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Research article

Improving dairy performance through molecular characterization of *SREBP-1* gene in Sarda sheep breed

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ABSTRACT

This research has two aims: (i) to characterize the coding sequence of the *SREBP-1* gene in dairy sheep in order to investigate possible relationships between single nucleotide polymorphisms (SNPs) and milk traits; and (ii) to investigate possible relationship between *SREBP-1* gene expression and nucleotide variation. Four hundred adult and multiparous lactating Sarda breed ewes were selected from two farms. Milk samples were collected from Day 30 to Day 150 of lactation to determine the mean yield, somatic cell count, lactose, fat, and protein content of the milk. RNA was extracted from the milk samples, after which the *SREBP-1* gene coding regions were amplified and sequenced to scan mutations. Whilst eight SNPs were identified, none had statistically significant association with the analysed milk traits. Moreover, the identified expression patterns were not affected by the SNP or combined genotypes. High *SREBP-1* gene expression levels were found to be correlated with high milk fat content ($P < 0.01$), indicating the crucial role of this gene in the milk fat synthesis. In conclusion, the polymorphisms found within SREBP-1 gene exhibited no significant associations with milk traits or with individual SREBP-1 mRNA expression patterns. The findings thus suggest that this small genetic variability may derive from the selection carried out in Sarda breed to improve milk yield.

1. Introduction

Sardinia, and Italian island located in the Mediterranean Sea, boasts the largest number of sheep and goat farms in the country (Mura et al., 2019). The region's native bred sheep (Sarda sheep), known as the most sought-after Italian dairy bred, produce an average of 300,000 tons of milk per year in Sardinia alone. This amount constitutes over 65% of Italy's sheep's milk production, and over 12% of total European production (ISTAT, 2017; EUROSTAT, 2017). Due to the large number of locally-reared sheep, Sardinia is considered among the main dairy sheep regions in the world, together with Spain, France, and Greece (de Rancourt and de Carrère, 2011). Traditional Sardinian sheep farming is a specialized, semi-extensive system which is based on well-structured and functional genetic selection of Sarda sheep spanning over a century. Additionally, it ensures high quality animal welfare, thus responding to the increasing consumer demands for eco-friendly animal products. Despite the traditional aspect of Sarda sheep farming, an increasing number of farms employ modern tools to improve the milk yield and quality, and therefore, farmer income (de Rancourt et al., 2006; Carta et al., 2009).

Investigations involving the genetic parameters of milk components and their relationships with dairy performance are now being utilised to improve quality traits in sheep. Among the different constituents of milk, fat is the major energy source of milk and influences milk processing and organoleptic characteristics of the dairy products (Lock and Bauman, 2004; Harvatine et al., 2009). The fatty acids composition and concentration in milk is affected by several factors including nutrition and genetic traits (Bauman et al., 2011). Since feeding is able to modify only a small part of the lipidic concentration in dairy species, in recent years, great attention has been devoted to genes involved in the fatty acids' biosynthesis (Palmquist et al., 1993; Schennink et al., 2007). There is a growing interest in *SREBP* genes due to their role in the synthesis of mammary fatty acids (Nafikov et al., 2013). Many of these studies are conducted in dairy cows, given the greater economic importance of cattle breeding and global consumption of dairy cow products. In cows, some variations in the *SREBP-1* gene sequence have been associated with milk quality traits (Hoashi et al., 2007; Cecchinato et al., 2012; Rincon et al., 2012), whilst its expression levels are also known to play a pivotal role in the regulation of milk fat synthesis (Bionaz and Loor, 2008).

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In recent years, several studies have been conducted on genes involved in improving milk quality among sheep and goats (Daga et al., 2013; Luridiana et al., 2014). Despite the importance of the *SREBP-1* gene in the regulation of milk fat synthesis in the ruminants' mammary gland, little is known about its role in dairy sheep. Currently only a preliminary study has been conducted in Sarda sheep within which the findings suggested that the different expression levels of this gene were associated with the milk fat yield (Carcangiu et al., 2013).

The relationship between polymorphisms and gene expression explains the role of *SREBP-1* gene in milk synthesis, and allows researchers to draw links between genotypes and phenotypes. Given the importance of Sarda sheep in nationals and global dairy production, it is important to study in more detail the involvement of this gene in dairy performances. The first aim of the present research was to characterize the coding sequence of the *SREBP-1* gene in Sarda sheep, and highlight possible relationships between single nucleotide polymorphisms (SNPs) and milk traits. Additionally, we evaluated if there was any relationship between SREBP-1 gene expression and nucleotide variation.

2. Materials and methods

2.1. Animals and experimental design

The study was performed on private farms, the owners of which provided their consent to engage their animals. A specific ethical approval was not necessary, as the methods used were common management procedures performed by vets on the National Veterinary Services in accordance with the Animal Welfare Act. Blood samples used for extracting DNA were taken from each subject by veterinarians of the National Veterinary Service during the official routinely controls. Milk samples were taken in the dates specified below during the morning daily milking (at 6.30 a.m.). 400 adult lactating Sarda ewes were selected from two farms (200 from each farm), which both had the same management and feeding conditions, and were located in North Sardinia (40°48′N 8°16′E). Each farm raised approximately 800 sheep; 200 of which were replacement lambs, and roughly 25 were rams. All ewes were multiparous (aged 3–5 years), had lambed in November, and had suckled their lambs until Day 21 after lambing. Only ewes at least at their third lambing were engaged for this study, as they were supposed to exhibit their effective milk production level. The subjects were fed on natural extensive pasture, and each received a of 300g daily supplement of commercial pellets (crude protein 20.4% and 12.5 MJ ME/kg DM). The ewes were penned at night in a sheepfold, where they received *ad libitum* hay (crude protein 11.1% and 7.2 MJ ME/kg DM) and water.

2.2. Blood and milk sampling

Individual blood samples ($n = 400$) were taken from the jugular vein of the chosen ewes 28 days after lambing using EDTA vacuum tubes (BD Vacutainer Systems, Belliver Industrial Estate, Plymouth, U.K.) in order to carry out the genomic analyses. Individual milk yield was recorded monthly from Day 30 to Day 150 of lactation, yielding a total of five recordings per ewe. The ewes were milked twice a day by a milking machine. In order to assess the mean level of milk production, individual milk yield was weighed in the morning (6.30 a.m.) and evening milkings (6.30 p.m.) on the days above specified. On the same dates, individual 10mL milk samples were taken from the morning milkings, to quantify somatic cell count (SCC), lactose, fat, and protein content. At Day 30 and Day 90 after lambing (corresponding to early and mid-lactation; based upon standard Sarda breed lactation length of 180 days) an additional 150mL milk sample was obtained from the morning milking from each ewe to conduct RNA extraction from somatic cells. An automatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark) was used to assess SCC according to the International Dairy Federation (IDF) 148–2:2006 method (IDF, 2006). To quantify lactose, fat and protein content, milk samples were analysed by infrared spectrophotometry according to IDF standard 141:2013 (ISO-IDF, 2013) (MilkoScan FT6000, Foss Electric, Hillerød, Denmark).

2.3. Genomic DNA preparation and amplification

A commercial DNA extraction kit (NucleoSpin®Blood, Macherey–Nagel, Düren, Germany) was used to obtain genomic DNA from 200 μl of whole blood following the supplier's protocol. The DNA samples were kept at -20 °C until use.

The primer set was synthesized by Life technologies, and reported in Table 1 together with annealing temperature. All primers were designed on the *SREBP-1* gene bovine sequence taken from ENSEMBL genome browser (http://www.ensembl.org) with accession number ENSBTAG00000007884. Primers were used for the amplification through a polymerase chain reaction (PCR) of the coding sequence of the *SREBP-1* gene (according to the latest genome version Oar_rambouillet_v1.0 - GenBank assembly accession number: GCA_002742125.1). Amplified fragments were named F1–F19, corresponding to exons from 2 to 20, respectively. Each 25 μL PCR mixture (polymerase chain reaction) consisted of the following reagents, expressed in their final concentrations: 1X PCR Buffer (minus $MgCl_2$) (20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM Sodium Phosphate, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50% (v/v) glycerol), the $MgCl_2$ varied based on the characteristics of the individual fragments: 1.2mM of $MgCl_2$ for F8 and F12, 1 mM for F19 and 1.5 mM for all the other fragments. Moreover, 0.2 μM of each dNTPs, 0.4 μM of each primer for F1 and F5, while for all the other fragments was 0.32 μM, and 1U of *Taq* DNA polymerase (Platinum *Taq* DNA Polymerase Invitrogen, Carlsbad, CA, USA).

The PCR reaction was performed on a Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany). Amplicons were separated by electrophoresis on 1.5% (w/v) agarose gel (GellyPhor, EuroClone S.p.A., Pero, Milano, Italy), compared with a 100 bp DNA marker (Invitrogen, Carlsbad, CA, USA) in 1X TAE buffer at a stable voltage of 100 V for 30 min. Afterwards, electrophoresis agarose gels were stained with ethidium bromide and visualized by an ultraviolet transilluminator (UVItec, Cambridge, U. K.).

2.4. DNA sequencing

Amplicons obtained from the 19 fragments were purified using a commercial DNA purification kit (ChargeSwitch® PCR Clean-Up kit, Invitrogen, Carlsbad, CA, USA) and sequenced using a commercial service (Bio-Fab Research srl, Roma, Italy) in forward and reverse directions. Nucleotide sequence alignments, translations, and comparisons were performed using the Bioedit software (www.mbio.ncsu.edu/bioedit/bioedit.html). The NCBI (National Center for Biotechnology Information) Blast algorithm was used to search the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) for homologous SNP discovery, genotyping, and protein sequence alignment. Multiple alignment of SREBP-1 protein sequences from mammalian species was performed using the Web-based Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/EMBL-EBI) (Sievers and Higgins, 2014).

2.5. RNA extraction and real-time quantitative PCR

Sequencing and sequence alignment highlighted nucleotide polymorphisms (SNPs) in the 19 analyzed fragments, allowing for the identification of possible genotypes for each fragment. Precisely eight SNPs were found, corresponding to 19 different genotypes (only those that occurred in more than five subjects were analysed). Five randomly chosen samples from each genotype (totalling 95 samples) were used to assess, from extracted RNA, the expression patterns of the *SREBP-1* gene at Day 30 and 90 after lambing, in order to relate polymorphisms with gene expression level. Total RNA was extracted from whole milk by the 150 mL samples taken at Day 30 and 90 after lambing, in the same manner as was done in Mura et al. (2013). In brief the milk samples were

Table 1. *SREBP-1* gene primer sequence, fragments' length and annealing temperature.

Fragments name	Primer sequence (5′ to 3′)[a]	Length (bp)	Annealing T (°C)
F1	F: CCCAGTTTCCGAGGAACTTTTC R: GGCCCTGACGCACCTTCTAT	221	60.5
F2	F: ACGGCTGCTCACGGCTTT R: AGCCTGCAAACTCCTCTACCA	523	64.3
F3	F: AGCCCCAGCCTTCATCTCT R: TCCCTGATGCCAGCCAGAC	238	62.0
F4	F: CCTCCCAGATACAGCAGGTC R: GGCAGAGTTAGCAGGTGGAC	333	64.0
F5	F: CCTGACGACCATGAAAACAG R: TATTAGGGCCTCAGCCCACA	436	61.0
F6	F: CTCTGCCCTCTTGCTTCAGT R: AACTTCCAGGGACACCAG	228	54.7
F7	F: CCTGGTGTCCCTGGAAGTT R: CCCTCAGCCTTGTCTTTCTTC	495	59.0
F8	F: GCTGAAGGGTTCCCACAGTA R: CACAGGACGGGATCCACATA	365	63.1
F9	F: GATCTTGTCCTGTGGGCTTG R: AGCACCTTCCCAGGCACT	327	67.0
F10	F: CCCAAGATGGAGGAGTAGCA R: TGGAAGATAAGAGGGCGTGA	398	62.0
F11	F: ATGGGTATGCGGGTGAGG R: GCTGTTGAGGAGGGAATGG	387	61.0
F12	F: GTGAGGGCTGCACAGAAAG R: AGGCAAGGGACAAGACACTG	388	65.0
F13	F: GGTGCGTGTGCAAAGGAG R: CCCAGAGAGGAACCGAAATG	284	56.0
F14	F: AGCCATGTTGACCGCCTGT R: GCAGAAACTCAGCCACACTG	222	61.0
F15	F: GCTGAGTTTCTGCCTCCTGT R: CTCTGCCCTGGTTCTGGAT	276	60.1
F16	F: ATCCAGAACCAGGGCAGAG R: CATCCAGGGAGTGGAAAGG	287	64.0
F17	F: TTGTGAGGCAGGTGCAGTG R: AGTCGGGCAGTGGCTTCAT	455	64.0
F18	F: GGGACAGGCATGAGGTGT R: CATCTTACGGTCTCCCTCTG	246	62.1
F19	F: CTTCTGGACCGTAGCCTGAG R: AGCTGGAGGTCACAGTGGTC	603	57.0

[a] F is forward primer and R is reverse primer.

centrifuged at 448g for 15 min at 6 °C. The supernatant was removed and the remaining cell pellet was washed twice with 10 mL of PBS (Phosphate Buffered Saline), pH 7.2, and supplemented with 0.5 mM of EDTA (Ethylene-D-amine-Tetra-Acetic-Acid) and DEPC 0.1% (D-Ethyl--Pyro-Carbonate) to inhibit RNase. This prevented the formation of casein micelles and fat globules. 404 μL of 5 mM TCEP (tris (2-carboxyethyl) phosphine) + lysis solution was added to the cell pellet and homogenized by vortex for 2 min. A commercial kit (PerfectPure RNA Tissue Kit, 5 PRIME GmbH, Hamburg, Germany) was used to extract total RNA. RNeasy Mini Kit columns (Qiagen, Hilden, Germany) were used to remove genomic DNA from RNA. The obtained RNA was eluted in 50μL of elution solution and then frozen at −80 °C. The quality of the extracted RNA was evaluated by spectrophotometer reading (Eppendorf Bio-Photometer, Eppendorf AG, Hamburg, Germany). By electrophoresis in denaturing agarose gel was assessed the RNA integrity of 28S and 18S rRNA subunits.

Following the manufacturer's protocol, approximately 500 ng of the extracted RNA were used to synthesize cDNA, using a commercial kit (iScript cDNA Synthesis Kit, Bio-Rad Laboratories, Hercules, CA, USA): 5X iScript reaction mix, 4 μL; iScript reverse transcriptase, 1 μL; RNA template, 7 μL (corresponding to 500–600 ng, approximately); Nuclease-free water, 8 μL. The reaction was performed in an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) gradient according to the kit's protocol: 25 °C for 5 min, at 42 °C for 30 min, 85 °C for 5 min, held at 4 °C.

The cDNA was sequenced in forward and reverse direction, and compared with sequences deposed in the NCBI-BLAST public database (http://www.ncbi.nlm.nih.gov/BLAST). The qPCR was performed using RealMasterMix SYBR ROX (5 PRIME GmbH, Hamburg, Germany) starting with mixing in one individual tube (1.0 mL) of 2.5X RealMasterMix SYBR ROX and 125 μL of SYBR ROX solution. The qPCR reaction was carried out in triplicate in a 12 μL final volume containing 4 μL cDNA diluted 1:3 (using DNase/RNase free water), and 8 μL of qPCR mastermix, composed as follows: 2.5X RealMasterMix SYBR ROX (0.05U/μL HotMaster Taq DNA Polymerase, 10 mM Magnesium Acetate (4.0 mM final), 1.0 mM dNTPs with dUTP (0.4 mM final) with the addition of 20X SYBR solution as above specified) 5 μL; 10 pmol/μL of each primer, 0.4 μL of each primer (333 nM final); DNA/RNA-free water, 2.2 μL. Samples were placed in 96-well Eppendorf plates in Eppendorf RealPlex ep gradient S (Eppendorf AG, Hamburg, Germany). Primers for *SREBP* and *UXT* genes were taken from Bionaz and Loor (Bionaz and Loor, 2007, 2008). UXT is a ubiquitously expressed trancript that can be used as internal control for mammary gland expression profiles as *UXT* is one of the genes having the most stable expression ratio across animal and time (Bionaz and Loor, 2007). For this reason, only *UXT* was used as reference gene because its invariant expression was already proven by previous studies (Bionaz and Loor, 2007, 2008). The qPCR conditions were: 95 °C for 2 min; followed by 40 cycles shared in denaturation at 95 °C for 15 s and annealing + extension at 60 °C for 1 min. The presence of a single

PCR product was verified by the dissociation protocol using incremental temperatures of up to 95 °C for 15 s and 65 °C for 15 s (repeated for 20 min), and then up to 95 °C for 15 s. The relative quantities of *SREBP* mRNA in somatic cell samples in the two different lactation stages were determined using the ΔΔCT method (Livak and Schmittgen, 2001), the ΔCT value was obtained by subtracting the average reference gene *UXT* CT value from the average target *SREBP* CT value, using GenEx software. A reference sample with SSC <100,000, and fat percentage of 5.37 was used as calibrator, whilst PCR-normalized data are presented as n-fold change relative to this sample.

2.6. Statistical analysis

GENEPOP population genetics software version 4.1 (http://kimura.univ-montp2.fr/~rousset/Genepop.htm) (Raymond and Rousset 1995; Rousset 2008) was used to calculate the genotypic and allelic frequencies, and the Hardy-Weinberg equilibrium. Statistical analysis was performed using R-Project software (R Core Team, 2018, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). In order to evaluate the association between *SREBP-1* gene polymorphisms or haplotype and milk yield, fat and protein contents, the General Linear Model (GLM) was used. The linear model used was the following as reported in model (1):

$$Y_{jklm} = \mu + F_j + G_k + N_l + D_m + E_{jklm} \quad (1)$$

where Y_{jklm} was the trait measured on each animal, μ the overall mean, F_j is the fixed effect of the farm, G_k is the fixed effect of the k^{th} genotype (or haplotype), N_l is the fixed effect of number of lactations, D_m is the fixed effect of DIM, and E_{jklm} is the error effect. P value < 0.05 was considered statistically significant. Pearson's correlation analysis was used to determine the relationship between fold change and the considered milk traits (fat yield, fat, proteins and lactose content). The Anderson-Darling test was used to determine if the data was normally distributed, after which an ANOVA (one-way analysis of variance) was applied to analyse the association between genotypes and expression levels of *SREBP-1* gene. The following linear model was applied as reported in model (2):

$$Y_{jk} = \mu + F_j + G_k + e_{jk} \quad (2)$$

where Y_{jk} is the trait measured on each animal (*SREBP-1* expression), F_j is the fixed effect of the farm, G_k is the fixed effect of the k^{th} genotype and e_{jk} is the error effect.

3. Results

3.1. Ewes' milk yield and composition

Table 2 summarizes the descriptive statistics of milk yield and composition, and the results from the linear model; additionally, the average concentration of the milk quality traits, for each sampling time are shown. Although phenotypic differences were found in milk yield among ewes, the mean values of production level in each of the five recording periods were not statistically significant. The highest mean daily milk yield was recorded at 90 days after lambing, whilst the lowest was recorded at 150 days after lambing; although no statistical differences were detected between these ranges. The fat, protein, and lactose concentrations showed no significant variations among the five recording periods.

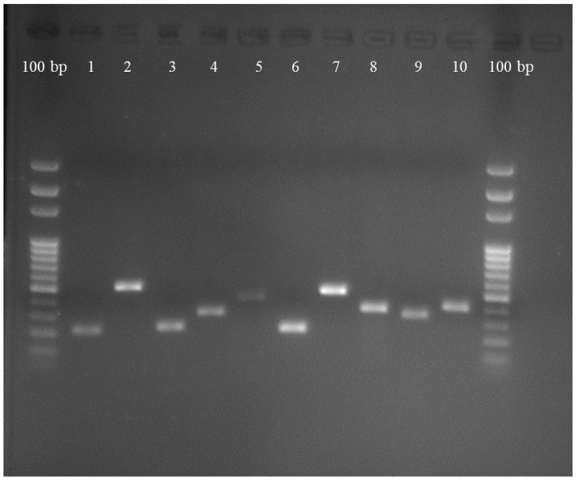

Figure 1. Electrophoresis on a 1.5% agarose gel of the amplified fragments of the *SREBP-1* gene in Sarda breed sheep. Lanes 1 and 12: 100bp marker; Lanes 2–11: fragments F1–F10. For a full-size image of the gel, see Figure S1 in the Supplementary files.

3.2. DNA analysis results

The length in base pair (bp) of the obtained amplicons, and confirmed by sequencing, is shown in Table 1. In Figures 1 and 2 the electrophoretic migration patterns of all the amplified fragments (F1–F19) are shown (the full-size figure gels are shown in Supplementary files Figure S1 and Figure S2, respectively). Each fragment consisted of the entire exonic sequence and a small part of the adjacent introns; except for the F19 which includes the final part of the exon 19, the entire intron 19 and the coding region of the exon 20. Nucleotide variations were found in the F3, F5, F11, F15, F16 and F19 fragments. An important result from the present research is the detection of eight novel SNPs in the ovine *SREBP-1* gene. Through sequencing, seven nucleotide substitutions were found at the exonic level, and one at the intronic level (Table 3). The found variations are expressed based on their chromosome position, according to the latest genome version Oar_rambouillet_v1.0 (GenBank assembly accession number: GCA_002742125.1). The g.28669555G>T and g.28664527G>A variations exhibited an amino acid change, *Gly/Val* and *Gly/Ser*, respectively. The exons' sequence and the relative SNPs we found, have been deposited in GenBank under accession number KF360085.2. The population was in Hardy-Weinberg equilibrium for all the analysed genotypes. Table 4 shows the SNPs position and the obtained allelic and genotypic frequencies. All of the identified SNPs showed a clear predominance of one of the found alleles (Table 3). In the g.28664767C>T and g. 28664558G>A SNPs, no homozygous T/T and A/A, respectively, were found. Variations at position g.28669556 and g.28669555 of the exon 4 were consistently observed together.

Table 2. Average (±SD) milk production and composition of Sarda ewes (n = 400) in the five sampling times.

Days of lactation	Milk yield g/die	Fat %	Protein %	Lactose %	SCC X10^3
30	975.4 ± 261.5	6.5 ± 0.7	6.6 ± 0.6	4.8 ± 0.3	78.6
60	1002.8 ± 252.4	6.2 ± 0.6	6.4 ± 0.5	4.8 ± 0.4	76.4
90	1087.3 ± 275.2	6.1 ± 0.7	6.5 ± 0.6	4.9 ± 0.4	79.2
120	932.6 ± 239.6	6.2 ± 0.8	6.4 ± 0.4	4.8 ± 0.3	78.6
150	855.9 ± 267.1	6.3 ± 0.7	6.5 ± 0.6	4.8 ± 0.3	79.2

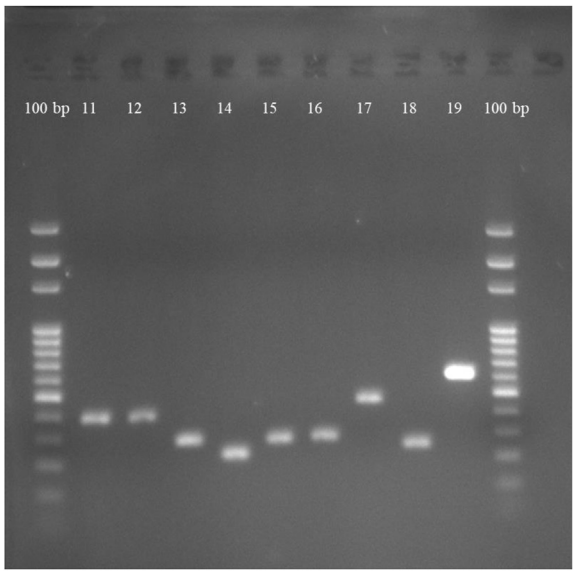

Figure 2. Electrophoresis on a 1.5% agarose gel of the amplified fragments of the *SREBP-1* gene in Sarda breed sheep. Lanes 1 and 11: 100bp marker; Lanes 2–10: fragments F11–F19. For a full-size image of the gel, see Figure S2 in the Supplementary files.

The results obtained by GLM did not shown a significant association between the identified SNPs and the analysed milk traits (milk yield, fat, proteins and lactose content) (Table 4). Through data processing, 12 haplotypes were identified, among which only those with a frequency greater than 0.02 were considered. In Table 5, the milk yield, fat, and protein content for each haplotype are shown. Statistical analysis did not highlight significant associations between haplotypes and the analysed milk traits.

3.3. SREBP-1 expression patterns result

The fragment used for the gene expression analysis correspond to the final part of exon 17 and the first part of exon 18 of the *SREBP-1* gene. This fragment produced a single 67-bp band after PCR of the cDNA, and a 514 bp band after PCR of the genomic DNA. Table 6 shows the RNA concentration and SCC in the two lactating stages examined (at 30 and 90 days of lactation). The amount of the RNA obtained from each sample was different, depending on the SCC content. In particular, an increase in the number of somatic cells corresponded to a greater amount of extracted RNA content, although this correlation was not significant. Correlations among fold change, fat yield, and concentration of fat, protein, and lactose are shown in Table 7. The statistical analysis showed a significant correlation between the fold change and fat yield at 30 and 90 days of lactation ($P < 0.01$). The other considered milk traits were not significantly correlated with fold change. The statistical analysis did not show associations between the identified polymorphisms and the expression of the *SREBP-1* gene at 30 and 90 days of lactation (Table 8).

4. Discussion

The present study allowed for the characterization of the coding sequence of the *SREBP-1* gene in Sarda sheep for the first time. This result is of great importance for the knowledge of the regulatory genes that control the secretion of milk fat in dairy sheep. The coding sequence of this gene was deposited into GenBank (accession number: KF360085.2), and was confirmed to be correct and complete (for CDS) for ovine specie. The sequence alignment with the latest genome version Oar_rambouillet_v1.0, showed correspondence between our fragments and the isoform X2 (NCBI accession number XM_027974784.1). It is important to specify that the *SREBP-1* gene isoform X2 includes 20 exons, but the first exon resulted in a non-coding exon, thus our fragments, here named F1 to 19, correspond to Exon 2 to 20, in the above sequence. The location of the ovine *SREBP-1* gene, within the Chromosome 11, extends from c.28680451 to c.28662503 and consists of over 17,900 nucleotides. The sequence emerged from the present study is similar to the bovine sequence, deposited in database (98% homology), and correspond to the *SREBP-1a* variant; the *SREBP-1c* sequence variant, which presumably occurs in humans, mice, and related species, was not found (Shimomura et al., 1997).

An important finding is the highlighting of eight different SNPs for the first time, specified as follows: 3 G>T transversions, at positions g.28669556, g.28669555 and g.28663190; 3C > T transition, at positions g.28668850, g.28666130 and g.28664767; and 2 G>A transition at positions g.28664558 and g.28664527. The SNPs at position g.28669555 and g.28664527 respectively also exhibited amino acid changes: *Gly/Val* and *Gly/Ser*. The number of SNPs found in Sarda sheep is quite low compared to the variation registered in other species in the NCBI dbSNP database. However, the ovine genome sequence is constantly evolving, thus it remains difficult to find a perfect correspondence between all of the data present in the databases. Moreover, no studies have been carried out as of yet on the possible association between *SREBP-1* gene SNPs and milk production traits in sheep. The Sarda sheep has been subjected to strong genetic selection in the last century (Luridiana et al., 2014), perhaps leading to the reduction of variations. Furthermore, in other dairy species such as buffalo and goats, few variations in the sequence have been highlighted (Deng et al., 2017; Manunza et al., 2012). The low variation in the *SREPB-1* gene found in different species evidences the important role of this gene in the milk fat secretion. Additionally, the animals carrying variations which were negatively linked to milk traits seem to have been eliminated from the flock over time. In Sarda sheep, additional genes have been involved in milk production, such as *POU1F1*,

Table 3. Position and amino acid change effect of the identified SNPs in the SREBP-1 gene, in Srada breed sheep (n = 400). The SNPs are ordered according to their positions in the latest genome version (Oar_rambouillet_v1.0, GenBank assembly accession number: GCA_002742125.1).

Fragment	SNP Position	SNP Location[a]	SNP alleles[b]	Amino acid change
F3	g.28669556	Ex4	GT	
F3	g.28669555	Ex4	GT	Gly/Val
F5	g.28668850	Ex6	CT	
F11	g.28666130	In12	CT	
F15	g.28664767	Ex16	CT	
F16	g.28664558	Ex17	GA	
F16	g.28664527	Ex17	GA	Gly/Ser
F19	g.28663190	Ex20	GT	

[a] Ex is for Exons and In is for Introns.
[b] Sequence is in a reverse orientation on the above specified genome version, so that nucleotide substitution appears in the reverse form compared to the present study.

Table 4. *SREBP-1* gene SNPs position, allele and genotype frequencies and associations with milk traits (n = 400).

SNP position	Allele frequency (%)	Genotype frequency (%)	Yield (g/die)	*P*-value	Fat (%)	*P*-value	Protein (%)	*P*-value
g.28669556G>T	G 90	GG 83	974.9 ± 268.1	0.1	6.3 ± 0.7	0.4	5.8 ± 0.5	0.1
	T 10	GT 15	875.3 ± 99.5		6.5 ± 0.7		6.2 ± 0.5	
		TT 02	986.6 ± 263.4		6.4 ± 0.5		5.9 ± 0.6	
g.28669555G>T	G 90	GG 83	974.9 ± 268.1	0.1	6.3 ± 0.7	0.4	5.8 ± 0.5	0.1
	T 10	GT 15	875.3 ± 99.5		6.5 ± 0.7		6.2 ± 0.5	
		TT 02	958.2 ± 238.7		6.3 ± 0.6		6.1 ± 0.6	
g.28668850C>T	C 70	CC 49	993.9 ± 276.7	0.3	6.3 ± 0.8	0.9	5.8 ± 0.4	0.1
	T 30	CT 46	918.6 ± 222.5		6.3 ± 0.7		5.9 ± 0.5	
		TT 5	1091.0 ± 265.3		6.3 ± 0.5		5.9 ± 0.6	
g.28666130C>T	C 60	CC 35	924.7 ± 236.9	0.6	6.2 ± 0.6	0.3	5.9 ± 0.5	0.9
	T 40	CT 52	986.1 ± 269.8		6.3 ± 0.8		5.8 ± 0.5	
		TT 13	987.3 ± 243.4		6.6 ± 0.8		5.9 ± 0.5	
g.28664767C>T	C 98	CC 95	936.1 ± 254.4	0.8	6.3 ± 0.7	0.7	5.9 ± 0.5	0.5
	T 2	CT 5	989.7 ± 280.9		6.4 ± 0.7		5.7 ± 0.4	
		TT 0						
g.28664558G>A	G 80	GG 68	880.6 ± 188.6	0.1	6.1 ± 0.5	0.2	6.0 ± 0.5	0.2
	A 20	GA 32	1003.3 ± 271.8		6.3 ± 0.8		5.8 ± 0.5	
		AA 0						
g.28664527G>A	G 85	GG 75	1060.7 ± 355.0	0.3	5.8 ± 0.4	0.1	5.7 ± 0.6	0.1
	A 15	GA 21	882.2 ± 268.5		6.0 ± 0.8		5.6 ± 0.5	
		AA 4	983.3 ± 244.2		6.4 ± 0.7		5.9 ± 0.5	
g.28663190C>T	C 83	CC 70	1020.7 ± 298.0	0.3	5.9 ± 0.4	0.1	5.8 ± 0.5	0.1
	T 17	CT 26	975.2 ± 283.5		6.1 ± 0.7		5.7 ± 0.6	
		TT 4	982.3 ± 267.2		6.2 ± 0.8		5.8 ± 0.5	

P > 0.05 was not significant.

Table 5. Haplotypes frequency of *SREBP-1* gene, mean of the analysed milk traits in Sarda sheep (n = 400).

Haplotype	Frequency (%)	Yield (g/die)	Fat (%)	Protein (%)
GGCTCGG	28	1021.1 ± 261.0	6.5 ± 0.6	5.7 ± 0.4
GGCCCGA	12	967.0 ± 250.4	5.9 ± 0.5	5.5 ± 0.3
GGCCCGG	19	1019.0 ± 243.1	6.4 ± 0.7	5.6 ± 0.5
GGCCTGG	3	1030.4 ± 253.0	6.4 ± 0.6	5.5 ± 0.4
GGTCCAG	15	862.9 ± 251.7	6.2 ± 0.5	6.0 ± 0.5
GGTCCGG	13	1069.2 ± 242.9	6.6 ± 0.6	5.8 ± 0.4
TTCTCGG	8	977.3 ± 221.8	6.4 ± 0.7	6.0 ± 0.3

Table 6. Mean of RNA concentration and Somatic Cells Count (SCC) at 30 and 90 days of lactation (n = 95).[a]

Days of lactation	RNA (μg/mL)	SCC ($x10^3$ cells/mL)
30	63.3	92.3
90	55.6	84.7

[a] (n = 95) corresponds to 5 animals, randomly chosen, for each of the found genotype.

Table 7. Correlation among fold change and milk traits at 30 and 90 days of lactation (n = 95).[a]

Fold change[b]	Fat yield	% fat	% protein	% lactose
Day 30 of lactation	0.56***	-0.03	-0.25	0.05
Day 90 of lactation	0.54***	-0.01	-0.11	-0.32

*** = $P < 0.001$.

[a] (n = 95) corresponds to 5 animals, randomly chosen, for each of the found genotype.

[b] Fold change is the measure of the change in the level of expression of the *SREBP-1* gene.

Table 8. Association of mean (±SD) gene *SREPB-1* expression and genotypes in Sarda sheep (n = 95).[a]

SNP position	Genotype	Fold change 30 days	*P*-value	Fold change 90 days	*P*-value
g.28669556G>T	GG	4.85 ± 0.14	0.16	4.91 ± 0.15	0.20
	GT	5.01 ± 0.12		5.15 ± 0.19	
	TT	4.73 ± 0.19		4.82 ± 0.17	
g.28669555G>T	GG	4.85 ± 0.15	0.16	4.91 ± 0.15	0.20
	GT	5.01 ± 0.14		5.15 ± 0.19	
	TT	4.73 ± 0.13		4.82 ± 0.17	
g.28668850C>T	CC	4.78 ± 0.18	0.14	4.68 ± 0.16	0.19
	CT	5.12 ± 0.15		4.96 ± 0.18	
	TT	4.92 ± 0.17		5.09 ± 0.16	
g.28666130C>T	CC	4.57 ± 0.16	0.59	4.79 ± 0.15	0.33
	CT	4.84 ± 0.15		4.88 ± 0.18	
	TT	4.75 ± 0.18		4.97 ± 0.14	
g.28664767C>T	CC	5.10 ± 0.15	0.28	5.08 ± 0.13	0.20
	CT	5.21 ± 0.16		5.18 ± 0.15	
g.28664558G>A	GG	4.83 ± 0.15	0.51	4.95 ± 0.17	0.41
	GA	4.72 ± 0.18		4.86 ± 0.15	
g.28664527G>A	GG	4.86 ± 0.19	0.17	4.98 ± 0.18	0.22
	GA	4.54 ± 0.14		4.88 ± 0.16	
	AA	4.46 ± 0,16		4.67 ± 0.14	
g.28663190C>T	CC	4.82 ± 0.17	0.20	4.95 ± 0.16	0.18
	CT	4.64 ± 0.18		4.81 ± 0.18	
	TT	4.56 ± 0.15		4.74 ± 0.19	

$P > 0.05$ was not significant.

[a] (n = 95) corresponds to 5 animals, randomly chosen, for each of the found genotype.

exhibited few variations compared to other European breeds (Bastos et al., 2006; Mura et al., 2012). Another study, on the *INSIG-2* gene in Sarda sheep, exhibited a small number of mutations in the gene sequence, all of which were identified at intronic level (Luridiana et al., 2014). The latter gene cooperates with the *SREBP-1* gene in the regulation of the fatty acids' synthesis also at the mammary gland level. It can thus be hypothesized that the low variability of the *SREBP-1* gene is aimed to preserve the high milk fat synthesis, which is typical of the Sarda breed sheep.

Although we found several SNPs, some of which have caused amino acid changes, no associations were found between these variations and milk traits. These results are in agreement with the findings of Deng et al. (2017) and Manunza et al. (2012) regarding buffalo and goats, respectively. Conversely, a deletion of 84bp at Intron 5 was found to influence the milk fatty acids composition among cattle. Moreover, in cattle, one polymorphic site at position 66 of the Exon 14 was shown influence the carcass fat compositions, and the expression of other genes involved in the milk synthesis (Rincon et al., 2012; Hoashi et al., 2007). In the present study no deletion at intron 5 and all the animals carried C/C genotype at position 66 of the exon 14. This genotype occurs in cattle exhibiting a reduced milk fat synthesis (Rincon et al., 2012), whilst, within this study, it was not associated to milk fat yield. Thus, we can hypothesize that in ovine specie this site is unrelated to the milk fat synthesis. Moreover, we can speculate that in cattle the C/C genotype, in the above location, is correlated to other variants within the nucleotide sequence, causing the milk fat-decreasing effect. Since SREBP-1 regulates a wide range of about 30 other genes and triggers lipid synthesis in the mammary gland, small variations in its sequence, without modification of functional domains, are unable in sheep to influence the production process of the milk fat.

Recently, it has been found that in the goat mammary gland, the *SREBP-1* gene regulates the expression of the acyl-CoA short chain synthetase (*ACSS2*) gene; this indicates the important role played by the *SREBP-1* gene in the synthesis of fatty acids (Xu et al., 2018). Additionally, the acetate absorbed by the cells is linked to the coenzyme A (CoA), from the *ACSS2* gene, to form acetyl-CoA, which is used for the synthesis of fatty acids, also in the mammary tissue. In ruminants, approximately half of the milk fatty acids are derived from de novo synthesis. Acetate, which is produced from ruminal carbohydrate fermentation, is the major carbon source for de novo synthesis (Bauman et al., 2006). Hence, as mentioned above, variations in the sequence of the *SREBP-1* gene causing alterations in the fat production process may have been eliminated over time by breeders, since the milk had poor organoleptic qualities.

The use of milk somatic cells as RNA source (instead of mammary gland tissue) exhibited its efficiency by allowing repeated controls during lactation, avoiding mammary gland trauma and preventing the animals from experiencing distress or pain; this is another important step towards a greater respect to animal welfare. Our results on the *SREBP-1* gene expression patterns during early and mid-lactation, evidenced the important role of this gene in the secretion of milk fat in dairy animals. The main body of studies on *SREBP-1* in dairy animals is carried out on cattle, indicating its pivotal role in the milk fat synthesis regulation (Bauman et al., 2006; Bionaz and Loor, 2008; Ma and Corl, 2012; Li et al., 2014). This central role of the *SREBP-1* gene has been documented also among yak, goat and sheep (Carcangiu et al., 2013; Xu et al., 2016; Lee et al., 2017).

A very large increase in expression from pregnancy to lactation was found in goat mammary tissue (approximately 30-fold), similarly providing additional support for its key role also in small ruminants (Xu et al., 2016). In our study the highest *SREBP-1* expression level was found in high milk fat producing ewes at Day 90 of lactation, suggesting that this gene also plays a central role in the expression levels of sheep. These differences in gene expression levels, in addition to variations related to the applied methodologies, can be linked to the high level of fat content in the milk of sheep, goats, yaks, and even more so in buffaloes. Therefore, our initial hypothesis that the different individual *SREBP-1* gene expression level found, which could be due to the polymorphisms in the coding sequence of this gene, was not confirmed by data analysis. Also, the analysis of genotypic combination showed no statistical correlation with *SREBP-1* mRNA expression. This finding reinforces the notion that *SREBP-1* is a very stable gene, due to its important role in milk fat

synthesis; this is further confirmed by its higher expression patterns in ewes producing the greater milk fat yield (g/die).

Our data supported previous findings regarding the need for high expression level of *SREBP-1* gene for the milk fat yield, however, other genes, involved in the fat uptake, are most likely to be actively engaged in the regulation of the fat concentration (Carcangiu et al., 2013). However, in order to confirm that the data obtained here, it may be necessary to extend sampling to a larger number of animals, and to assess the expression of the *SREBP-1* gene over the entire course of lactation.

5. Conclusion

In conclusion the findings contributed to the current understanding of exonic and intronic regions of the *SREBP-1* gene in dairy sheep, and highlighted the presence of different polymorphic sites in its nucleotide sequence. The importance of this gene in the milk fat yield is identified, and a positive relationship between *SREBP-1* gene expression and the milk fat yield in sheep has been highlighted. The polymorphisms found within *SREBP-1* gene exhibited no associations with milk traits, or with individual *SREBP-1* mRNA expression patterns among Sarda sheep, suggesting that the small variability may depend on the genetic selection aimed to improve the milk fat yield in this breed. Further efforts are required to evaluate the exact role of the identified SNPs in a larger sample size, as well as among different dairy sheep breeds, to expand the knowledge regarding this gene and to evaluate *SREBP-1* gene polymorphisms in breeds with different purposes.

Declarations

Author contribution statement

V. Carcangiu; S. Luridiana; M.C. Mura: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

L. Pulinas; M.V. Di Stefano; G. Cosso: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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