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Polymorphisms of *β-defensin* genes in Valle del Belice dairy sheep

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Abstract The aim of this work was to study *β-defensin 1* (*SBD1*) and *β-defensin 2* (*SBD2*) genes in Valle del Belice dairy sheep in order to identify polymorphisms that can be utilized as markers of the analyzed genes, and search for the functional effects and roles of the identified polymorphisms (variation of the amino acid sequence of the protein and stability of mRNA molecule). The study was conducted on 300 randomly selected animals belonging to four flocks. A total of seven SNPs were identified, two in *SBD1* and five in *SBD2*. The two SNPs identified in *SBD2* coding region, at position 1659 and position 1667, were non-synonymous, leading to amino acid changes in the protein product. Nevertheless, the functional effects predicted by the two SNPs demonstrated that amino acid substitutions may not have effect on *β*-defensin 2 protein function. Moreover, we demonstrated that *SBD2* mutant sequence shows changes in mRNA secondary structure. These results suggest that identified SNPs could play a role in the modulation of the immune response.

Keywords *β*-Defensin · SNPs · Sheep · Valle del Belice

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Abbreviations

SBD1	*β*-Defensin 1 gene
SBD2	*β*-Defensin 2 gene
AMPs	Antimicrobial peptides family
SCC	Somatic cell count

Introduction

Defensins are a family of small peptides belonging to the antimicrobial peptides (AMPs) group. They represent the major family of AMPs found in invertebrates, vertebrates and plants [1–5]. The defensin family includes peptides ranging from 18 to 45 amino acids, which contain cysteines, have three intramolecular disulfide bonds, cationic net charge due to arginine and lysine residues, and tertiary structures dominated by *β*-sheets [6, 7]. Defensins are classified into *α*-, *β*-, and *θ*- based on structure, size, and disulfide bonds pattern [7, 8]. These peptides are involved in the innate immunity mechanisms and act directly against bacteria, viruses, and fungi, due to their bactericidal and cytotoxic activity [4]. Moreover, they have been suggested as effector molecules in host defense, interacting with many target cells and tissues [9]. Defensins have been reported to promote secondary responses that may be critical in the regulation of acute inflammation, recruitment of adaptive immune cells, angiogenesis, and wound healing [10]. Defensins may be therefore considered as link molecules between the innate and the acquired immune response [11].

The genes, encoding for defensins are arranged in clusters [12, 13] and expressed in epithelial cells lining various organs [14, 15] and in leukocytes [16].

In recent years, *β-defensin* genes have been studied in several domestic species such as cattle, pig, and goat, due

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to their important role in the immune response. To date, 13 neutrophils and two epithelial (i.e., the tracheal antimicrobial peptide (TAP) and the lingual antimicrobial peptide (LAP)) β-defensins have been described in cattle [16–18]. Moreover, Cormican et al. [19] recently reported a novel group of bovine *β-defensins*. In pigs, β-defensin 1 has been characterized by Shi et al. [20]. Sang et al. [21] have also reported the identification and initial characterization of 11 novel *β-defensins* in pigs. In goats, the precursors for *β-defensin 1* and *β-defensin 2* have been characterized by Zhao et al. [22]. Sharma et al. [23] have also cloned and characterized the mRNA sequence of a gene encoding the caprine LAP.

In sheep, only two *β-defensin* genes have been described so far: *β-defensin 1* (*SBD1*) and *β-defensin 2* (*SBD2*). Both genes have been mapped on chromosome 26 and consist of two exons and one intron of approximately 1,500 bp [24]. The first 58 bp exon encodes the signal sequence and the second 296 bp exon encodes the pro-peptide and the mature peptide [25]. There are several studies published on *β-defensin* polymorphisms in human and cattle; which have been generally associated to diseases [26, 27] and production traits [28–30]. However, literature does not report results on *β-defensin* polymorphisms in sheep. Therefore, the aim of this work was to study *SBD1* and *SBD2* in Valle del Belice dairy sheep in order to identify polymorphisms that can be utilized as markers of the analyzed genes, and search for the functional effects and roles of the identified polymorphisms (variation of the amino acid sequence of the protein and stability of mRNA molecule).

Materials and methods

Animals and sample collection

A total of 300 samples of Valle del Belice dairy sheep randomly chosen from four flocks were included in the analysis. Blood samples from each animal were collected, and genomic DNA was extracted from buffy coat using a salting out DNA isolation method [31].

β-Defensin genes amplification

Four specific primer pairs were designed to amplify *SBD1* and *SBD2* exons on the basis of the sequences available in the GenBank database (Acc. no. U75250 and U75251, respectively). Primer sequences, size of amplified fragments, and annealing temperatures for *SBD1* and *SBD2* are reported in Table 1. PCR reactions were performed in a thermal cycler GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 µl containing 50 ng of genomic DNA, 10 µM of each primer and 2× PCR Master Mix (Fermentas, Burlington, ON, Canada). Thermal cycling conditions were: 94°C for 3 min, 35 cycles of 94°C for 30 s, 62–68°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 5 min. PCR products were checked by electrophoresis on 2% agarose gel stained with ethidium bromide.

Sequencing of *β-defensin* genes

In total, 100 out of the 300 sheep sampled, were analyzed by direct sequencing, in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). PCR products of *SBD1* and *SBD2* were purified using Exonuclease I and Shrimp Alkaline Phosphatase according to manufacturer's protocol (Fermentas, Burlington, ON, Canada). DNA sequencing reaction was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same primers used in the PCR reaction. Cycle sequencing reaction was performed according to manufacturer's instructions following Ethanol/EDTA/Sodium Acetate precipitation.

Single nucleotide primer extension assay

A single nucleotide primer extension assay was set up to analyze the remaining 200 sheep. Seven specific primers

Table 1 Primer sequence, amplified fragments, and annealing temperature for *SBD1* and *SBD2*

Gene	Region	Primer sequence 5′–3′	Amplified fragment (bp)	Annealing temperature (°C)
SBD1	Exon1	Fw-CAGCCTCTTCTCCAGCATCA	282	66
		Rev-GAATTTTGCAGGACGGTTCT		
	Exon2	Fw-ATTGTCATGAAGCCGTGTCCG	423	68
		Rev-ATTCACCTGGGATCAGACACCACA		
SBD2	Exon1	Fw-CAGCCTCTTCTCCAGCATCA	279	62
		Rev-AAATTTTGCAGGACAGTTCT		
	Exon2	Fw-GTTGTCATGAAGCCGTGTCCA	392	66
		Rev-ACCTCAATGACCAGTGGGCAAGAT		

Table 2 Primer sequences, length, and final concentration (μM) for the single nucleotide primer extension assay

SNP position	Primer sequence	Length (bp)	(μM)
SBD1-1747	5′-CAGAAGTCTAAGACGCGGCC-3′	20	0.09
SBD1-1757	3′-CCTCATTCTTTGACGCGGGA$(A)_5$-5′	25	0.09
SBD2-89	5′-$(A)_{15}$CATCACCTACTCCTCGTGCT-3′	35	1.5
SBD2-1659	5′-$(T)_{10}$AGGAATCTGTGTGCTGACCA-3′	30	0.2
SBD2-1667	3′-CCTGATACTCTGTCTAACCGTGGA$(T)_{21}$-5′	45	0.8
SBD2-1750	5′-$(T)_{20}$CAGAAGTCTAAGACGCGGCC-3′	40	0.2
SBD2-1761	3′-CTCAGTCTTTGACGCAGGAA$(T)_{30}$-5′	50	1

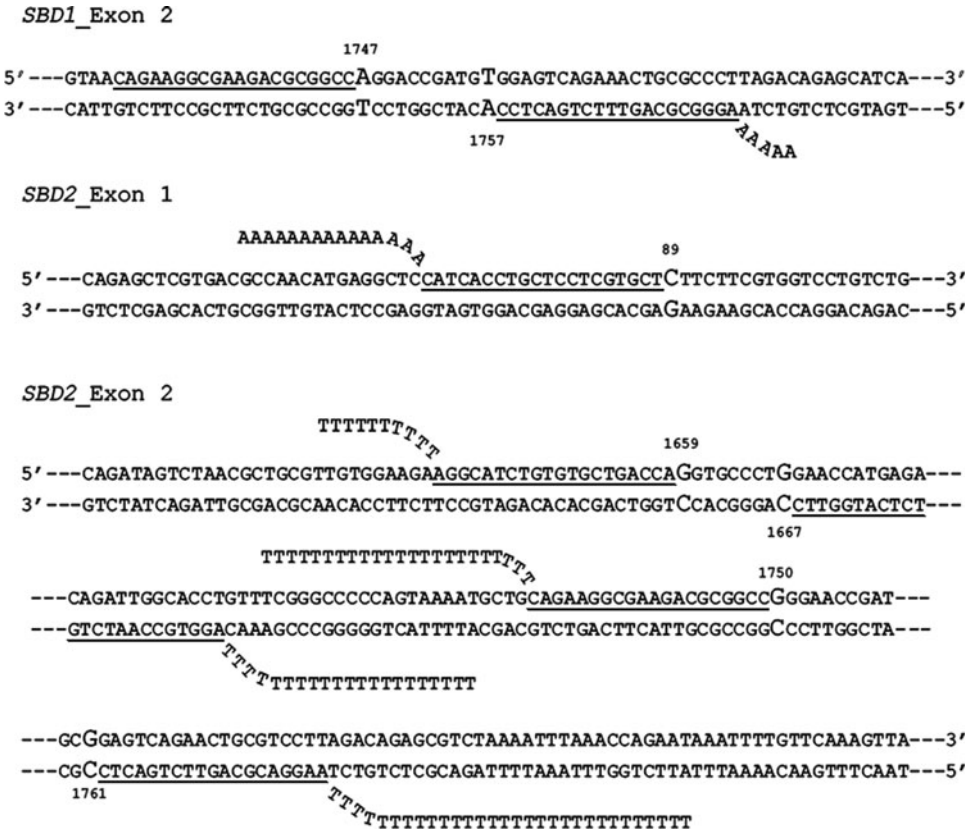

Fig. 1 Primers design for single nucleotide primer extension assay. *Larger letters* indicate SNPs. *Underlined* nucleotides indicate primers with tails of variable length, used for polymorphisms detection

were designed: two for *SBD1* and five for *SBD2*. The primers sequence, length, and concentration used in the reaction are reported in Table 2. Primer sequences were chosen in order to have the appropriate length and melting temperature. Primers were designed complementary to the negative (−) DNA strand when the positive (+) DNA strand was difficult to assay (Fig. 1). Poly(dT) or poly(dA) of variable length were added at the 5′ end of the primers in order to modify the length, and to avoid overlapping of peaks in the electropherograms obtained by capillary electrophoresis. Following template amplification, 15 μl of the PCR product were incubated at 37°C for 1 h with 2 U of Exonuclease I and 5 U of Shrimp Alkaline Phosphatase (Fermentas, Burlington, ON, Canada). Two multiplex primer extension reactions were used: the first one for genotyping SNPs in *SBD1* exon 2 and in *SBD2* exon 1, and the second one for genotyping SNPs in *SBD2* exon 2. Single nucleotide primer extension reaction was carried out in 5 μl reaction mixture containing 2.5 μl of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA), 0.5 μl of primers mix, 0.5 μl of deionized water and 1.5 μl of pooled PCR products previously purified. Thermal cycling conditions for extension reactions were: 25 cycles of 96°C for 10 s, 55°C for 5 s, and 60°C

for 30 s using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). To remove unincorporated labeled ddNTPs, 0.5 U of Shrimp Alkaline Phosphatase (Fermentas, Burlington, ON, Canada) were added to the primer extension product and incubated at 37°C for 1 h followed by 75°C for 15 min. Finally, 2 µl of the primer extension product mixed with 7.5 µl of Hi-Di Formamide and 0.5 µl of GeneScan 120 LIZ Size Standard (Applied Biosystems, Foster City, CA, USA) were denaturated for 5 min at 95°C. Capillary electrophoresis was performed in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Bioinformatics analyses

SeqScape v2.5 software (Applied Biosystems, Foster City, CA, USA) was used to analyze the nucleotide sequences, and the Clustal W software [32] to align the sequences. Results of the primer extension assay were analyzed by GeneMapper v3.7 software (Applied Biosystems, Foster City, CA, USA). GENEPOP v4.0 software [33] was used to calculate genotypic frequencies. The evaluation of Hardy–Weinberg equilibrium was carried out with chi-square analysis from the HWE software program (Linkage Utility Programs, Rockefeller University, New York, NY, USA). Estimation of the likelihood of the nonsynonymous coding SNPs to cause a functional impact on the protein was evaluated with PANTHER (Protein ANalysis THrough Evolutionary Relationships) software [34]. This software calculates the subPSEC (substitution Position-Specific Evolutionary Conservation) and a probability ($P_{deleterius}$) score based on an alignment of evolutionarily related proteins. The probability that a given variant will cause a deleterious effect on protein function is estimated by $P_{deleterious}$, such that a subPSEC score of -3 corresponds to a $P_{deleterious}$ of 0.5. SIFT (Sorting Intolerant From Tolerant) software [35] was used to predict whether the amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids. SIFT is a multistep procedure in the sense that, given a protein sequence, the software firstly searches for and chooses similar sequences, then makes an alignment of these sequences, and finally calculates scores based on the amino acids appearing at each position in the alignment. The amino acid substitution is predicted as deleterious if the score is ≤ 0.05, and tolerated if the score is >0.05. The analysis was carried out using the default value of median conservation of sequences.

The RNAstructure v4.6 software was used for the prediction and analysis of SBDs mRNA secondary structure [36], using default options. This software uses a structure prediction algorithm and the most current thermodynamic parameters to predict the secondary structure based on RNA sequence. For this analysis, wild type and 3′-UTR mutated β-defensin mRNA sequences were used.

Results

SNPs identification and genotypic frequencies

Alignment and analysis of *SBD1* and *SBD2* sequences revealed the presence of 7 polymorphic sites. All point mutations were SNPs. The SNPs were submitted to the Single Nucleotide Polymorphism database—dbSNP—(http://www.ncbi.nlm.nih.gov/projects/SNP/). No SNPs were found in the *SBD1* exon 1, whereas two SNPs were found in the exon 2: a transition A → G at position 1747 (RefSNP number 119102887) and a transition T → C at position 1757 (RefSNP number 119102888) in the 3′-UTR. Mutation at position 1747 creates a cutting site for HpaII restriction enzyme. Our results showed that all individuals presenting the A → G change at position 1747, presented the T → C change at position 1757 as well; suggesting that SNP alleles were tightly linked. The tight linkage between the two mutation sites was also confirmed by primer extension. Based on these results, sheep were only genotyped for the SNP at position 1757.

In *SBD2*, SNPs were found in both exons. A transition C → T, located at position 89 in the coding region (RefSNP number 119102892) was the only SNP found in the exon 1. However, this synonymous substitution does not change the amino acid residue within the protein sequence. The four SNPs detected in the exon 2 were all transitions G → A. The G → A at position 1659 (RefSNP number 119102893) determines an amino acid change Arg^{42} → Lys^{42}, whereas the one at position 1667 (RefSNP number 119102889) causes the switch Gly^{45} → Arg^{45}. The SNPs at position 1750 (RefSNP number 119102890) and 1761 (RefSNP number 119102891) were located in the 3′-UTR. The substitution at position 1750 disrupts a cutting site for HpaII restriction enzyme.

Genotypic frequencies for each SNP are reported in Table 3. As expected, SNPs in *SBD1* showed the same frequency in wild type, heterozygote, and mutated homozygote condition. Among the seven SNPs, those at positions 89, 1667, 1750, and 1761 were not found in mutated homozygote condition, whereas the other SNPs (i.e., 1747 and 1757 in *SBD1* and 1659 in *SBD2*) occurred with low frequency in this condition. The *SBD2* genotypes at positions 89, 1667, and 1761 occurred more frequently in wild type condition. Moreover, mutation at position 1667 occurred more rarely compared to the others, as it is characterized by the lowest frequency value in heterozygote condition (0.03). All SNPs did not fit Hardy–Weinberg equilibrium.

Table 3 SNPs positions in *SBD1* and *SBD2* and genotypic frequencies

Gene	SNP position	Genotypic frequencies		
		Wild type	Heterozygote	Mutated homozygote
SBD1	1747 A → G	AA (0.78)	GA (0.16)	GG (0.06)
	1757 T → C	TT (0.78)	TC (0.16)	CC (0.06)
SBD2	89 C → T	CC (0.72)	CT (0.28)	TT (0)
	1659 G → A	GG (0.39)	GA (0.56)	AA (0.05)
	1667 G → A	GG (0.97)	GA (0.03)	AA (0)
	1750 G → A	GG (0.49)	GA (0.51)	AA (0)
	1761 G → A	GG (0.83)	GA (0.17)	AA (0)

In silico SNPs analyses

The PANTHER and SIFT software's were used to predict the functional effects of SNPs determining an amino acid change in the sheep β-defensin 2 protein. The predictions performed by PANTHER gave the subPSEC scores of -1.81977 ($P_{deleterious} = 0.23501$) and -2.63999 ($P_{deleterious} = 0.41096$) for the amino acid change at position 42 and 45, respectively. Both values were lower than the cut-off value ($P_{deleterious} = 0.5$). The output of SIFT software for SBD2 substitutions showed that they are to be tolerated with a score of 0.20 and 0.24 respectively; indicating that the aforementioned mutations may not have a functional effect.

The SNPs located in the 3′-UTRs (i.e., at position 1747 and 1757 in *SBD1* and at position 1750 and 1761 in *SBD2*) were analyzed with RNAstructure software to determine their influence on the SBDs mRNA structure. The results obtained after comparing the wild type (Fig. 2a) and mutated *SBD2* (Fig. 2b, c, and d) sequences show that the SNPs at position 1750 and 1761 change the tridimensional mRNA secondary conformation (Fig. 2b and c, respectively). However, when the two mutations are both present (Fig. 2d), the mRNA folds as well as when only the SNP at position 1750 is present. It seems, therefore, that this mutation has a greater effect on the RNA folding. The SNPs found in the sequence of *SBD1* did not affect mRNA shape.

Discussion

Recent studies have indicated that β-defensins play an important role in innate immune responses showing an immunomodulatory activity as well [37]. Due to their important biological function the nucleotide sequences which encode for these peptides were well characterized in mammals. Moreover, attention was recently focused on the alterations in overall charge, length, and sequence of these peptides associated with variations in the nucleotide sequence [38, 39]. *β-defensin* genes have been shown to present high polymorphism in the human [26, 27, 40] and bovine [28–30, 41] sequences. Nevertheless, current knowledge regarding *β-defensin* polymorphism in sheep is limited. In cattle, Ryniewicz et al. [28] reported that polymorphisms of *defensin* genes could be used as markers for udder health traits and susceptibility to inflammations. Moreover, Bagnicka et al. [30] showed that the polymorphism of *β4-defensin* gene might be used in breeding programs as molecular marker in the selection of dairy cattle with increased resistance to mammary gland infection. The inflammation of the mammary gland is indeed one of the most common health problems affecting dairy species [42]. Nevertheless, work involving the association of *β-defensin* SNPs identified in this study with somatic cell count (SCC), as indirect indicator of mastitis is required to resolve the applicability of the aforementioned SNPs in marker assisted selection for mastitis resistance in Valle del Belice dairy sheep.

The SNP identified in *SBD2* exon 1 does not cause any change in the amino acid sequence. Nevertheless, several studies have shown that mutations which are predicted to be translationally silent can alter gene expression by influencing splicing accuracy/efficiency or codon biasness [43, 44].

The two missense mutations identified in *SBD2* have been reported previously by Luenser et al. [25] in two species belonging to the Caprini tribe. The first of these substitutions ($Arg^{42} \rightarrow Lys^{42}$) was described in *Ovis ammon* (Argali sheep), and is located in a site which presents a moderate conservation level and determines the change of a residue with similar physico-chemical features (both amino acids involved in the substitution are basic). The second mutation ($Gly^{45} \rightarrow Arg^{45}$), detected in the *Ovis orientalis cycloceros* (Afghan urial), is associated to the switch from nonpolar to polar amino acid resulting in the substitution of residues with different features. This change could have an effect on the interaction between the protein and the host membrane; indeed the physical driving force behind antibacterial activity of these antimicrobial molecules is known to involve the spatial arrangement of polar and hydrophobic residues [45]. The similar mutation

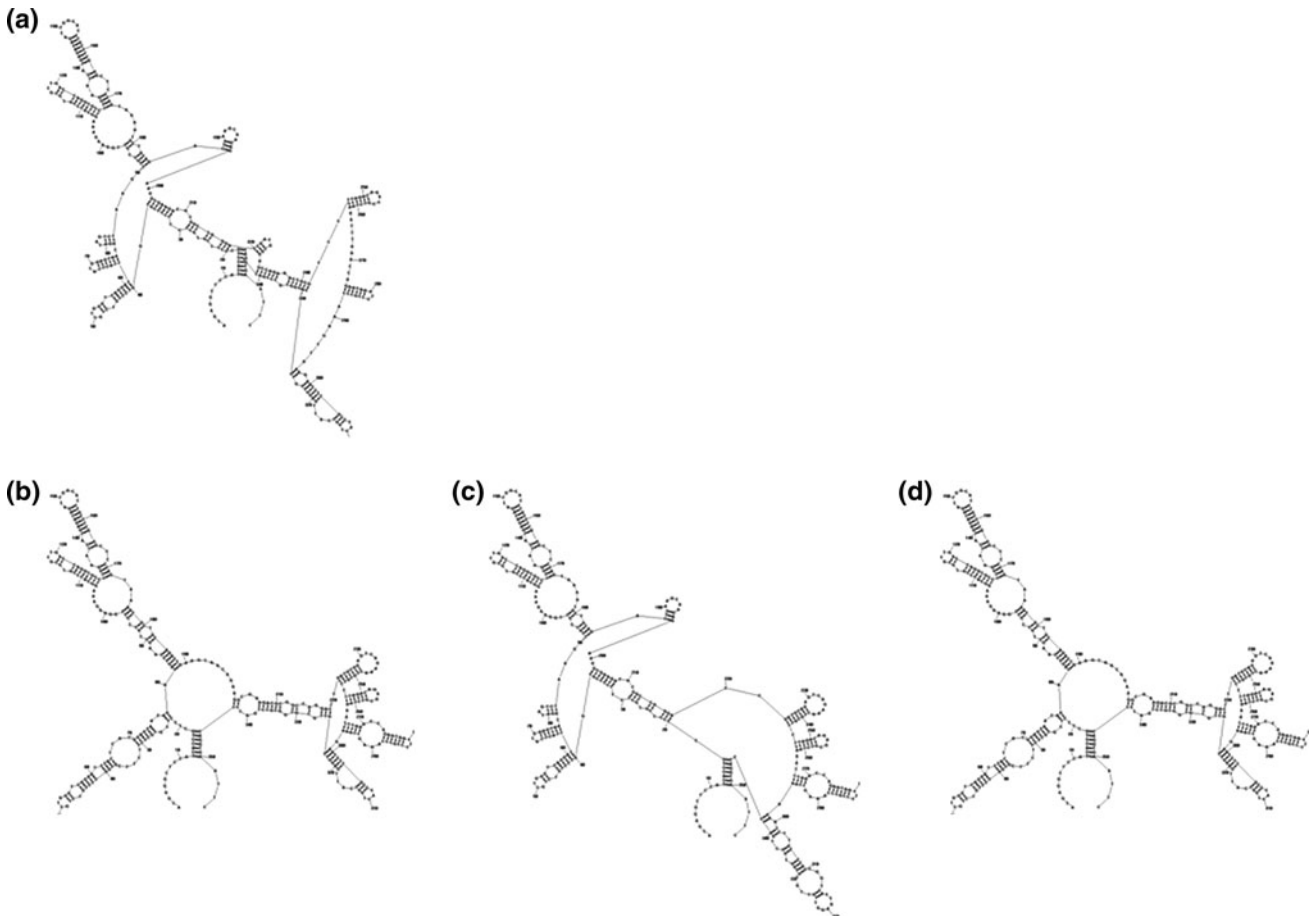

Fig. 2 SBD2 tridimensional mRNA secondary structure. Structure of the wild type (**a**) and mutated mRNA in relation to the 3′-UTR SNP at position 1750 (**b**), 1761 (**c**), and 1750 and 1761 (**d**)

profile observed in the Valle del Belice breed in this study, most likely reflects a recent common ancestry for the two Asian breeds and *Ovis aries* species. These results are in agreement with the theory based on the existence of a common ancestral *β-defensin* gene, which after duplication became subject to lineage-specific evolution by mutation and natural selection [12, 46].

The analysis with PANTHER and SIFT software's showed that the coding SNPs in *SBD2* do not affect protein function, as the amino acids involved in the substitution at position 42 have the same physical and chemical features. This change, therefore, does not affect the net charge of the peptide. On the other side, the switch at position 45, which leads to the substitution between amino acid residues with different features, could have an influence on the protein structural characteristics and consequently on SBD2 function. The predictions from both packages demonstrated that the aforementioned substitution is not deleterious. Nevertheless the substitution does modify the net charge of the mutated protein (i.e., from a nonpolar amino acid to a polar one), and hence could affect its function. Some studies reported the existence of structure–function correlations [47] showing that small changes of the primary structure have an effect on their function [48–50]. However, further studies will be necessary to verify if these SNPs compromise SBD2 function.

The SNPs located in 3′-UTR were analyzed for their possible effect on the mRNA secondary structure. The results of this study demonstrated that the mRNA conformation in mutated condition of SBD1 was the same as in wild condition. Nevertheless, SBD2 structure changed in presence of mutations in the 3′-UTR both whether they were present in combination or not. The structural difference in SBD2 messenger may be related to a possible role of the SNPs in SBD2 mRNA translation efficiency and consequential modulation of protein production. The 3′-UTRs are indeed known to play crucial roles in the post-transcriptional processes including the modulation of the transport of mRNAs outside of the nucleus and of translation efficiency [51], the subcellular localization [52], and the stability [53]. Changes in the 3′-UTR sequences could alter the *cis-acting* regulatory elements, primary sequence

motifs that bind to proteins leading to stabilization or destabilization of mRNAs or binding sites for microRNA, which are involved in post-transcriptional gene expression regulation mechanisms. The importance of UTRs in regulating gene expression is underlined by the finding that mutations altering UTRs can lead to pathology [54] and the effects caused by SNPs located within these regions were reported in different studies [55, 56]. Kalus et al. [40] have recently presented a study that correlates a 5′-UTR SNP (−44 C/G SNP) in the human *DEFB1* gene with changes in the gene expression of two β-defensins: hBD-1 and hBD-3, but this relationship was not observed when considering the peptide expression. They have proposed a post-transcriptional regulation of *DEFB1* through changes of mRNA secondary structure caused by the 5′-UTR SNP haplotypes. Other authors reported that the same SNP could change a putative NFKB binding site, proposing the influence of this polymorphism in the regulation of *DEFB1* gene expression at the transcription stage [57]. Moreover, a SNP in the 3′-UTR of human *DEFB1* gene has been associated with the increased susceptibility to periodontal diseases and has been suggested to influence the microRNA regulation, probably modifying the putative 3′ binding site of a specific miRNA [58].

Given the relevance of the biological role of β-defensin peptides in the immune response, the SNPs identified in this study could therefore play a role in the modulation of the immune response against microbes, through increasing the antibacterial protein production, or sequence diversity, enhancing the pathogen recognition spectrum. Nevertheless, further studies are necessary to verify whether the mutations identified in the 3′-UTR affect the mRNA stability and the expression of these immunity genes.

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