The single nucleotide polymorphism g.133A>C in the stearoyl CoA desaturase gene (SCD) promoter affects gene expression and quali-quantitative properties of river buffalo milk

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A variant in the *SCD* gene promoter affects buffalo milk

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The *SCD* plays an essential role in the biosynthesis of monounsaturated fatty acids (FAs). It has been extensively investigated in farm animals for its influence on milk fat quality. Among ruminants, the buffaloes produce milk with a higher fat content; therefore, the improvement of this trait is one of the main goals for the progress of the species. We have elucidated the biological role of a variant found in the *SCD* promoter combining mRNA profile analysis, electromobility shift assay and q-PCR. Our data revealed no influence of the SNP on gene transcripts; however, this variant affects the binding of a Sp1 transcription factor, the gene expression and milk FAs content.

The SNP g.133 A>C in *SCD* promoter affects gene expression and quali-quantitative properties of river buffalo milk

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A VARIANT IN THE *SCD* GENE PROMOTER AFFECTS BUFFALO MILK

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ABSTRACT

The stearolyl-CoA desaturase (*SCD*) gene has been deeply investigated in ruminants because of its effect on milk fat composition. In river buffalo, the SNP g.133A>C at the gene promoter has been associated with milk quality and yield. However, the biological reason for such effects remained unexplored. In this study, we have combined mRNA profile analysis, electromobility shift assay and q-PCR to elucidate the role of this SNP on gene transcription and its effects on milk fat traits. A preliminary genotyping of the SNP g.133A>C was carried out on a group of 303 river buffaloes to choose the individuals for the downstream applications. Allele frequencies showed an increase of the minor allele C (0.25) compared with previous findings (0.16). Six animals (two for each genotype) were chosen for cloning and a total of 216 positive cDNA recombinant clones for *SCD* (72 per genotype) were analyzed by PCR. All clones showed the same length on agarose gel, therefore random clones were chosen for the sequencing. No qualitative differences were found and all gene transcripts resulted correctly assembled. Electrophoretic mobility shift assay was performed to evaluate the binding of the transcription factor Sp1 to DNA sequences including the SNP g.133A>C. The genotype CC showed a higher binding affinity than the genotype AA in two different conditions (EB: 201.77 ± 4.06 *vs* 141.65 ± 3.77 and PB: 95.90 ± 1.15 *vs* 67.30 ± 2.14). The following q-PCR confirmed the up-regulation of the CC compared with AA and AC genotypes. The association study with milk fat traits revealed a favourable effect of the allele C. The heterozygous genotype had the highest values for MUFAs, oleic acid (C18:1 c9), PUFAs and odd branched-chain fatty acids (OBCFAs), and the lowest values for SFAs, atherogenic and thrombogenic indices, and significantly differed from the AA genotype. The AC genotype had been previously associated with higher milk yield. Therefore, the SNP g.133A>C is a marker with a dual impact and it is an interesting candidate for assisted selection programs in river buffalo. These data clarified the biological role of the SNP g.133A>C in the *SCD* promoter and how it affects the gene function, providing important knowledge on the genetic background of lipid metabolism, including the future possibility of selecting alleles with quantitative or qualitative favourable effects.

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2 Keywords: River buffalo, Stearoyl-CoA, Transcripts, Gene expression, Sp1 transcription factor

INTRODUCTION

In the last decades the riverine buffalo population (*Bubalus bubalis*) in Italy (Mediterranean river buffalo breed) has highly increased, reaching more than 385,000 heads in 2016 (FAO, 2016), with over 59,000 buffaloes in 272 farms (ANASB, 2017) officially recorded for production. This positive trend facilitated the development of the whole buffalo dairy industry, which currently involves 102 cheese factories officially registered for Buffalo Mozzarella PDO (EC Reg. nos. 1107/1996 and 510/2006), producers association (https://www.mozzarelladop.it/consortium/dop-in-numbers) and more than 20,000 operators, with a consolidated turnover estimated in about 720 million of euros (ISMEA, 2016).

Considering the high market demand for buffalo dairy products, the main breeding goal of the Italian National Association of Buffalo Breeders (ANASB) is the improvement of milk yield. However, the quality of milk and dairy products are fundamental prerequisites to satisfy the requirements of national and international consumers. In this context, fatty acid (FA) composition has become a crucial aspect for the assessment of milk nutritional value. Animal feeding is considered the main source of variation of milk FA composition. However, several studies have demonstrated that genetic variants found in key genes involved in FAs metabolism may be responsible for changes in milk FAs profile (Bionaz and Loor, 2008, Thering et al., 2009).

In this respect, one of the most investigated genes in ruminants belongs to the stearoyl-CoA desaturase (*SCD*) gene family, which in cattle is composed of two different isoforms, *SCD*1 and *SCD*5 (Lengi and Corl, 2007). The *SCD*1 gene, mapped on bovine chromosome 26, codes for the stearoyl-CoA desaturase, an endoplasmic reticulum enzyme which plays an essential role in cellular biosynthesis of monounsaturated fatty acids (MUFAs). The stearoyl-CoA desaturase is responsible for catalysing the insertion of a double bond between the carbons 9 and 10 of the FAs, activity carried out also in the mammary gland (Bionaz and Loor, 2008). In addition, the enzyme is involved in the endogenous production of the cis-9, trans-11 isomers of conjugated linolenic acid (CLA),

which are generally found in ruminant milk and considered healthy in the human diet (Bhattacharya et al., 2006).

The *SCD1* gene has been intensively studied in cattle, where a total of 8 SNPs forming two haplotypes (A and B) have been found (Medrano et al., 1999). Furthermore, associations between a missense polymorphism at the exon 5 (GenBank AY241932: g.10329C^{Ala}>T^{Val}) and the concentration of some individual FA and FA unsaturation indices in the milk fat have been reported in several cattle breeds (Mele et al., 2007, Moioli et al., 2007, Schennink et al., 2008).

In a previous study, we reported a great genetic diversity also within the river buffalo *SCD* gene. In particular, a total of 15 SNPs spread from the gene promoter to the 3' UTR have been found (Pauciullo et al., 2010). Among them, the transversion FM876222:g.133 A>C in the promoter has been considered of particular interest because it falls between two Sp1 binding sites. The bioinformatic analysis showed that the allelic form C creates a novel consensus sequence for the transcription factor Sp1, and at the same time, it generates a putative clusterization of three consecutive Sp1 binding sites (Pauciullo et al., 2010).

A preliminary analysis on the association with the total desaturation index (DI) of milk fat (on 22 samples) showed the highest value for the homozygous CC buffaloes (Pauciullo et al., 2010). The same marker has been associated also with daily milk yield, with the genotype AC showing an over-dominance effect (approximately 1.2 kg/d) constant across lactation stages (Pauciullo et al., 2012).

The variability found in the buffalo *SCD* Sp1 cluster was suggested as responsible for the quali-quantitative variation of the gene transcripts, and consequently of *SCD* activity. However, despite the great impact of this SNP on both milk quality and yield, the biological reason for such effects has remained so far unexplored.

Therefore, in present study we have combined the *SCD* transcript analysis, electromobility shift assay and q-PCR to assess the role of the SNP g.133A>C on Sp1 clusterization and gene expression. In addition, we investigated the possible associations of the genotypes g.133A>C with

milk fat traits for their potential application to the quali-quantitative improvement of the productive efficiency of the breed.

MATERIAL AND METHODS

Sample collection and nucleic acid isolation

Fresh milk and blood samples were collected from 303 unrelated lactating Mediterranean river buffaloes reared in 14 farms in southern Italy. Samples were collected in collaboration with ANASB (Caserta, Italy).

Milk samples were used for fatty acid analysis as described in Cosenza et al. (2017), whereas blood samples were treated for genomic DNA isolation according to Sambrook and Russell, (2001). All DNA samples were diluted with 100µl TE buffer pH 7.6 (10mM Tris, 1mM EDTA).

Further individual milk samples were collected from 6 river buffaloes after the g.133A>C genotyping (2 for each genotype). The animals belonged to the same farm located in province of Salerno (Italy), were comparable for age, feeding system, number of lactation (third), lactation stage (4th month) and were free of clinical mastitis. These samples were used to perform the quali-quantitative transcript analysis. For this purpose, the total RNA was isolated from milk somatic cells using Trizol (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to manufacturer's instruction. Traces of contaminating DNA were removed using DNase I treatment (Thermo- Fisher Scientific).

DNA and RNA concentrations were measured with the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

PCR conditions and genotyping by TaqI PCR-RFLP

The entire panel of 303 animals was genotyped for the SNP g.133A>C according to the protocol of Pauciullo et al. (2012) with a slightly modification for the annealing (65.4°C) and the extension time (20 s). In fact, new primers reported in table 1 were used to shorten the amplified

fragment to 201 bp. Product specificity was confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis.

Digestion of 5µl of each amplicon was accomplished with 1µl of FastDigest *TaqI* endonuclease (T↓CGA) in 1X buffer for 10 min at 65°C as suggested by the firm (Thermo Fisher Scientific). The digestion products were analysed by electrophoresis in 2% agarose gel in 0.5X TBE buffer and stained with ethidium bromide.

RT-PCR, cloning and sequencing

The reverse transcription of total RNA for the 6 Mediterranean river buffaloes with different genotype for the SNP g.133A>C was conducted using an oligo dT_{18}. The mix was set up in a final volume of 20µl using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) according to the standard protocol recommended by the firm. The PCR reaction was performed by using the primers in table 1.

The PCR reaction mix (50 µl) comprised: 50 ng of total cDNA, 1x PCR Buffer (Promega), 2.5 mM $MgCl_2$, 5 pmol of each primer, dNTPs each at 200µM, 1 U of *Taq* DNA Polymerase (Promega). PCR was performed under the following thermal conditions: 95°C for 4 min, 35 cycles at 95°C for 45 s, 62°C for 45 s, 72°C for 90 s, and the final extension at 72°C for 5 min.

The amplified products were first analysed by electrophoresis on 2 % agarose gel in 0.5X TBE buffer and then cloned into pGEM®-T Easy Vector (Promega). The ligation products were transformed into JM109 High-Efficiency Competent Cells (Promega) following the manufacturers' guidelines. White recombinant clones were randomly chosen and screened by PCR according to Pauciullo and Erhardt (2015) using the following combination of primers: M13 For 5'-GTAAAACGACGGCCAGT-3' and M13 Rev 5'-AACAGCTATGACCATG-3'.

Ten amplicons were chosen from each genotype, purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and sequenced in both directions in out-sourcing (Microsynth AG, Switzerland) using Sanger DNA sequencing technologies.

Agarose Electrophoretic Mobility Shift Assay (EMSA)

According to the genotype, 45-mer single stranded DNA sequences (table 1) corresponding to the nucleotides -488 to -444 of the *SCD* promoter were co-denatured at 95°C for 5 min in 0.2X SSC buffer and annealed at room temperature for 2 hours with their complementary sequence to obtain double strand probes (homozygous AA and CC). Concentrations and OD ratios were measured with the Nanodrop ND-1000 spectrophotometer as reported previously and dilutions were prepared to a final concentration of 80ng/µl.

Electrophoretic Mobility Shift Assay reactions were set up according to Ream et al., (2016) using agarose instead of polyacrilamide gel. Trial tests were arranged to assess the performance of two binding buffers and to evaluate the best concentration of Sp1 transcription factor (Enzo Life Science Inc., Farmingdale, NY, USA). Binding reactions were set-up by incubating 0.5 BFU of Sp1 transcription factor in 1X Enzo buffer (EB) with 40 ng DNA probe. The same Sp1 and DNA concentration was used also with 1X binding buffer (PB) suggest by Poletto et al., (2016). After incubation at 37°C for 20 min, the samples were loaded in 0.7% TB agarose gel and run at 30V for 100 min. Staining was performed in 1X SYBR Green.

Quantitative real-time PCR analysis (qPCR)

Comparative *SCD* gene expression was examined in the 6 Mediterranean river buffaloes divided into three groups (2 AA, 2 AC and 2 CC) according to the genotype of SNP g. 133A>C. Quantitative PCR was performed using 2-fold diluted cDNA products with PowerUp™ SYBR® Green Master (Thermo Fisher Scientific). Analysis was carried out with a StepONE cycler (Applied Biosystems, Foster City CA, USA) for 40 cycles at 95°C for 20 s and amplification at 60°C for 1

min. Amplification specificity was checked using melting curve following the manufacturer's instructions. Primers used for *SCD,* β-actin and 18S rRNA expression are listed in table1. Each sample was analysed in triplicate (technical replicates) and relative gene expression was determined using β-actin and 18S as endogenous controls. Results are expressed as fold-change relative to the mean.

Bioinformatic and statistical analysis

The allele frequency and Hardy-Weinberg equilibrium (χ^2 test) were calculated for the sample of 303 buffaloes. Homology searches, comparison among sequences, and multiple alignments were accomplished using DNAsis-Pro (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). Sp1 binding was measured by band intensity values (mean ± SEM) using Image J software (National Institute of Health; Bethesda, MD, USA). Analysis of variance (ANOVA) was used to examine Sp1 binding intensity and gene expression, followed by a Student-Newman-Keuls test using SPSS software. A *P*-value of less than 0.05 was considered statistically significant.

Associations between *SCD* genotype, fat percentage, single fatty acid percentage and fatty acid classes (supplementary table 1) were tested using the following mixed linear model:

$$y_{ijklmn} = \mu + AGE_i + DIM_j + Month_k + SCD_l + HERD_m + C_n(SCD) + e_{ijklmn}$$

where:

y_{ijklmn} = dependent variable* (supplementary table 1);

μ = overall mean;

AGE_i = fixed effect of the *i*th class of animals age at calving expressed in years (6 levels: 1= <4yrs., 2=4 yrs., 3=5 yrs., 4=6 yrs., 7=5 yrs., 6 = >7 yrs.);

DIM_j = fixed effect of the *j*th stage of lactation (10 levels of 30 days each);

$Month_k$ = fixed effect of the *k*th month of calving (12 levels);

SCD_l = fixed effect of the *l*th *SCD* genotype;

$HERD_m$ = Random effect of the *m*th herd

C_n = Random effect of individual buffalo cow nested within *SCD* genotype

e_{ijklmn} = Random residual

Pairwise comparisons among different levels of fixed effects included in model were performed using a Bonferroni adjusted test.

RESULTS

In order to assess the effect of the g.133A>C mutation on the transcription of *SCD* gene, and in order to further validate the preliminary association with FAs, we genotyped by PCR-RFLP a population of 303 Mediterranean river buffaloes as shown in table 2. The minor allele frequency (g.133C) was 0.25 and the population resulted in disequilibrium for Hardy-Weinberg (χ^2 =7.96).

For the qualitative transcript analysis of the 6 samples with different genotype (2 AA, 2 AC and 2 CC), a total of 216 positive cDNA recombinant clones (72 clones for each genotype group) were screened by PCR and agarose gel electrophoresis. All the analyzed cDNA clones showed the same length. Ten clones per genotype were chosen for the sequencing and no qualitative transcript variability was found among the cDNAs of the three investigated genotypes. In fact, the comparative sequence analysis indicated only completed assembled cDNAs from exon 1 to exon 6 for all the clones.

Electrophoretic mobility shift assay was performed to assess the binding of Sp1 transcription factor to DNA sequences including the SNP g.133A>C. Specific probes corresponding to homozygous genotypes were tested with two different binding buffers. The two genotypes revealed positive binding reactions in both experimental conditions, however the complex genotype CC-Sp1 showed a stronger intensity than the genotype AA for both EB (201.77 ± 4.06 *vs* 141.65 ± 3.77 – $P< 0.000$) and PB (95.90 ± 1.15 *vs* 67.30 ± 2.14 – $P<0.000$) (figure 1).

Furthermore, a Real-time PCR experiment was performed to confirm the putative effect of the g.133A>C genotype on the amount of transcribed mRNA. Two housekeeping genes were used (β-actin and 18S rRNA) and gave the same normalization results. Relative *SCD* expression from the same group of samples showed the highest values for the genotype CC, while the heterozygote AC showed the lowest expression. The genotype CC was significantly up-regulated compared with both AA and AC genotypes ($P<0.01$), and the genotype AA showed upregulation compared to AC genotype ($P<0.05$) as shown in figure 2 (β-actin normalization).

The analysis of the relationship between the *SCD* genotype and the FA profile showed significant association with different groups of FAs (table 3). In particular, heterozygous buffalo cows had the highest value for MUFAs ($P=0.010$), oleic acid (C18:1 c9) ($P=0.005$), PUFAs ($P=0.030$) and odd branched-chain fatty acids (OBCFAs) ($P=0.047$), as well as the lowest content for the saturated FAs ($P=0.007$), and lowest atherogenic (AI) ($P=0.012$) and thrombogenic (TI) ($P=0.003$) indices compared to the AA genotype (table 3).

DISCUSSION

Genetic variability of *SCD* in Italian Mediterranean buffaloes has been investigated in our previous studies and the SNP g. 133A>C has been identified and associated with milk yield (Pauciullo et al., 2012) and, in a preliminary way, with the total desaturation level of milk fat (Pauciullo et al., 2010). These associations were supposed to be a consequence of a novel Sp1 binding site discovered *in silico*, which generates a clusterization of three Sp1 consensus sequences in the gene promoter. In the present work, a comparative quali-quantitative approach has been applied to establish the role of this SNP on *SCD* gene transcription. Furthermore, an association study with milk FAs has been carried out to elucidate the effect of the genotype on this trait in river buffaloes.

A preliminary genotyping on 303 milking buffaloes was necessary to identify their genotype at the locus g.133A>C and to select the groups for the quali-quantitative analysis. Genotype

distribution indicated that the variant C is less frequent (0.25) in the population. This value is slightly higher than frequencies estimated in our previous studies (0.16) (Pauciullo et al., 2010, Pauciullo et al., 2012). This might be due to several reasons including an indirect selection for other traits that favoured the C allele, a non-random matings, or simply the higher number of farms involved in the present study.

After the genotyping, six buffalo cows (2 per each genotype) were chosen for investigating the effect of the SNP g.133A>C on the RNA through a comparative quali-quantitative analysis.

The qualitative analysis of cDNA populations did not evidence differences in the primary transcripts. In fact, only correctly assembled cDNAs (from exon 1 to exon 6) were found.

It is known that the first element affecting the intricate mechanism of the RNA maturation, which reflects the occurrence of splicing events, is the extremely split architecture of the genes (Heyn et al., 2015). In this respect, our result is not surprising. In fact, differently from other genes expressed in the mammary gland and well-studied from a transcript point of view (Ramunno et al., 2005, Gu et al., 2017), the *SCD* gene consists of 6 exons only (Pauciullo et al., 2010). However, the lack of splicing events is in conflict with our previous findings. In fact, Pauciullo et al. (2007) reported at least 5 different cDNA populations, among which the most represented transcript (~52%) was the correctly assembled, followed by minor transcripts. In particular, a very short cDNA (only 565 bp long) was observed, deleted of great part of the exons as a consequence of an alternative splicing. Such a difference with the present results might be attributable to the diverse sampling criteria, achieved by the selection of milk samples with extreme fat content (Pauciullo et al. 2007).

The lack of qualitative differences among the *SCD* transcripts led to investigate the interaction of the polymorphism g.133A>C with the Sp1 transcription factor and then to assess its effect on the quantity of transcripts.

An electrophoretic mobility shift assay experiment was set up to address the first point. EMSA is a powerful method used to test the ability of proteins to bind selectively specific double stranded

DNA sequences. The technique compares the electrophoretic mobility of a free DNA sequence with the mobility of the same DNA sequence incubated with a protein or a mixture of proteins. Specific protein-DNA interaction results in reduced migration rate of this complex compared to the free DNA (Garner and Revzin, 1981). In this respect, two specific DNA constructs (homozygous AA and CC) corresponding to the promoter region of *SCD* were generated and each treated with a recombinant Sp1 transcription factor in two different binding buffers. The Sp1 functionally interacted with the promoter of both DNA constructs. This confirms, *de facto,* the bioinformatics results reported in the previous studies, where at least two Sp1 binding sites were presumed to characterize the region -473/-448 of the *SCD* promoter (Pauciullo et al., 2010, Pauciullo et al., 2012). In addition, the genotype CC showed a higher binding affinity to Sp1 irrespective of the buffer used (figure 1). The sequence analysis suggests the formation of a further consensus site falling between two existing Sp1 binding sites. However, the coexistence of three active sites in such short DNA region seems functionally difficult for steric bulk. In fact, DNA has one helical turn every 10.5 bp and the binding of another Sp1 transcription factor would completely straighten the molecule; furthermore Sp1 transcription factors have a footprint that extends beyond their binding sites (Pascal and Tjian, 1991). Therefore, a higher band intensity of the genotype CC and lack of a "super shift" in the EMSA assay suggests only a higher affinity of the allele C for Sp1 binding sites (figure 3).

The Sp1 motifs are well-known enhancer elements for the basal expression of many genes, including *SCD,* where they cover conserved promoter regions, fundamental for the gene expression (Keating et al., 2006, Pauciullo et al., 2012). Furthermore, these motifs often occur in clusters generated by variable number tandem repeats (VNTR) (Fürbass et al., 2006, Wang et al., 2008). Their variability might regulate the transcription rate of the gene, as demonstrated for instance in other key genes involved in milk triacylglycerol (TAG) synthesis or related to lipid droplet formation and secretion. For instance, the *DGAT1* VNTR polymorphism in cattle results in a different number of a potential binding motif for the transcription factor Sp1 with potential

functional effects (Fürbass et al., 2006). Besides, the alteration of Sp1 expression in goat mammary epithelial cells changes the expression of other lipogenic genes as *DGAT1, DGAT2, TIP47* and *ADFP* (Zhu et al., 2016) and affects the expression level of peroxisome proliferator-activated receptor-γ (*PPARγ*) and liver X receptor α (*LXRα*), both crucial in regulating fatty acid metabolism together with SRBP1 (Zhu et al., 2015).

The highest affinity of Sp1 transcription factor for the C allele led to investigate also the effect of the SNP on the *SCD* transcription level. The q-PCR showed a significant up-regulation of the genotype CC compared with both AA and AC genotypes ($P<0.01$), whereas the heterozygote showed the lowest expression.

In a previous study, the genotype AC showed an over-dominance effect when associated with milk yield and buffaloes with this genotype showed higher milk yield *vs* AA and CC (Pauciullo et al., 2012). It is well known that fat and milk yield are negatively correlated traits in buffalo (Kholif, 1997, Yadav et al., 2013) as in other dairy species, which might be consistent with the lowest AC expression.

The highest expression of the genotype CC is in agreement with the results of the EMSA experiments. In particular, the C allele induced a higher Sp1 binding affinity, which consequently affected the gene expression. In addition, the association study with different groups of FAs indicated the AC and CC as genotypes showing the highest values for the unsaturated classes of FAs (MUFAs, mainly due to the oleic acid C18:1 c9, PUFAs and OBCFAs). This profile is confirmed by a lower content of saturated FAs, which consequently led to lower atherogenic (AI) and thrombogenic (TI) indices (table 3). The latter indices are considered an important way of classifying milk FAs because they measure the benefits of omega-3 FAs in the prevention of heart disease (Ulbricht and Southgate, 1991). On average, our data agree with the AI and TI values recently reported by Santillo et al. (2016) on buffalo milk. Furthermore, to our knowledge, this study represents one of the first indications of genetic association with these traits for the river buffalo.

The lack of correlation between *SCD* mRNA expression and desaturase indices (i.e. apparent *SCD*/Δ9-desaturase activity assessed by comparing product to precursor fatty acid ratios) was unexpected. However, in this respect, the literature is often contradictory. For instance, in dairy cows the pattern of *SCD* mRNA was not significantly correlated with any of the Δ9 desaturase indices, and was nearly opposite to the overall Δ9 desaturase index when considering the lactation cycle (Bionaz and Loor, 2008). Conversely, a positive correlation of *SCD* mRNA with oleic acid (Barber et al., 2000) and desaturase indices (Bernard et al., 2005) was observed in lactating sheep and goat, respectively. These conflicting data evidence more intricate desaturation mechanisms at level of mammary gland, which even under the same feeding condition, likely depend also on other factors including mobilization of TAG from adipose tissue, selective uptake of stearic acid from blood VLDL (Very Low Density Lipoprotein), plasma level of oleic acid, etc. (for a review Barber et al., 1997).

Therefore, in the present study, the allele C revealed a better desaturation profile, and in a previous work, the genotype AC showed a greater milk yield (Pauciullo et al., 2012), showing a putative positive correlation under the control of the same genotype. In this respect, our data confirm the positive correlation of unsaturated C18 FA with milk yield found by Stoop et al., (2008) in dairy cows. Despite the greater desaturase activity was hypothesized to correspond to fewer nutrients directed toward milk yield (Macciotta et al., 2008), our findings agree with previous reports, demonstrating that prolonged selection for increased milk yield did not alter the content of the majority of individual milk fatty acids (Kay et al., 2005).

The association of the allele C with the OBCFAs (P=0.047) also deserves attention. Recently, other fundamental genes for milk traits have been positively associated to OBCFAs in river buffalo. For instance, Cosenza et al., (2017) found higher contents of these FAs associated to the SNP g.129C>T (P<0.0006) at the oxytocin receptor gene (*OXTR*), whereas the marker g.11188A>G at the prolactin receptor gene (*PRLR*) tended to have a significant effect (P<0.06) on the same traits (Cosenza et al., 2018). This class of FAs in milk fat are mostly derived from rumen bacteria,

although linear OCFAs might be synthesized *de novo* in the mammary gland (Vlaeminck et al., 2006). In ruminants, OBCFAs are mainly used as biomarkers of rumen functionality (Craninx et al., 2008, Stefanov et al., 2010), whereas in human, OBCFAs showed anti-carcinogenic effects (Wongtangtintharn et al., 2004) and therefore are considered as an emerging class of bioactive FAs, comparable to that of conjugated linoleic acid (Bainbridge et al., 2016).

In this context, the *SCD* g.133A>C is a good candidate for increasing the milk FAs desaturation level and, together with SNPs in other genes, may contribute to the improvement of river buffalo milk quality.

CONCLUSIONS

The quality of buffalo milk is essential condition to satisfy the requests of an increasing market demand of the related dairy products. Therefore, genes involved in fat metabolisms are important targets of study to achieve this goal. In the present study, we elucidated the role of the *SCD* g.133A>C on gene transcription and we investigated the occurrence of possible associations with milk fat traits. No alternative splicing events were found in the 3 investigated genotypes, therefore the SNP had no influence of transcript quality. However, EMSA demonstrated that the substitution of the allele A to C generated a higher binding affinity of the Sp1 transcription factor to the gene promoter. The stronger Sp1 binding produced a quantitative difference in the gene expression, although not directly linked to desaturation activity of *SCD* in mammary gland. The allele C was associated with a different fatty acid profile than the A allele. Therefore, the SNP g.133A>C is a marker with a dual impact on milk yield and FAs desaturation level, thus becoming an interesting candidate for assisted selection programs of the species.

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Table 1. Oligonucleotide primers sequences, positions and applications.

Application	Gene	Region		Primer sequence (5' to 3')	EMBL ID	Size (bp)
Genotyping	*SCD*	Promoter	*Forward* *Reverse*	ATTTGCGAATTGCCCGGGG GCGCAATCTGCTGTTCCCTCT	FM876222	201
cDNA amplification	*SCD*	Exon 1 to 6	*Forward* *Reverse*	CAGCGGAAGGTCCCGA TGGGTTAACGTCATCTTTAGCA	NM_001290915	1333
Realtime-PCR	*SCD*	Exon 1	*Forward* *Reverse*	CAGCGGAAGGTCCCGA CAAGTGGGCCGGCATC	FN395259	157
	β-actin	Exon 1	*Forward* *Reverse*	TCCTCCCTGGAGAAGAGCTA AGGAAGGAAGGCTGGAAGAG	NM_001290932	101
	18S rRNA	Exon 1	*Forward* *Reverse*	CGTTCTTAGTTGGTGG GTAACTAGTTAGCATGC	NR_036642	76
			Genotype	**Probes (5' to 3')**		
EMSA	*SCD*	promoter	AA CC	CGCCTCCGAGGCGGCAGGGTGCCCGGT**A**GAGGCCCAGCGGCCGGT CGCCTCCGAGGCGGCAGGGTGCCCGGT**C**GAGGCCCAGCGGCCGGT	FM876222	45

Primers were designed by means of DNAsis software (Hitachi Software Engineering Co., San Bruno, CA).

Table 2. Genotypes, allele frequency and relative frequencies for the SNP g.133A>C at the *SCD* gene in river buffalo.

	Genotype distribution				Allele frequency		Relative frequencies used in the model		
	AA	**AC**	**CC**	**TOT**	**A**	**C**	**Genotype**	**No. of cows**	**No. of farms**
Obs.	180	95	28	303	0.75	0.25	AA	59.4	
Exp.	170.81	113.37	18.81				AC	31.3	14
							CC	9.3	
							TOT	100	14

χ^2=7.96; d.f. = 1

Table 3. Least Squares Means (± standard error) for the three different genotypes of the SNP g.133A>C for significant fat related traits.

FAs[1]	P[2]	AA	AC	CC
SFAs	0.007	72.00 ^{A} ± 0.59	70.69 ^{B} ± 0.74	71.90 ^{AB} ± 0.82
MUFAs	0.010	24.81 ^{A} ± 0.52	25.97 ^{B} ± 0.56	24.93 ^{AB} ± 0.74
C18:1 c9	0.005	18.80 ^{A} ± 0.46	19.92 ^{B} ± 0.49	19.17 ^{AB} ± 0.65
PUFAs	0.030	3.08 ^{a} ± 0.10	3.23 ^{b} ± 0.10	3.08 ^{ab} ± 0.13
OBCFAs	0.047	3.87 ^{ab} ± 0.11	3.95 ^{a} ± 0.11	3.75 ^{b} ± 0.13
AI	0.012	3.04 ^{A} ± 0.11	2.82 ^{B} ± 0.11	3.01 ^{AB} ± 0.15
TI	0.003	2.35 ^{A} ± 0.07	2.20 ^{B} ± 0.08	2.31 ^{AB} ± 0.09

[1] SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids; OBCFAs = odd-branched chain fatty acids; C18:1 c9 = oleic acid; AI = atherogenic index [C12:0 + (4× C14:0) + C16:0]/[(PUFAs) + (MUFAs)]; TI = thrombogenic index (C14:0 + C16:0)/[(0.5 × MUFAs) + (0.5 × n6) + (3 × n3) + (n3:n6)].
[2] = significance level of the SNP effect.
^{a,b} = means with different superscripts within row differ (P<0.05).
^{A,B} = means with different superscripts within row differ (P<0.01).

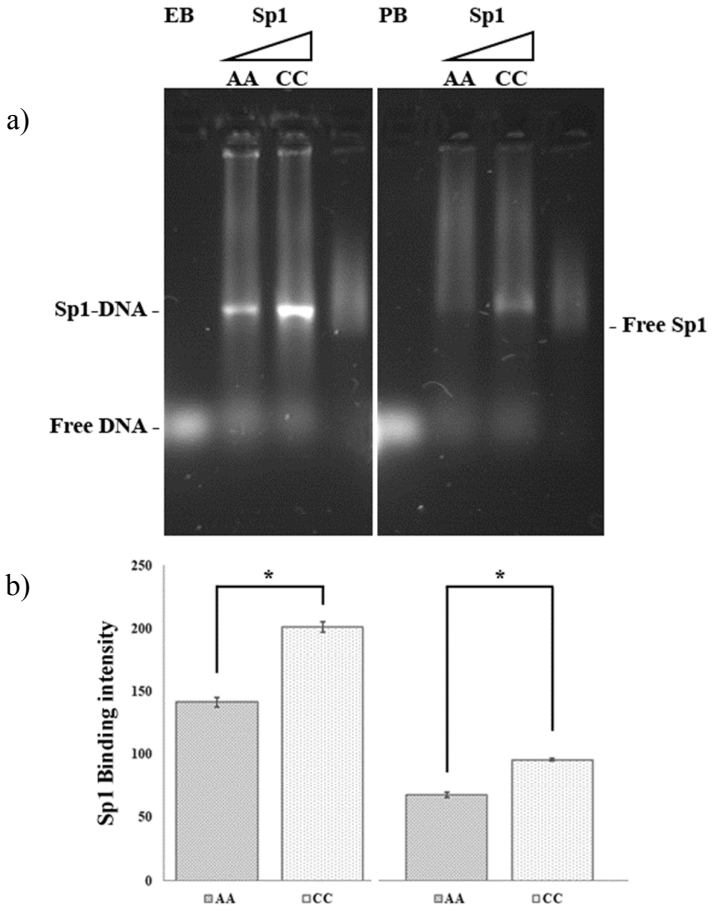


Figure 1. a) Agarose electrophoresis mobility shift (EMSA) assay measuring the Sp1 binding activity to *SCD* promoter constructs (homozygous AA and CC) for two different binding buffer EB and PB. b) Densitometric quantification of the DNA/protein complex is reported at the bottom of the picture. Sp1 binding intensity values are shown as mean ± standard deviation for each genotype and each buffer. Asterisks (*) indicate significant differences ($P<0.000$).

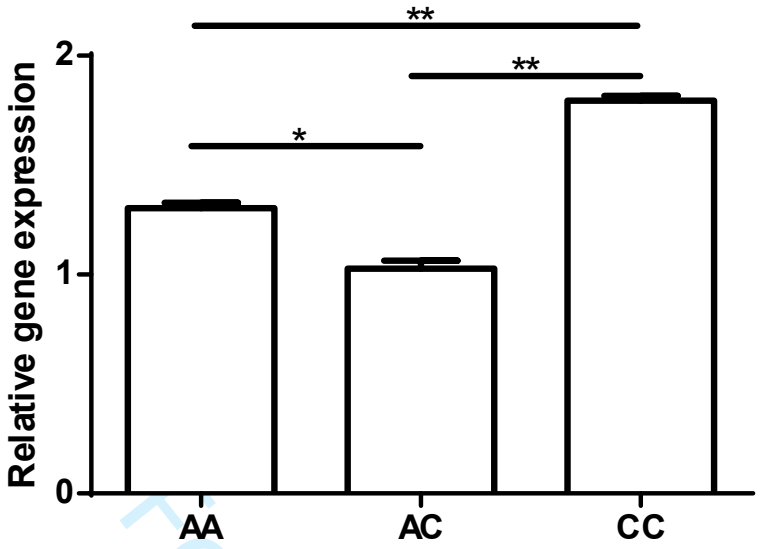
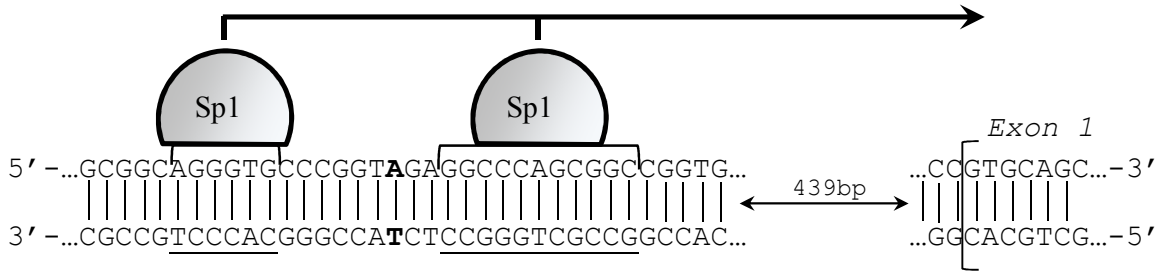


Figure 2. Relative expression values (mean ± standard deviation) of two subjects for each g.133A>C genotype. Unpaired Student's t-test, *P<0.05; **P< 0.01

g.133 AA



g.133 CC

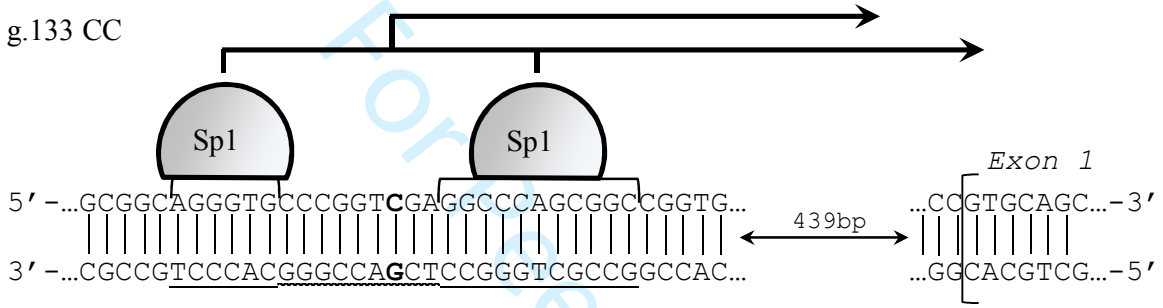


Figure 3. Schematic representation of the Sp1 motif cluster and binding. The sequence analysis of the homozygous CC showed an additional Sp1 motif generated by the SNP g.133 A>C in the promoter region of *SCD* (EMBL ID: FM876222). The SNP likely increases Sp1 binding affinity to the promoter region by adding an additional Sp1 binding-site. This results in higher gene expression of the CC allele in the mammary gland.

Supplementary table 1. *Average fat content (%), fatty acid composition (%) and fatty acid classes (%) of 303 dairy buffaloes milk samples measured in 14 herds located in Southern Italy. These phenotypes were used in the mixed linear model to assess associations with the *SCD* genotypes for the SNP g.133A>C.

Herd	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean	SE
Fat (%)	7.44	7.99	7.94	7.28	7.00	7.80	6.93	8.03	9.34	8.43	8.58	6.24	7.42	7.59	7.72	0.21
Fatty acid (%)																
C4:0	3.47	4.38	3.85	3.90	3.51	4.10	4.35	3.88	3.86	3.65	4.42	3.93	3.80	3.60	3.91	0.08
C6:0	1.61	1.96	2.04	1.86	1.71	1.69	1.83	1.62	1.49	1.52	1.93	1.54	1.64	1.52	1.71	0.05
C8:0	0.8	0.98	1.14	0.95	0.90	0.78	0.86	0.77	0.66	0.73	0.95	0.69	0.79	0.72	0.84	0.04
C10:0	1.73	2.05	2.53	2.03	1.96	1.60	1.77	1.65	1.33	1.47	1.95	1.39	1.65	1.49	1.76	0.09
C12:0	2.38	2.72	3.30	2.63	2.60	2.11	2.34	2.28	1.76	1.96	2.57	1.88	2.22	1.98	2.34	0.11
C14:0	11.07	12.55	12.74	11.65	11.18	10.16	10.81	10.62	9.49	9.26	11.56	9.95	10.50	9.61	10.80	0.29
C14:1	0.73	0.84	0.93	0.59	0.71	0.59	0.85	0.57	0.46	0.50	0.81	0.61	0.56	0.52	0.66	0.04
C15:0	1.26	1.24	1.24	1.23	1.19	1.10	1.28	0.97	0.89	1.00	1.05	1.03	1.09	0.92	1.11	0.04
C16:0	37.68	39.00	33.50	33.93	33.36	33.83	35.65	33.50	34.45	32.17	35.26	33.65	35.21	34.29	34.68	0.48
C16:1	2.31	2.34	2.19	1.57	1.83	1.76	2.52	1.60	1.49	1.55	2.29	1.78	1.60	1.53	1.88	0.10
C17:0	0.55	0.47	0.54	0.57	0.48	0.48	0.56	0.48	0.43	0.50	0.44	0.58	0.48	0.46	0.50	0.01
C17:1	0.23	0.16	0.21	0.18	0.16	0.16	0.24	0.15	0.12	0.13	0.17	0.22	0.15	0.14	0.17	0.01
C18:0	9.07	8.46	9.21	11.54	11.64	11.20	9.72	12.67	14.24	14.22	8.86	12.15	12.14	12.71	11.27	0.52
C18:1 c9	18.60	15.65	17.75	18.55	19.81	20.90	18.58	21.10	20.66	20.63	19.77	21.40	19.15	20.17	19.48	0.42
C18:1 *t11* (VA)	0.78	0.67	0.77	0.98	0.84	1.11	0.96	0.94	1.15	1.21	0.68	1.05	1.11	1.71	1.00	0.07
CLA c9,*t11* (RA)	0.38	0.33	0.36	0.39	0.38	0.43	0.46	0.36	0.39	0.45	0.35	0.45	0.43	0.67	0.42	0.02
C18:2 *n6* (LA)	1.42	1.28	1.56	1.34	1.95	1.51	1.32	1.36	1.62	2.51	1.58	1.31	1.36	1.55	1.55	0.09
C18:3 *n3* (ALA)	0.33	0.22	0.22	0.19	0.22	0.26	0.19	0.18	0.21	0.41	0.26	0.16	0.22	0.17	0.23	0.02
C20:4 *n6* (ARA)	0.10	0.08	0.13	0.11	0.13	0.09	0.12	0.08	0.07	0.12	0.09	0.12	0.09	0.10	0.10	0.01
C20:5 *n3* (EPA)	0.03	0.02	0.02	0.03	0.02	0.02	0.02	0.01	0.02	0.03	0.02	0.04	0.02	0.03	0.02	0.00
C22:5 *n3* (DPA)	0.04	0.02	0.04	0.04	0.04	0.03	0.03	0.02	0.03	0.04	0.03	0.05	0.04	0.03	0.03	0.00
C22:6 *n6* (DHA)	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C14 index	0.06	0.06	0.07	0.05	0.06	0.05	0.07	0.05	0.05	0.05	0.07	0.06	0.05	0.05	0.06	0.00
C16 index	0.06	0.06	0.06	0.04	0.05	0.05	0.07	0.05	0.04	0.05	0.06	0.05	0.04	0.04	0.05	0.00
C18 index	0.67	0.65	0.66	0.62	0.63	0.65	0.66	0.62	0.59	0.59	0.69	0.64	0.61	0.61	0.64	0.01
CLA index	0.33	0.33	0.32	0.28	0.31	0.28	0.32	0.28	0.25	0.27	0.34	0.30	0.28	0.28	0.30	0.01
DI	0.27	0.24	0.27	0.27	0.29	0.30	0.28	0.29	0.28	0.29	0.29	0.30	0.27	0.28	0.28	0.00
Fatty acid classes (%)																
SCFA	7.74	9.50	9.72	8.87	8.22	8.26	8.94	7.99	7.39	7.44	9.37	7.61	7.96	7.40	8.32	0.22
MCFA	58.79	61.80	57.64	54.74	54.11	52.54	57.24	52.74	51.42	49,50	56.47	52.28	54.08	51.54	54.64	0.90
LCFA	33.47	28.70	32.64	36.38	37.67	39.21	33.82	39.27	41.19	43.06	34.17	40.10	37.96	41.06	37.05	1.08
SFA	72.09	76.18	72.96	72.61	71.03	69.18	71.97	70.84	70.75	68.80	71.12	69.22	71.59	69.26	71.26	0.52
MUFA	24.69	21.22	23.86	24.15	25.31	27.43	25.13	26.36	26.13	26.54	25.80	27.61	25.27	26.98	25.46	0.45
PUFA	3.14	2.53	3.07	3.04	3.50	3.16	2.82	2.67	3.02	4.47	2.94	3.02	2.92	3.51	3.13	0.13
OBCFA	4.14	4.01	4.45	3.87	3.73	4.14	4.10	4.01	3.70	4.83	3.78	3.33	3.46	4.03	3.97	0.10
PUFA n3	0.42	0.28	0.29	0.28	0.30	0.33	0.26	0.22	0.26	0.49	0.32	0.26	0.28	0.24	0.30	0.02
PUFA n6	1.64	1.44	1.84	1.56	2.23	1.72	1.58	1.55	1.80	2.80	1.78	1.58	1.58	1.79	1.78	0.09
Ratio and index																
n6/n3	4.25	5.31	6.41	5.64	7.47	5.30	6.26	6.98	7.47	5.70	5.55	6.17	5.61	7.46	6.11	0.26
AI	3.15	3.98	3.33	3.09	2.88	2.61	3.07	2.73	2.62	2.30	2.95	2.51	2.86	2.48	2.90	0.11
TI	2.67	2.97	2.33	2.37	2.04	2.15	2.39	2.05	2.00	1.90	2.32	2.05	2.32	1.96	2.25	0.08