Effects of diet on casein and fatty acid profiles of milk from goats differing in genotype for α_{S1}-casein synthesis

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European Food Research and Technology
Zeitschrift für Lebensmittel-Untersuchung und -Forschung A

ISSN 1438-2377

Eur Food Res Technol
DOI 10.1007/s00217-013-2069-8

Springer

Eur Food Res Technol
DOI 10.1007/s00217-013-2069-8

ORIGINAL PAPER

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Received: 3 May 2013 / Revised: 17 July 2013 / Accepted: 18 July 2013

Abstract This study investigated the interactions between nutrition and the genotype at α_{S1}-CN loci (*CSN1S1*) in goats, evaluating the impact of fresh forage-based diets and an energy supplement on the casein and fatty acid (FA) profiles of milk from Girgentana goats. Twelve goats were selected for having the same genotype at the α_{S2}-CN, β-CN, and κ-CN loci and differing in the *CSN1S1* genotype: homozygous for strong alleles (AA) or heterozygous for strong and weak alleles (AF). Goats of each genotype were divided into three groups and, according to a 3 × 3 Latin square design, fed ad libitum three diets: sulla fresh forage (SFF), SFF plus 800 g/day of barley (SFB), and mixed hay plus 800 g/day of barley (MHB). The SFB diet led to higher-energy intake and milk yield. The energy-supplemented diets (SFB, MHB) reduced milk fat and urea and increased coagulation time. The fresh forage diets (SFF, SFB) increased dry matter (DM) and crude protein (CP) intake and milk β-CN. Diet had a more pronounced effect than *CSN1S1* genotype on milk FA profile, which was healthier from goats fed the SFF diet, due to the higher content of rumenic acid, polyunsaturated, and omega-3 FAs. The AA milk had longer coagulation time and higher curd firmness, higher short- and medium-chain FAs (SMFA), and lower oleic acid than AF milk. Significant diet by genotype interactions indicated the higher milk yield of AA goats than AF goats with the higher-energy SFB diet and the lower synthesis of SMFA in AF than in AA goats with the SFF diet.

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Keywords Goat milk · *CSN1S1* genotype · Nutrition · Casein fraction · Milk fatty acid

Introduction

In goats, genetic variants for α_{S1}-casein (α_{S1}-CN) synthesis greatly influence several milk production traits, especially casein content and the cheese making ability of milk [1].

With regard to polymorphisms at α_{S1}-CN loci (*CSN1S1*), 18 alleles have been detected and classified according to their rate of milk casein synthesis: strong (A, B1, B2, B3, B4, B′, C, H, L, M), intermediate (E and I), weak (D, F, and G), and null (O1, O2, and N) alleles that synthesize high (3.5 g/L), medium (1.1 g/L), low (0.45 g/L), and no amounts of α_{S1}-CN, respectively [2, 3].

Goats with strong alleles have a greater ability to synthesize α_{S1}-CN than goats with weak alleles; they also produce milk higher in casein, fat, calcium, and phosphorus, with smaller casein micelles and higher coagulation time (*r*) and curd firmness (a_{30}) [1, 4].

The *CSN1S1* genotype also affects the milk fatty acid (FA) composition; specifically, goats that are homozygous for strong alleles (AA) have more short- and medium-chain FAs (SMFA) and less delta-9-desaturase activity than goats homozygous for weak alleles (FF) [5, 6].

Because feed also exerts a great influence on the yield and properties of goat milk, there is interest in how nutrition might interact with the genetic polymorphism at α_{S1}-CN. Recent researches showed how AA goats, compared with FF goats, more efficiently utilize dietary protein [7–9] and respond to high-energy diets by utilizing nutrients more efficiently and achieving a higher milk yield [10].

In a more recent research [11], goats homozygous for strong alleles at *CSN1S1* loci (AA) and those

Published online: 02 August 2013

heterozygous for a weak allele (AF), which are associated with high and low levels of α_{S1}-CN synthesis, respectively, were compared on the basis of their feeding behavior, metabolic, and hormonal responses, and milk production resulting from different nutrient intake. The choice of the AF genotype depended on the high frequency of heterozygous goats at *CSN1S1* loci in the farms, but also on the small number of researches focused on the heterozygous *CSN1S1* genotype. In that study, the AA goats confirmed, also in comparison with AF goats, the more efficient energy and protein utilization, already evident at the digestive level, and the better productive responses to high-nutrition diets.

Casein and FA play a fundamental role in the nutritional and technological properties of milk. Thus, to further investigate the interactions between nutrient intake and the *CSN1S1* genotype in goats, this paper reports a successive study, conducted within the same research [11], evaluating the impact of a fresh forage diet and/or an energy supplement on casein fractions and FA profile of milk produced by Girgentana goats with different genetic abilities to synthesize α_{S1}-CN. Goats that were homozygous (AA) and heterozygous (AF) for *CSN1S1* alleles were fed diets based on fresh sulla (*Hedysarum coronarium* L.), a legume forage common in Mediterranean areas [12–14], with or without a barley supplement.

Materials and methods

Animals and experimental design

The present experiment was carried out on a farm in Sicily (Santa Margherita Belice, Agrigento) for a period of 11 weeks, from March to May. A total of 40 milking goats were genotyped at the *CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3* loci, codifying for α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN, respectively, using specific PCR protocols at the DNA level [15–18].

Twelve goats in their third or fourth lactation, with 50 or 120 days in milking (DIM) and averaging 37.2 ± 3.5 kg of live weight, were selected for having the same genotype at the *CSN1S2* (AA), *CSN2* (AA), and *CSN3* (AA) loci and a different *CSN1S1* genotype: six goats were homozygous for a strong allele (AA) and the other six were heterozygous for strong and weak alleles (AF).

During the entire experiment, the goats were housed in individual large pens placed inside a closed shed. After a 2-week period of adaptation to their changed housing conditions, the six goats of each *CSN1S1* genotype (AA and AF) were allocated homogeneously, based on DIM, to three groups and fed three diets in succession, according to a 3 × 3 Latin square design with three experimental periods of 21 days each (14 days for adaptation to the diets and 7 days for measuring and sampling).

The three experimental diets consisted of sulla (*Hedysarum coronarium* L.) fresh forage ad libitum (SFF), SFF ad libitum plus 800 g/day of barley meal (SFB), and mixed hay ad libitum plus 800 g/day of barley meal (MHB).

The sulla forage was mowed daily in the morning, cut roughly, and supplied to goats in the feeding trough twice a day, at 10 a.m. and 5 p.m., while the barley meal was divided into two meals.

Sampling and analysis

At the beginning and at the end of each experimental period, all goats were weighed and checked for their body condition score (BCS).

During the last 7 days of each experimental period, the offered and refused forage and barley of each goat were weighed daily and sampled twice to estimate the amount and quality of feed intake. Individual milk yield was recorded daily at morning (7 a.m.) and evening (4 p.m.) milking and sampled three times on days 3, 5, and 7 of the sampling week in each period.

The samples of barley and forage were analyzed for the determination of dry matter (DM), crude protein (CP) [19], and NDF [20]. Their energy content, expressed in Mcal of net energy for lactation (NE_L), was estimated using equations of the National Research Council [21]. In addition, freeze-dried samples of sulla forage were analyzed by spectrophotometer for condensed tannins using the butanol-HCl method [22] and delphinidin as the reference standard [23].

Individual milk samples were analyzed for fat, protein, casein, and somatic cell count using the infrared method (Combi-foss 6000, Foss Electric, Hillerød, Denmark), pH using a HI 9025 pH-meter (Hanna Instruments, Ann Arbor, MI, USA), titratable acidity using the Soxhlet-Henkel method (°SH/50 mL), and urea by enzymatic method using the difference in pH (CL-10 Plus, Eurochem, Roma, Italy).

Individual milk samples were also evaluated for their clotting ability by measuring coagulation time (*r*, min), curd firming time (k_{20}, min), and curd firmness after 30 min (a_{30}, mm), according to Zannoni and Annibaldi [24], in 10 ml milk at 35 °C with 0.2 mL of a diluted solution (1.6:100) of rennet (1:15,000; Chr. Hansen, Parma, Italy), using the Formagraph (Foss Electric).

Milk casein fractions

Milk caseins (α_{S1}-CN, α_{S2}-CN, β-CN, and k-CN) were separated and quantified in individual milk samples collected on day 7 at the end of sampling week in each experimental period. This was done by direct analysis with RP-HPLC

(reversed-phase high-performance liquid chromatography), according to Bonizzi et al. [25].

Purified α_S-CN (purity 90 %), β-CN (purity 98 %), and κ-CN (purity 98 %) fractions used as standards, and HPLC-grade trifluoroacetic acid, water, acetonitrile, and other chemicals were purchased from Sigma-Aldrich (Milano, Italy).

Single-fraction mother solutions were prepared by dissolving 249.4 mg purified α_S-CN, 255.2 mg purified β-CN, and 51.7 mg purified κ-CN in 10 mL of a denaturing solution containing 8 M urea, 165 mM Tris, 44 mM sodium citrate, and 0.3 % (v/v) β-mercaptoethanol. A mixed standard solution was prepared by mixing 1 mL of each single concentrated solution and adding 2 mL of the denaturing solution, so that the dilution factor at this step was 5 for all casein fractions. Then, a set of four mixed concentration standards was obtained from the mixed mother solution by applying the dilution scheme reported by Bonizzi et al. [25]. Because α_{S1}-CN and α_{S2}-CN are not available as single proteins, the corresponding values were calculated from the α_S-CN by applying the 4:1 proportion reported in the literature [25]. The resulting standard solutions were analyzed to construct the α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN calibration curves.

Milk samples were lyophilized and preserved frozen at −4 °C until analysis. Each milk sample was weighed before and after lyophilization to determine the water percentage content. Before analysis, the lyophilized milk sample was solubilized by adding a corresponding volume of distilled water and then it was homogenized by Vortex and the fat removed by centrifugation at 1,000×*g* for 10 min at 4 °C. A volume of 400 μL of skimmed milk was diluted with 1.6 mL of the denaturing solution described above. The diluted sample was filtered through a 0.45-μm-pore cellulose membrane (Phenomenex, Torrance, CA, USA) and directly analyzed twice.

The chromatographic system (Shimadzu, Kyoto, Japan) used to perform the analyses consisted of an LC-20AT liquid chromatographer, a DGU-20A 5 degasser, a SIL-20A HT autosampler, a CTO-20A column oven, and a SPD-20A UV/VIS detector, run using LC Solutions software.

Chromatographic separation was performed in reversed-phase mode using a Jupiter C_4 column (250 mm × 4.6 mm, 300 Å pores, 5 μm particles; Phenomenex) kept at room temperature. The detection wavelength was 220 nm.

The analyses were carried out by applying a binary gradient profile to the mobile phase composition, according to a modified gradient program developed recently, as reported by Bonizzi et al. [25]. Eluent A was HPLC-grade water containing 0.1 % (v/v) trifluoroacetic acid, and eluent B was HPLC-grade acetonitrile containing 0.1 % (v/v) trifluoroacetic acid.

The gradient elution program was run at a constant flow rate of 0.8 mL/min and was set as follows: 0–40 min linear gradient from 30 % B to 50 % B; 40–42 min linear gradient from 50 % B to 100 % B; 42–43 min isocratic elution 100 % B; 43–46 min linear gradient from 100 % B to 30 % B, followed by a 5-min isocratic elution at the initial conditions. The total duration of a single run, including column re-equilibration, was 51 min.

The quantification of milk casein fractions was performed by comparing the corresponding peak areas in the chromatogram of the sample with those of the standard solutions used for the construction of the calibration curves.

Milk FA composition

Milk FAs were determined from individual milk samples collected at the end of each experimental period.

FAs in lyophilized milk samples (100 mg) were directly methylated with 1 mL hexane and 2 mL 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 [26].

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-ionization detector (Agilent Technologies, Santa Clara, CA, USA). FAME from all samples were separated using a 100 m length, 0.25 mm i.d., 0.25 μm capillary column (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 He flow of 45 mL/min. The initial oven temperature was held at 70 °C for 1 min, increased 5 °C/min to 100 °C, held for 2 min, increased 10 °C/min to 175 °C, held for 40 min, then finally increased 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. Conjugated linoleic acid (CLA) isomers were identified using a commercial mixture of methyl esters of the C18:2 *c9 t11* and C18:2 *c10 t12* (Sigma-Aldrich). The Health Promoting Index was calculated as suggested by Chen et al. [27]: total unsaturated FA/[C12:0 + (4 × C14:0) + C16:0].

Statistical analysis

Statistical analysis was carried out using the MIXED procedure in SAS 9.1.2 [28]. Experimental phase (1, 2, 3), DIM (50 and 120 days), diet (SFF, SFB, MHB), genotype (AA and AF), and the diet by genotype interaction were fixed factors, and the goat was considered a random factor and used as an error term. Somatic cell count values were

transformed in logarithmic form ($\log_{10}$). Means were compared using Tukey's test ($P < 0.05$).

Results and discussion

Feed intake and milk production

At the end of the experimental period, the live weight and BCS of the goats did not show changes as a function of diet or *CSN1S1* genotype, as previously observed [11].

The DM and main nutrients intake were strongly influenced by diet, while it did not reveal a significant effect of *CSN1S1* genotype and diet by genotype interaction (Table 1). Similar results were found by Bonanno et al. [29] and Pagano et al. [10]. In particular, Bonanno et al. [29] reported no difference in DM intake between goats with strong (AA) and heterozygous (AF) genotypes, like in the present study, although they observed a lower feed intake in goats with a weak (FF) genotype.

With regard to diet, the sulla fresh forage increased the DM intake compared to hay, regardless of the energy supplementation with barley (Table 1). This confirms the positive effect of sulla forage on voluntary feed intake [12, 30] attributed to the high protein percentage, the low NDF content, and the high ratio of nonstructural-to-structural carbohydrates of sulla [31]. Intake of protein, as well as condensed tannins, increased with increasing levels of fresh forage ingested. The SFF diet resulted in the maximum NDF intake, followed by the MHB diet, whereas the SFB diet, because of its lower NDF intake, corresponded to the highest energy intake.

Like feed intake, milk production was affected by diet (Table 1). In fact, the daily milk yield increased from the SFF diet to the MHB diet, culminating with the SFB diet.

With regard to the effect of diet on milk composition, the energy supplement with barley reduced the contents of milk fat and urea (Table 1). This reduction in fat was certainly due to the lower forage/concentrate ratio of the supplemented diets and thus to the lower cellulose intake. The reduction in urea was presumably a consequence of the more balanced protein/energy ratio in the diets with barley supplementation, which favoured the conversion of dietary nitrogen into microbial protein in the rumen [32].

Moreover, the sulla fresh forage, independent of the barley supplement, resulted in an increase in the percentages of milk protein and casein. This was probably due to the higher intake of condensed tannins (Table 1), secondary metabolites contained in sulla forage in moderate amounts (<6 % DM) [33]. These tannins are able to reduce protein degradability in the rumen and consequently enable a greater amount of amino acids to be absorbed in the intestinal tract [34]. This contributes to improving the efficiency of dietary protein utilization for milk casein synthesis in the udder.

Regardless of genotype, diet affected the titratable acidity and coagulation time of milk, which were higher and lower, respectively, when goats received the SFF diet (exclusively sulla fresh forage) than the other diets (Table 1). This result is in line with Todaro et al. [35], who found a negative correlation between titratable acidity and the coagulation time of goat milk. However, generally, the relationship between diet and milk coagulation ability is quite complex, even though diet has been shown to affect milk titratable acidity and the coagulation process [36].

For milk yield, there was no influence of genotype, whereas there was a significant interaction between diet and genotype (Table 1). In this regard, the literature has frequently shown the lack of an effect of *CSN1S1* genotype on goat milk yield. For example, many researchers have found no significant difference between goats with AA and FF genotypes at *CSN1S1* loci [5, 7, 9, 37]; only Avondo et al. [38] reported increased milk production in goats with the strong genotype (AA) compared to the weak genotype (FF). Moreover, the milk yields of goats with the AA and AF genotypes do not differ significantly, and both genotypes result in more milk production than the FF genotype [29]. However, Pagano et al. [10] showed a higher milk yield in AA goats compared to AF and FF goats, which did not differ.

These discrepancies can be attributed to the different milking responses of goats to nutrients in accordance with their *CSN1S1* genotype. As evidence of this assertion, in the current study, a significant interaction between diet and genotype emerged, because the superior production of AA goats compared to AF goats occurred when the goats were fed with more energy SFB diet (1,720 vs. 1,606 g/day, $P < 0.05$). Moreover, the milk yield of AA goats fed the SFB diet was 350 g/day more than that of goats fed the other diets, whereas the differences among diets were markedly lower in AF goats. These results clearly show the existence of relationships between nutrition and α_{S1}-CN polymorphism, as supported by other authors [9, 10], and particularly confirm the better milking response of goats with strong alleles at *CSN1S1* loci, compared with FF goats, when fed higher-energy diets balanced for energy and protein content [8–10].

The *CSN1S1* genotype did not significantly influence milk composition. In this regard, several authors [9, 37–39] have reported that the milk of goats with the strong *CSN1S1* genotype (AA) has a higher percentage of casein than that of goats with the weak *CSN1S1* genotype (FF); casein levels in the milk of heterozygous goats (AF) are intermediate and statistically different from those of either AA or FF goats [10, 29], contrary to the results of this trial.

Even though the *CSN1S1* genotype did not significantly influence the milk casein content, the milk of goats with strong alleles had a longer coagulation time and greater

Table 1 Effects of diet and *CSN1S1* genotype of goats on nutrient intake and milk yield, composition, and clotting ability

Genotype (G)				AA	AF	AA			AF			SEM	Significance		
Diet (D)	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
Intake															
DM, g/day	1,820^{a}	1,807^{a}	1,655^{b}	1,746	1,776	1,769	1,776	1,692	1,872	1,837	1,618	86.8	**	ns	ns
CP, g/day	321^{a}	290^{b}	203^{c}	272	270	322	286	209	320	293	197	15.3	***	ns	ns
NDF, g/day	632^{a}	483^{c}	539^{b}	535	568	592	463	550	673	503	527	37.4	***	ns	ns
Condensed tannins, g/day	47.2^{a}	35.6^{b}	3.50^{c}	29.1	28.5	47.7	35.7	3.70	46.6	35.4	3.31	1.64	***	ns	ns
NE_L, Mcal/day	2.40^{b}	3.03^{a}	2.34^{b}	2.60	2.58	2.36	3.05	2.37	2.44	3.01	2.31	0.088	***	ns	ns
Milk traits															
Milk yield, g/day	1,353^{c}	1,664^{a}	1,423^{b}	1,487	1,473	1,356^{cd}	1,720^{a}	1,384^{cd}	1,348^{d}	1,606^{b}	1,465^{c}	44.3	***	ns	**
Fat, %	3.59^{a}	3.17^{b}	2.95^{c}	3.17	3.31	3.52	3.06	2.92	3.66	3.28	2.99	0.21	***	ns	ns
Protein, %	3.34^{a}	3.28^{a}	3.21^{b}	3.29	3.26	3.35	3.26	3.24	3.33	3.29	3.17	0.10	**	ns	ns
Urea, mg/dL	35.4^{a}	32.1^{b}	30.9^{b}	33.8	31.8	35.8	33.4	32.3	35.0	30.9	29.5	2.19	***	ns	ns
SCC, $\log_{10}$ n/mL	5.27	5.28	5.27	5.13	5.42	5.15	5.09	5.14	5.39	5.46	5.41	0.15	ns	ns	ns
pH	6.63	6.65	6.65	6.66	6.64	6.64	6.67	6.65	6.63	6.63	6.65	0.018	ns	ns	ns
Titratable acidity, °SH/50 mL	2.81^{a}	2.67^{b}	2.62^{b}	2.59	2.82	2.81	2.56	2.66	2.81	2.79	2.58	0.11	**	ns	ns
Coagulation time (r), min	13.8^{b}	15.0^{a}	14.9^{a}	15.2^{a}	13.9^{b}	14.6	15.8	15.3	13.1	14.2	14.5	0.55	*	*	ns
Curd firming time (k_{20}), min	2.67	2.82	2.65	2.79	2.63	2.82	2.94	2.62	2.53	2.70	2.67	0.33	ns	ns	ns
Curd firmness (a_{30}), mm	32.2	34.2	30.9	35.9^{a}	29.0^{b}	37.3	37.5	32.8	27.0	31.0	29.1	2.09	ns	***	ns

Genotypes are as follows: AA = homozygous for strong alleles and AF = heterozygous for a weak allele

Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/day barley meal, and MHB = mixed hay plus 800 g/day barley meal

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns = not significant. Means within a row with different superscripts differ ($P \leq 0.05$)

curd firmness (Table 1). Since generally these clotting responses are related to a higher casein level [40], they could be linked to a more favorable partition among the casein fractions compared to in AF goats. Because in this trial the genotypes differed only for the variants of α_{S1}-CN synthesis, this result implicates α_{S1}-CN as key in variations in milk coagulation.

In previous trials [29, 37], milk from goats with the AA genotype at *CSN1S1* loci showed greater curd consistency in comparison with milk of FF goats, whereas the coagulation ability of milk from AA goats did not differ from that of milk from AF goats.

Milk casein fractions

The analysis of casein components, such as κ-CN, α_{S2}-CN, α_{S1}-CN, and β-CN, showed a higher α_{S1}-CN percentage in the milk of AA goats than AF goats, as expected (Table 2). In Spanish goat breeds, genotypes with strong alleles (BB) also displayed significantly increased levels of milk α_{S1}-CN in comparison with heterozygous genotypes (BF) [41].

Figures 1 and 2 show the chromatograms obtained by RP-HPLC from milk samples of goats with genotypes expressing a high (AA) and low (AF) level of α_{s1}-CN synthesis, respectively.

The levels of k-CN and α_{S2}-CN were not affected by either diet or genotype, whereas the percentage of β-CN, which is the most represented casein fraction, was significantly influenced only by diet. β-CN, in fact, was mostly synthesized with the fresh forage diets, presumably as a consequence of the favorable effects of the higher content in the protein and condensed tannins of the sulla forage [34].

When milk casein profiles were analyzed for the daily production of the various fractions, the effect of genotype

Table 2 Effects of diet and *CSN1S1* genotype of goats on percentage in milk and daily yield of casein fractions

Genotype (G)				AA	AF	AA			AF			SEM	Significance		
Diet (D)	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
κ-CN, %	0.34	0.36	0.37	0.36	0.35	0.36	0.36	0.36	0.31	0.36	0.38	0.022	ns	ns	ns
α_{S2}-CN, %	0.68	0.68	0.73	0.69	0.70	0.70	0.67	0.71	0.66	0.69	0.76	0.043	ns	ns	ns
α_{S1}-CN, %	0.58	0.53	0.53	0.67^{a}	0.42^{b}	0.74	0.63	0.64	0.42	0.42	0.41	0.062	ns	**	ns
β-CN, %	1.33^{a}	1.28^{a}	1.21^{b}	1.21	1.33	1.31	1.18	1.15	1.35	1.37	1.27	0.086	**	ns	ns
κ-CN, g/day	4.56^{b}	5.98^{a}	4.89^{b}	5.26	5.03	5.00	6.13	4.64	4.12	5.83	5.14	0.58	**	ns	ns
α_{S2}-CN, g/day	9.22^{b}	11.2^{a}	9.62^{b}	9.96	10.1	9.76	11.2	8.91	8.68	11.3	10.3	1.24	*	ns	ns
α_{S1}-CN, g/day	7.80^{ab}	8.90^{a}	7.01^{b}	9.77^{a}	6.03^{b}	10.10	10.90	8.31	5.50	6.89	5.71	1.35	*	*	ns
β-CN, g/day	18.0^{b}	20.9^{a}	15.6^{b}	17.4	18.9	18.5	19.3	14.5	17.5	22.5	16.8	2.20	**	ns	ns

Genotypes are as follows: AA = homozygous for strong alleles and AF = heterozygous for a weak allele

Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/day barley meal, and MHB = mixed hay plus 800 g/day barley meal

* $P \leq 0.05$; ** $P \leq 0.01$; ns = not significant. Means within a row with different superscripts differ ($P \leq 0.05$)

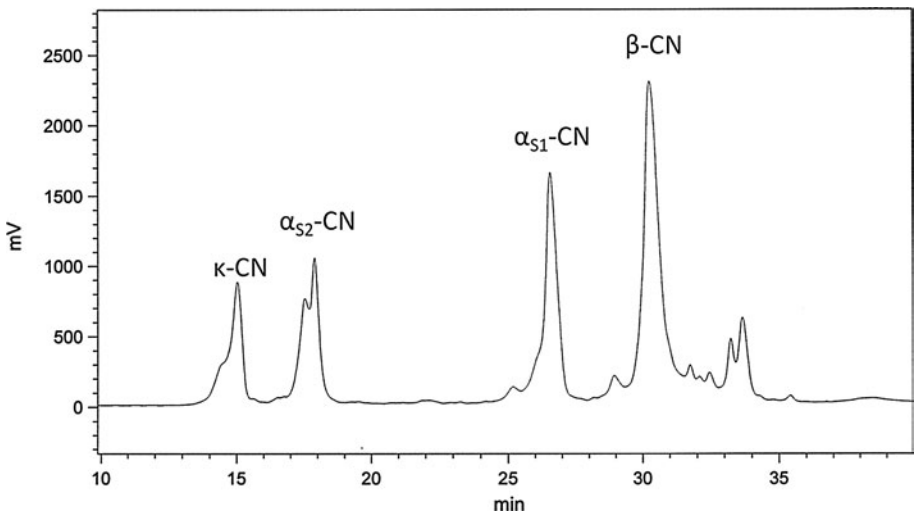

Fig. 1 Chromatogram obtained by RP-HPLC from a milk sample of a goat with AA genotype at *CSN1S1* loci, showing a high expression of α_{S1}-CN synthesis

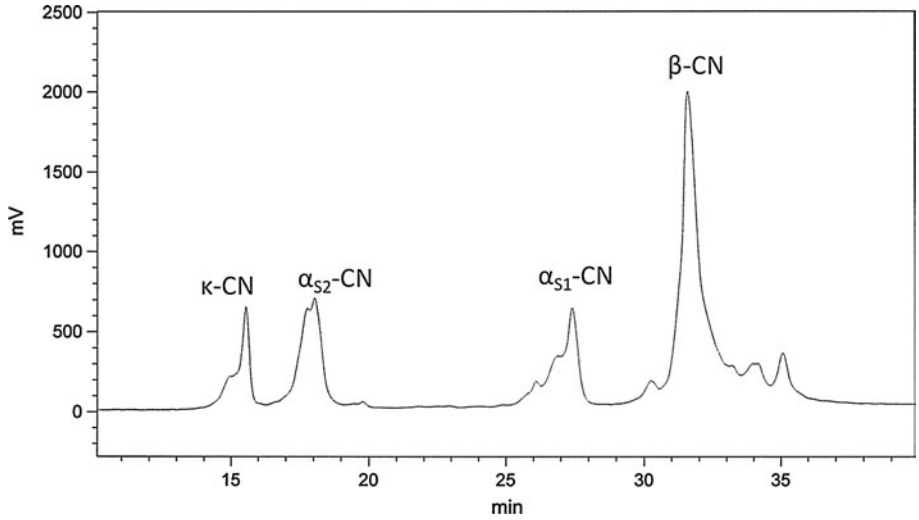

Fig. 2 Chromatogram obtained by RP-HPLC from a milk sample of a goat with AF genotype at *CSN1S1* loci, showing a low expression of α_{S1}-CN synthesis

was again significant for α_{S1}-CN, which was higher in AA than in AF milk (Table 2). Moreover, all casein fractions showed an effect of diet, irrespective of genotype; their production, in fact, was favoured by the higher-energy and more balanced diet based on sulla forage supplemented with barley.

With regard to the effect of diet on the casein profile of goat milk, researchers have compared animals with strong (AA) and weak (FF) alleles at *CSN1S1* loci [9, 39]. In line with the results of the present trial, De la Torre Adarve et al. [9] detected a higher incidence of α_{S1}-CN in the milk of goats with strong than weak alleles regardless of dietary protein intake. However, these same authors also observed an increase in the percentage of α_{S2}-CN in goats with the strong genotype and an increase in α_{S1}-CN and α_{S2}-CN daily yield in goats with the weak genotype when fed a diet rich in protein.

Valenti et al. [39] observed that goats with a strong genotype for α_{S1}-CN responded to a higher-energy diet, increasing both milk casein content and daily casein yield, and that this increase was due to only α_{S1}-CN. Instead, in the present trial, the increase in milk α_{S1}-CN percentage in AA goats was independent of diet, and the daily α_{S1}-CN yield with the higher-energy SFB diet increased similarly in goats with the AA and AF genotypes.

Ultimately, with regard to the incidence of casein fractions, the diet affected the level of β-CN similarly in goats of both genotypes, whereas the AA genotype at *CSN1S1* loci was linked exclusively to the increase in α_{S1}-CN synthesis, regardless of diet. Therefore, the milk of goats of these genotypes differed only in the level of α_{S1}-CN. Considering the response by genotype in terms of milk coagulation previously described (Table 1), this result shows that in this trial, α_{S1}-CN was solely responsible for the coagulation properties of the milk, particularly for curd firmness (a_{30}).

Milk FA composition

As can be seen in Tables 3 and 4, the milk FA composition was influenced strongly by nutrients intake and only marginally by the polymorphism at *CSN1S1* loci and the interaction between diet and genotype.

Both the sulla fresh forage and the hay supplemented with barley induced an increase in the levels of SMFA in milk (from C10:0 to C16:0, Table 3; Σ C4–C14, Table 4).

Moreover, the milk obtained with the SFB diet showed the highest content of linoleic acid (C18:2 n-6, LA) (Table 4), certainly due to the contribution of both feeding sources, sulla forage and barley.

Conversely, the diet based exclusively on green forage (SFF) resulted in an increase in most of the odd and branched chain FA in milk (C14:0 *iso*, C15:0 *iso*, C15:0 *anteiso*, C15:0, C17:0 *anteiso*, and C17:0, Table 3), grouped under the acronym OBCFA in Table 4. The OBCFA, to which a certain anticancer activity is recognized, derive mainly from the biosynthesis of rumen bacteria; therefore, their presence is considered as an indicator of microbial fermentations in the rumen and is favoured by a higher incidence of the forage component in the diet [42].

The SFF diet also resulted in an increase in many FAs with 18 carbon atoms (Table 4), such as stearic (C18:0), vaccenic (C18:1 *t11*, VA), oleic (C18:1 *c9*), and rumenic (CLA, C18:2 *c9 t11*, RA) acids. The incidence of sulla forage in the diet also strongly influenced α-linolenic acid content (C18:3 n-3, LNA), which was lowest in the hay-based diet, increased with the SFB, and then further increased with the sulla forage alone (Table 4). This trend was also found for total polyunsaturated and omega-3 FAs and then, in reverse, for the omega-6/omega-3 ratio (Table 4).

Like every other green forage, sulla fresh forage is rich in polyunsaturated FA, which can represent more than 70 % of the total FA, and consists mainly of LNA and LA

Table 3 Effects of diet and *CSN1S1* genotype of goats on short- and medium-chain fatty acid composition (g/100 g FAME) of milk

Genotype (G)				AA	AF	AA			AF			SEM	Significance		
Diet (D)	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
C4:0	0.97	0.84	0.95	1.01	0.83	1.15	0.93	0.96	0.80	0.75	0.94	0.13	ns	+	ns
C6:0	2.01	2.05	1.89	2.22	1.74	2.48	2.34	1.85	1.54	1.75	1.94	0.32	ns	+	ns
C8:0	2.52	2.89	2.28	3.02^{a}	2.10^{b}	3.31	3.51	2.24	1.73	2.26	2.32	0.44	ns	*	ns
C9:0	0.36	0.32	0.25	0.28	0.33	0.39	0.22	0.24	0.32	0.42	0.25	0.073	ns	ns	ns
C10:0	9.77^{b}	12.5^{a}	11.1^{ab}	12.0^{a}	10.2^{b}	11.3	13.7	11.0	8.25	11.3	11.1	0.83	**	*	ns
C11:0	0.37	0.48	0.49	0.47	0.42	0.42	0.49	0.50	0.33	0.46	0.48	0.052	+	ns	ns
C12:0	4.45^{b}	6.78^{a}	6.24^{a}	6.09	5.55	4.98	6.85	6.45	3.92	6.71	6.02	0.69	**	ns	ns
C13:0	0.18^{b}	0.26^{a}	0.30^{a}	0.23	0.26	0.18	0.23	0.27	0.17	0.30	0.32	0.033	**	ns	ns
C14:0 *iso*	0.18^{a}	0.13^{b}	0.13^{b}	0.14	0.15	0.20	0.10	0.12	0.17	0.16	0.14	0.026	*	ns	ns
C14:0	9.14^{b}	12.3^{a}	12.9^{a}	11.3	11.6	9.25	12.0	12.5	9.02	12.5	13.3	0.63	***	ns	ns
C15:0 *iso*	0.27^{a}	0.16^{c}	0.21^{b}	0.21	0.21	0.26	0.17	0.20	0.27	0.15	0.22	0.022	***	ns	ns
C15:0 *anteiso*	0.46^{a}	0.32^{b}	0.36^{b}	0.36	0.40	0.44	0.28	0.34	0.48	0.35	0.38	0.043	**	ns	ns
C14:1 *c9*	0.10^{b}	0.18^{a}	0.21^{a}	0.14	0.19	0.09	0.15	0.20	0.12	0.22	0.23	0.033	*	ns	ns
C15:0	1.59^{a}	0.86^{b}	1.00^{b}	1.08	1.22	1.42	0.80	1.03	1.77	0.92	0.97	0.13	***	ns	ns
C16:0 *iso*	0.28	0.28	0.25	0.26	0.28	0.28	0.29	0.23	0.29	0.27	0.28	0.032	ns	ns	ns
C16:0	23.3^{b}	28.4^{a}	31.1^{a}	27.7	27.5	23.1	28.0	32.0	23.6	28.8	30.2	1.46	***	ns	ns
C17:0 *iso*	0.36^{a}	0.26^{b}	0.38^{a}	0.31	0.35	0.33	0.26	0.35	0.38	0.26	0.41	0.033	**	ns	ns
C17:0 *anteiso*	0.37^{a}	0.21^{b}	0.20^{b}	0.24^{b}	0.28^{a}	0.33	0.20	0.19	0.41	0.23	0.21	0.027	***	*	ns
C16:1 *c9*	0.47	0.50	0.62	0.49	0.57	0.45	0.45	0.58	0.48	0.56	0.66	0.065	+	ns	ns
C17:0	1.17^{a}	0.78^{b}	0.77^{b}	0.89	0.92	1.08	0.75	0.85	1.27	0.82	0.68	0.073	***	ns	ns
C17:1	0.30^{a}	0.18^{b}	0.22^{b}	0.22	0.25	0.27	0.17	0.22	0.32	0.19	0.23	0.022	***	ns	ns

Genotypes are as follows: AA = homozygous for strong alleles and AF = heterozygous for a weak allele

Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/day barley meal, and MHB = mixed hay plus 800 g/day barley meal

$^{+}$ $P \leq 0.10$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns = not significant. Means within a row with different superscripts differ ($P \leq 0.05$)

Table 4 Effects of diet and *CSN1S1* genotype of goats on long-chain and grouped fatty acid composition (g/100 g FAME) of milk

Genotype (G)				AA	AF	AA			AF			SEM	Significance		
Diet (D)	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
C18:0	11.2^{a}	8.18^{b}	7.23^{b}	8.39	9.36	10.2	7.96	7.00	12.2	8.39	7.46	0.64	***	+	ns
C18:1 *t11*, VA	1.44^{a}	0.64^{b}	0.43^{b}	0.77	0.91	1.23	0.62	0.45	1.65	0.66	0.42	0.15	***	ns	ns
C18:1 *c9*	15.6^{a}	11.6^{b}	12.2^{b}	12.5^{b}	13.9^{a}	14.6	11.2	11.6	16.7	12.0	12.9	0.86	***	*	ns
C18:2 n-6 *c9 c12*, LA	1.67^{b}	2.32^{a}	1.89^{b}	1.87	2.05	1.76	2.19	1.65	1.58	2.44	2.14	0.18	**	ns	ns
C18:3 n-3 *c9 c12 c15*, LNA	1.94^{a}	0.97^{b}	0.41^{c}	1.11	1.10	1.88	0.88	0.56	2.00	1.05	0.26	0.17	***	ns	ns
CLA C18:2 *c9 t11*, RA	0.56^{a}	0.29^{b}	0.27^{b}	0.35	0.41	0.48	0.28	0.28	0.65	0.30	0.27	0.066	***	ns	ns
CLA isomers	0.27	0.24	0.18	0.23	0.23	0.29	0.25	0.16	0.25	0.23	0.20	0.1014	ns	ns	ns
C20:5 n-3, EPA	0.20	0.16	0.17	0.19	0.16	0.22	0.15	0.19	0.18	0.17	0.14	0.034	ns	ns	ns
C22:6 n-3, DHA	0.13	0.11	0.11	0.11	0.12	0.09	0.13	0.12	0.17	0.09	0.10	0.075	ns	ns	ns
C22:5 n-3, DPA	0.25	0.18	0.29	0.23	0.25	0.20	0.17	0.33	0.29	0.20	0.26	0.10	ns	ns	ns
Saturated FA	70.8^{b}	78.6^{a}	78.8^{a}	77.2^{a}	74.9^{b}	72.5	79.7	79.4	69.1	77.5	78.1	1.42	***	*	ns
Monounsaturated FA	21.8^{a}	15.8^{b}	16.5^{b}	17.1^{b}	18.9^{a}	20.3	15.2	15.9	23.3	16.5	17.0	1.08	***	*	ns
Polyunsaturated FA	6.35^{a}	4.78^{b}	3.91^{c}	4.85	5.17	6.25	4.44	3.88	6.46	5.12	3.94	0.37	***	ns	ns
Unsaturated FA	28.1^{a}	20.6^{b}	20.4^{b}	22.0^{b}	24.1^{a}	26.5	19.6	19.8	29.8	21.6	21.0	1.36	***	*	ns
Saturated/unsaturated	2.56^{b}	3.92^{a}	4.04^{a}	3.74^{a}	3.28^{b}	2.78	4.17	4.25	2.33	3.67	3.83	0.29	***	*	ns
Σ omega-6	2.53	2.88	2.50	2.55	2.72	2.69	2.70	2.27	2.37	3.06	2.72	0.23	ns	ns	ns
Σ omega-3	2.56^{a}	1.30^{b}	0.91^{b}	1.56	1.61	2.39	1.20	1.10	2.72	1.40	0.71	0.21	***	ns	ns
Omega-6/omega-3	1.14^{c}	2.29^{b}	3.96^{a}	2.53	2.40	1.40	2.28	3.91	0.88	2.31	4.01	0.58	***	ns	ns
OBCFA	6.58^{a}	4.71^{b}	5.37^{b}	5.32	5.79	6.25	4.30	5.42	6.92	5.12	5.32	0.39	***	ns	ns
Σ C4–C14	30.0^{b}	38.7^{a}	36.7^{a}	36.9^{a}	33.4^{b}	33.7^{b}	40.5^{a}	36.4^{ab}	26.3^{c}	36.8^{ab}	37.0^{ab}	1.73	***	*	+
C14:1/C14:0	0.009	0.014	0.016	0.012	0.013	0.008	0.013	0.016	0.010	0.014	0.015	0.003	+	ns	ns
C16:1/C16:0	0.020	0.018	0.020	0.018	0.021	0.020	0.016	0.019	0.021	0.020	0.022	0.003	ns	ns	ns
C17:1/C17:0	0.26	0.23	0.35	0.25	0.31	0.26	0.22	0.27	0.26	0.23	0.43	0.060	ns	ns	ns
C18:1/C18:0	1.45	1.52	1.75	1.55	1.59	1.51	1.43	1.70	1.39	1.59	1.79	0.15	ns	ns	ns
RA/VA	0.40^{b}	0.47^{b}	0.65^{a}	0.49	0.53	0.39	0.44	0.63	0.42	0.50	0.67	0.065	**	ns	ns
HPI	0.44^{a}	0.25^{b}	0.24^{b}	0.30	0.32	0.41	0.24	0.24	0.47	0.26	0.24	0.026	***	ns	ns

Genotypes are as follows: AA = homozygous for strong alleles and AF = heterozygous for a weak allele

Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/day barley meal, and MHB = mixed hay plus 800 g/day barley meal

VA vaccenic acid, *LA* linoleic acid, *LNA* α-linolenic acid, *RA* rumenic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *DPA* docosapentaenoic acid, *OBCFA* odd and branched chain fatty acids, *RA/VA* rumenic acid/vaccenic acid, *HPI* Health Promoting Index [27] = unsaturated fatty acids/[C12:0 + (4 × C14:0) + C16:0]

$^{+}$ $P \leq 0.10$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; *ns* not significant. Means within a row with different superscripts differ ($P \leq 0.05$)

acids (about 60 and 10 % of the total FA, respectively) [43]. Therefore, sulla fresh forage intake might have favoured the increase in polyunsaturated FA in the milk.

However, the intake of condensed tannins contained in the sulla forage could also have played a determining role in increasing the amount of polyunsaturated FA in the milk; the condensed tannins, in fact, would have been able to inhibit the activity of ruminal microorganisms in biohydrogenating the unsaturated FA, as demonstrated by Cabiddu et al. [44]. In this context, RA represents the first and VA the last of the intermediate products that are formed in the rumen during the saturation of LA and LNA to stearic acid (C18:0) [45, 46], and therefore, their levels increase as a consequence of the inhibiting action of the sulla condensed tannins.

Rumenic acid is the most abundant of the CLA isomers; these molecules have beneficial properties for human health and, because of their cytotoxic action against several tumor cell lines, are mainly used to prevent the occurrence of tumors [47, 48]. Rumenic acid originates not only from the biohydrogenation of LA and LNA in the rumen but also from the desaturation of VA in the mammary gland [45]. In this regard, the lower ratio of RA to VA (Table 4) in the SFF and SFB diets compared to the MHB diet would indicate a lower efficiency of the activity of the enzyme delta-9-desaturase in the mammary gland tissue for the conversion of VA to RA, an effect that probably is due to the higher level of VA. However, the ratios between saturated and unsaturated FAs of the same chain length (Table 4), used as indicators of FA desaturation in the mammary gland by delta-9-desaturase, were not influenced by diet.

Overall, the exclusive intake of sulla fresh forage by goats improved the FA profile of milk fat, making it more suitable to the health needs of consumers [46, 49, 50]. Indeed, the sulla forage enriched the milk in OBCFA, CLA (RA), and monounsaturated, polyunsaturated, and omega-3 FAs, thereby reducing the ratios of saturated/unsaturated FAs and omega-6/omega-3 FAs and improving the Health Promoting Index (Table 4), which expresses the health value of dietary fat [27].

Compared to diet, the effect of the genotype at *CSN1S1* loci on milk FA composition was weak. However, an effect of genotype was found, at varying levels of significance, for the short- and even-chain FAs (from C4:0 to C10:0) (Table 3), which were higher in AA goats, as well as for C17:0 *anteiso* (Table 3), stearic acid (C18:0), and oleic acid (C18:1 *c9*) (Table 4), which were higher in AF goats. Therefore, the FA profile of milk fat of goats with a greater ability to synthesize α_{S1}-CN was characterized by more saturated FA, especially for the contribution of SMFA (Σ C4–C14), and less monounsaturated FA, mainly due to the reduced incidence of oleic acid (C18:1 *c9*) (Table 4). Accordingly, the milk of goats with the strong genotype showed a higher saturated/unsaturated FA ratio, although the Health Promoting Index was not affected by genotype.

Only Todaro et al. [51], studying the effects of genotype at *CSN1S1* loci on the FA profile of milk from goats of Maltese breed, also evaluated animals with a heterozygous genotype for a weak allele (AF). They detected differences between the AF goats and goats with a weak genotype (FF) that were mainly due to the high presence of medium-chain FA in the milk of the latter goats. They did not find any differences between the AF and AA goats.

In agreement with Todaro et al. [51], in this trial, the level of RA did not differ by *CSN1S1* genotype, although it was slightly higher in heterozygous goats than in goats with the strong genotype, as was VA. Also, FA desaturation occurred in the mammary gland by the enzyme delta-9-desaturase, as indicated by ratios of saturated and unsaturated FAs of the same chain length (Table 4), did not appear to be affected by genotype. However, Chilliard et al. [5] found an increasing content of RA in the milk of goats with the weak genotype (FF), and in line with Valenti et al. [52], also found higher ratios of FA desaturation in comparison with milk of the strong genotype (AA).

When the goats carrying strong alleles at *CSN1S1* loci were compared with those homozygous for the weak alleles (FF), the effect of genotype for α_{S1}-CN was more pronounced than that detected in this trial and differences emerged mainly for SMFA (Σ C4–C14), which was higher with the genotype with strong alleles [5–7, 9, 51]. This shows that the proportion of SMFA is normally higher in animals with a high capacity for α_{S1}-CN synthesis, in line with the findings of this study.

With regard to the OBCFA, only Valenti et al. [6] found a higher content of C15:0 *anteiso* in the milk of goats with the strong genotype than the weak genotype, while no study in the literature reports an increase in C17:0 *anteiso* with the weak genotype as emerged in this current trial.

Furthermore, as in this study, Chilliard et al. [5] and De la Torre Adarve et al. [9] found a lower oleic acid (C18:1 *c9*) content in the milk of goats with the strong genotype. Since a negative energy balance increase in milk the amount of long-chain FA mobilized from adipose tissue, especially oleic acid (C18:1 *c9*) [5], this results would indicate that AA goats, compared to those with the heterozygous and weak genotypes, had less of a need to mobilize their body fat reserves. In this regard, Valenti et al. [52] observed that goats with the strong genotype for α_{S1}-CN did not show the increase in oleic acid (C18:1 *c9*) content that occurred in goats with the FF genotype when fed the lower energy diet, which further supports the greater efficiency of energy utilization in these animals.

In the present experiment, an interaction between diet and genotype emerged, at a tendency level, only for the sum of SMFA (Σ C4–C14, Table 4). These FA increased

when the goats with the low genetic capacity for α_{S1}-CN synthesis received the SFB and MHB diets with the energy supplement. Similarly, Valenti et al. [52] found a greater synthesis of SMFA in the milk of goats with the weak genotype when these animals were fed a higher-energy diet.

Finally, this study, as well as the other investigations discussed, points to the weak link between goat polymorphism at *CSN1S1* loci and milk FA composition. According to Leroux [53], the absence of a more pronounced effect of genotype may be justified by the fact that milk fat content does not seem to depend on a different expression of enzymes involved in lipogenesis. However, other enzymes seem to be involved in the de novo synthesis of SMFA in the udder tissue. In this regard, Ollier et al. [54] hypothesized that weak variants at *CSN1S1* loci may interfere negatively with the expression of genes coding for the enzyme that catalyzes the de novo synthesis of SMFA in the mammary gland.

Conclusions

In this study, Girgentana goats with genotypes associated with a high (AA) or low (AF) level of α_{S1}-CN synthesis were compared on the basis of milk casein and FA profiles deriving from different nutritional treatments.

The diet highest in energy, a combination of sulla fresh forage and barley (SFB), maximized the goats' energy intake and milk yield; however, milk production with SFB diet was more efficient in AA goats than in AF goats.

Regardless of *CSN1S1* genotype and the presence of a barley supplement, the fresh forage diets (SFF and SFB) increased DM and protein intake and milk β-CN content. The diet based exclusively on sulla fresh forage (SFF) improved the health properties of milk fat that was richer in CLA (RA), OBCFA, monounsaturated, polyunsaturated, and omega-3 FAs, and had lower saturated/unsaturated FAs and omega-6/omega-3 FAs ratios and a more favorable Health Promoting Index. These improvements were presumably the result of condensed tannins of sulla in inhibiting the biohydrogenation of unsaturated FA in the rumen.

With regard to genotype, AA goats differed from AF goats in terms of their superior ability to synthesize α_{S1}-CN, regardless of diet. Therefore, the higher α_{S1}-CN content in the AA milk was responsible for the improved milk clotting properties, as a result of the longer coagulation time and higher curd firmness, in comparison with the AF milk.

Compared to the AA goats, the heterozygous AF goats showed less of an ability to biosynthesize SMFA (Σ C4–C14) in the mammary gland tissue, but this effect disappeared when they received the energy supplement. Whereas the lesser exigency to mobilize body fat depots of AA goats, thus their more efficient energy utilization was confirmed by the lower content of oleic acid (C18:1 *c9*) in the milk.

Ultimately, this study confirms the better nutritional and productive efficiency and the higher capacity for α_{S1}-CN synthesis of goats with the strong genotype at *CSN1S1* loci in comparison with heterozygous AF goats. In addition, this study demonstrates that the milk production potential of AA goats, besides being higher than that of the FF goats that have the least ability to synthesize α_{S1}-CN, as reported in the literature, is also superior to that of heterozygous AF goats.

Moreover, the results provide evidence of the pronounced effect of diet on milk FA composition (i.e., the improved health properties of the milk of goats fed exclusively sulla fresh forage) and, in contrast, the weak influence of goat polymorphism at *CSN1S1* loci on milk FA composition.

Acknowledgments This research was supported by the Italian Ministry of Education, University and Research (MIUR) (Project of High National Interest PRIN 2007B4JBWN "Genetic polymorphism of caseins in goats. Effects of feeding on milk production and quality, feed intake, metabolic and hormonal responses in goats at different genetic potential to produce casein").

Conflict of interest None.

Compliance with Ethics Requirements During the experiment, the goats were managed according to the guidelines for accommodation and care of experimental animals of the European Union Directive 86/609/EEC and the recommendation of the Commission of the European Communities 2007/526/EC.

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