P*-value**
C4:0	21.4	22.0	22.5	0.93	0.693
C6:0	20.4	21.0	22.5	0.89	0.252
C8:0	19.3	20.0	21.7	1.00	0.222
C9:0	0.48	0.58	0.59	0.06	0.401
C10:0	61.7	65.6	71.0	3.69	0.203
C11:0	0.11	0.12	0.38	0.13	0.265
C12:0	37.7	41.0	43.0	2.56	0.305
C13:0	1.94	2.17	2.24	0.20	0.482
C14:0 *iso*	1.61	1.76	1.83	0.11	0.412
C14:0	112	120	124	4.87	0.186
C15:0 *iso*	2.32	2.59	2.81	0.17	0.127
C15:0 *anteiso*	4.45^{A}	5.14^{AB}	5.67^{B}	0.36	0.008
C14:1 *c9*	4.54	5.00	4.66	0.63	0.672
C15:0	12.0^{a}	13.6^{b}	14.0^{b}	0.51	0.031
C16:0 *iso*	3.08	3.38	3.49	0.18	0.263
C16:0	257	272	276	10.87	0.385
C17:0 *iso*	3.45	3.58	3.86	0.23	0.394
C17:0 *anteiso*	1.84	1.79	2.10	0.15	0.222
C16:1 *c9*	18.7	20.5	20.1	1.80	0.432
C17:0	7.66	8.13	8.41	0.32	0.184
C17:1 *c10*	3.12	3.15	3.29	0.16	0.621
C18:0	38.1	37.9	44.7	3.79	0.272
C18:1 *t11* , VA[2]	7.96^{a}	8.44^{ab}	10.6^{b}	0.83	0.031
C18:1 *c9*	124	112	131	9.02	0.222
C18:2 n-6 *c9 c12* LA[3]	14.4	14.6	17.4	1.45	0.236
C18:3 n-3 ALA[4]	4.26	4.39	4.46	0.33	0.894
CLA[5] C18:2 *c9 t11*, RA[6]	5.30^{a}	5.17^{a}	6.21^{b}	0.43	0.048
CLA isomers	6.14	6.08	7.06	0.44	0.103
C20:5 n-3, EPA[7]	0.42	0.36	0.42	0.09	0.845
C22:5 n-3, DPA[8]	0.27	0.12	0.35	0.10	0.201
Saturated FA	614	650	679	19.63	0.082
Monounsaturated FA	173	164	186	9.87	0.193
Polyunsaturated FA	33.18	32.45	37.76	2.44	0.175
Unsaturated FA	206	197	223	11.84	0.174
Total FA	821^{a}	849^{ab}	903^{b}	23.08	0.046
Saturated/Unsaturated	3.19	3.38	3.12	0.16	0.342
Σ omega-6	18.9	18.9	22.5	1.74	0.191
Σ omega-3	5.96	5.47	6.00	0.47	0.652
Omega-6/omega-3	3.27	3.76	3.95	0.32	0.325
BCFA[9]	18.4	20.0	21.6	1.10	0.114
Σ C4-C11	128	135	144	6.02	0.205
Σ C12-C16	472	505	518	18.43	0.175
Σ C17-C24	220	207	242	15.42	0.172
RA/(RA+VA)	0.40	0.39	0.37	0.01	0.102

a, b, means within a row with different superscripts differ (P≤0.05), A, B, means within a row with different superscripts differ (P≤0.01); [1]standard error of mean; [2]vaccenic acid; [3]linoleic acid; [4]α-linolenic acid; [5]conjugated linoleic acid; [6]rumenic acid; [7]eicosapentaenoic acid; [8]docosapentaenoic acid; [9]branched chain fatty acids.

Table 5
Cheese quality characteristics (least square means)

	FLP0	**FLP1**	**FLP2**	**SEM[1]**	***P*-value**
Bulk milk fat (g/kg)	80.9	83.3	85.3	7.7	0.921
Bulk milk protein (g/kg)	67.5	69.4	67.3	3.6	0.913
Cheese yield (kg cheese/100 l of milk)	19.1	22.5	21.3	1.88	0.484
Cheese DM[2] (kg cheese DM/100 l of milk)	62.5	60.8	63.1	1.30	0.474
Cheese protein (g/kg DM)	454	439	457	19.9	0.792
Cheese fat (g/kg DM)	410	449	414	31.2	0.653
Cheese total phenolic compounds (g GAE[3]/kg DM)	1.35[A]	7.47[B]	10.4[C]	1.22	0.011
TEAC[4] (mmol Trolox/100 g DM)	1.24[a]	2.94[b]	2.38[b]	0.47	0.051
Phenolic compounds recovery (%)	0.75[A]	3.27[B]	3.46[B]	0.23	0.001

a, b, means within a row with different superscripts differ (P≤0.05), A, B, means within a row with different superscripts differ (P≤0.01); [1]standard error of mean;[2]dry matter; [3]gallic acid equivalent; [4]Trolox equivalent antioxidant capacity.