

Article

Combining Nucleotide Sequence Variants and Transcript Levels of Immune and Antioxidant Markers for Selection and Improvement of Mastitis Resistance in Dromedary Camels

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Abstract: The immune and antioxidant genetic factors that could converse with mastitis susceptibility in dromedary camels were looked at in this research. Of 120 female dromedary camels (60 healthy, and 60 with mastitis) were utilised. Each camel's jugular vein was pierced to obtain five millilitres of blood. The blood was placed within tubes containing sodium fluoride or EDTA anti-coagulants to obtain whole blood and extract DNA and RNA. The immunological (*OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, and *C4A*) and antioxidant (*CAT*, *SOD3*, *PRDX6*, *OXSRI*, *NDUFS6*, *SERP2*, and *ST1P1*) genes' nucleotide sequence polymorphisms between healthy and mastitis affected she-camels were discovered using PCR-DNA sequencing. Fisher's exact test revealed that camel groups with and without mastitis had noticeably different odds of all major nucleotide alterations propagating ($p < 0.01$). Mastitic camels were significantly more likely to express the *OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, *C4A*, *OXSRI*, *SERP2*, and *ST1P1* genes ($p < 0.05$). However, *CAT*, *SOD3*, *PRDX6*, and *NDUFS6* genes elicited a different pattern. The results may be used to develop management strategies and support the significance of nucleotide differences and gene expression patterns in these markers as indicators of the incidence of mastitis.

Keywords: immunity; antioxidant biomarkers; transcript levels; mastitis; dromedary camels

1. Introduction

A vital component of desert and semi-desert eco-systems are camels (*Camelus* spp.) [1]. Contrary to many other domestic species, camels can survive in harsh climates, areas with little flora, and with little access to food and water [2]. The two primary subfamilies of camelids are *Camelinae* (Old World camelids) and *Laminae* (New World camelids), which make up the Camelidae family. Two domesticated species of camelids are found in the Old World: the one-humped dromedary (*Camelus dromedarius*) and the two-humped Bactrian camel (*Camelus bactrianus*) [3]. A third species of Old World camelids is the wild camel (*Camelus ferus*), a double-humped camel that is related to the Bactrian camel and inhabits central Asia and Africa [4]. The four principal species of the New World camelids, which inhabit the high elevations of South America, are two wild species (the *guanaco* and *vicua*) and two domesticated species (the *llama* and *alpaca*) [5]. In dry and arid areas, the camel (*Camelus dromedaries* or one-humped camel) is crucial to human life and utilisation [6]. They are viewed as safe-haven investments and protection against natural calamities, which frequently strike the desert and induce livestock mortality. The most difficult issue facing individuals who live in the desert is protecting the camels and the items they produce from mastitis [7]. Camels hold a significant place in the lives of these people. One of the key ingredients in the nomads' diet in semiarid and arid regions is camel milk. It is a necessary aliment for human survival and may be the only milk accessible in regions where it is impossible to keep other milking animals [8].

Mastitis infections, one of the most complicated and expensive endemic diseases of dairy animals, induce inflammation of the mammary gland and udder tissue. It manifests as an immune reaction to a bacterial invasion of the teat canal [9]. The udder may also experience thermal, mechanical, or chemical injury. Due to decreased milk production, early culling, veterinary expenses, and the possibility of drug residues, this disease causes enormous economic loss [10]. The economic benefits and incentives influence all decision-makers to perfect mastitis management [8]. Other restrictions include the genetic evolution of antibiotic-resistant mutant microorganisms. Vaccines are not 100% effective because of genetic drift and shift; therefore, changes in treatment, vaccination, and pathogen control no longer have any effect [11]. Therefore, mastitis must be decreased in order to improve productivity and health [12]. Mastitis is quite important from a zoonotic and financial standpoint. Multiple harmful impacts on human health and animal production are brought on by it [13]. In order to prevent and control mastitis in the study context, it is necessary to use complete procedures in order to enhance camel milk quality, reduce financial loss, and avoid serious threats to the public's health [14]. There is less published information on the etiological agents linked to camel mastitis than there is for bovine mastitis. Mastitis in domestic animals is thought to be mostly brought on by bacterial infections. According to some studies, *Streptococcus agalactiae*, coagulase-negative *staphylococci*, *Pasteurella haemolytica*, *Escherichia coli*, *Staphylococcus aureus*, *streptococcus* spp., *Micrococcus* spp., *Staphylococcus epidermides*, and *Corynebacterium* spp. have all been linked to mastitis in camels [15,16].

Both cellular and non-cellular elements of the immune system work together in a complicated network to provide efficient immune responses against pathogens [17]. While the camel immune system has been the subject of extensive research. Numerous recent publications have previously detailed the improvements made in the study of camel immunoglobulins [18,19]. Responses of the adaptive immune system, particularly cellular immunity, significantly impact camel resistance. A thorough understanding of the mechanisms behind camel adaptive immune reactions is essential for the development of vaccines to prevent illnesses and improve camel well-being [20]. The primary factor contributing to immunosuppression and increased disease vulnerability is oxidative stress [21]. The increased metabolic demands brought on by late pregnancy, delivery, and the beginning of nursing are known to cause reactive oxygen species (ROS) and lipoperoxidation to harm tissues, particularly immune cells [22]. Free radicals cause oxidative stress, which can be prevented or repaired by physical, antioxidant, and other defenses [23].

Despite the detailed characterization of the infectious organisms linked to prevalent diseases and the interpretation of their genomic sequences, controlling major infectious diseases in livestock remains difficult [24]. Additionally, new developments in the genetic and genomic studies of livestock have shown that host genetics has a significant impact on the spread of infectious diseases throughout populations [25,26]. Most infectious illnesses have host genetic variation in resistance, and this variability may be utilized in selection [27]. In order to improve disease resistance, a genetic strategy could be used in conjunction with current control methods [28]. The use of genomic selection in numerous nations and the discovery of genomic areas linked to disease resistance traits are the results of advancements in animal genome sequencing technologies [29]. This two-fold effect makes it easier to incorporate information about disease-resistant loci into animal breeding [30].

Even though camel mastitis has been extensively studied, all prior investigations on the condition concentrated on its aetiology, epidemiology, and therapy [2,31]. Mastitis can be prevented by using genetic marker-assisted selection for mastitis features because it produces greater homogeneity and more discriminating between phenotypes than traditional selection, which is lacking in information about the immunological and antioxidant genetic polymorphisms and expression profile of indicators associated with this disease [32,33]. The candidate gene approach has been used to verify mastitis resistance/susceptibility in livestock; nonetheless, details on immune and antioxidant biomarker polymorphisms and expression profiles for mastitis incidence in dromedary camels are scarcely reported. To overcome this limitation, the effectiveness of potential immunological and antioxidant genes as candidates for mastitis occurrence prediction and tracking in dromedary camels was evaluated in this investigation using real-time PCR and PCR-DNA sequencing techniques.

2. Material and Methods

2.1. Dromedary Camels and Research Samples

The current study was conducted on 120 dromedary she-camels aged 18 years with a mean body weight of 520 kg. On a private farm in the Egyptian district of Matrouh, camels are grown. The study was conducted from February 2023 to May 2023. According to their state of health, the investigated camels were split into two almost equal groups. Each she-camel underwent a thorough health assessment, including body temperature, pulse rates, respiration rates, and udder inspection before having raw milk. According to a prior investigation, all milk samples were tested for mastitis using the California mastitis test (CMT) [33]. The first group, known as the healthy group, was made up of camels with normal body temperatures, pulse rates, and respiration rates, as well as being clinically healthy. Milk samples and normal udder texture tested negative for CMT (the combination remained liquid). The second set of camels had clinical mastitis (elevated body temperature, pulse rate, and respiratory rate as well as low appetite), swollen, congested, hot, and uncomfortable udder was also revealed. A considerable decrease in milk production, with unusual consistency and appearance. CMT was detected in milk samples (the mixture soon thickened and inclined to form jelly). Five millilitres of blood were collected by puncturing the jugular vein of each camel. Samples were placed in tubes containing anticoagulants (EDTA or sodium fluoride) in order to collect entire blood and retrieve DNA and RNA. The University of Sadat City's Veterinary Medical School (Code VUSC-029-1-23) was in charge of overseeing all animal management procedures, test experimental collection, and sample eradication in accordance with IACUC guidelines.

2.2. DNA Extraction and Amplification

Total blood was utilised to extract DNA from the genome using the JET complete blood genomic DNA isolation kit and the manufacturer's instructions (Thermo Scientific,

Vilnius, Lithuania). Nanodrop was used to consider DNA with good purity and concentration. Immune (*OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, and *C4A*) and antioxidant (*CAT*, *SOD3*, *PRDX6*, *OXSRI*, *NDUFS6*, *SERP2*, and *STIP1*) genes has been invitro cloned. The primer sequences for amplification were constructed using the GenBank *Camelus dromedarius* genome. Table 1 lists the oligonucleotide sequences used for the PCR.

Table 1. Immune and antioxidant oligonucleotide primers for the investigation of genetic polymorphisms.

Marker of Research	Sense	Antisense	Annealing Temperature (°C)	Target Band Size (bp)
<i>OTUD3</i>	5'-TGCGAGGAGGAGTTCGTCAGC-3'	5'-AGGTGCGCAGGTGCTTCCGAGT-3'	60	452
<i>TLR2</i>	5'-TCAGGCCATTCTCTGTCTTG-3'	5'-AGATGAGTGATGTTCTGAATT-3'	58	540
<i>TLR4</i>	5'-TAGCTGTGGCTCTGATGACAG-3'	5'-CTAGAGATGCTAGGTTTGTCT-3'	60	405
<i>STAB2</i>	5'-GACTGAGTGCCAATCCTGCTC-3'	5'-TCTGGGCAGAGCAAGGTTGCA-3'	60	498
<i>MBL2</i>	5'-CGGCACGACTAATCATGTGTG-3'	5'-GTCCAATTCTGATCGCAGGGC-3'	60	389
<i>TRAPPC9</i>	5'-AGACTCTCCTCGTGGTGGTCCA-3'	5'-GCGACTCGATGAAGTCTCGAT-3'	58	423
<i>C4A</i>	5'-TCTGCAGAAGCCCAGTTGCT-3'	5'-TGATCCAGAGCAAAGACTCGGT-3'	60	444
<i>CAT</i>	5'-CGGGATCCAGCCAGCAGCAGCA-3'	5'-GTCCAGACCATATCTGGATC-3'	58	540
<i>SOD3</i>	5'-TGCTCTGTGCCTACCTGCTCA-3'	5'-GTCGCGCACGGCGAAGTTGCCG-3'	58	458
<i>PRDX6</i>	5'-TCGAGGCCAATACTACCGTG-3'	5'-CTGTCAGCTGGAGAGAGATAA-3'	60	471
<i>OXSRI</i>	5'-CTGCATCTGTTGTGCATGTA-3'	5'-AGTTCATCTAGGACTCCACT-3'	60	420
<i>NDUFS6</i>	5'-CGCCCGGTGCTTCGGGGTGCG-3'	5'-TAGTGGTGGTGTCTGTAAC-3'	60	303
<i>SERP2</i>	5'-TCCTGAATCAGAGCCGACCTGC-3'	5'-TAAGAGCTGAAGTGGTCTCCA-3'	58	373
<i>STIP1</i>	5'-AGCCCTGAGTGCTGGCAACAT-3'	5'-TCGCAGTTGCTCTATCAGCTC-3'	60	442

OTUD3 = OTU Deubiquitinase 3; *TLR2* = Toll-like receptor 2; *TLR4* = Toll-like receptor 4; *STAB2* = Stabilin 2; *MBL2* = Mannose binding lectin 2; *TRAPPC9* = Trafficking protein particle complex 9; *C4A* = Complement C4A; *CAT* = Catalase; *SOD3* = Superoxide dismutase 3; *PRDX6* = Peroxiredoxin-6; *OXSRI* = Oxidative stress responsive kinase 1; *NDUFS6* = NADH:ubiquinone oxidoreductase subunit S6; *SERP2* = Stress associated endoplasmic reticulum protein family member 2; and *STIP1* = Stress-induced phosphoprotein 1.

A PCR thermal cycler (Bio-Rad, Hercules, California, USA) containing a concluding volume of 150 µL was used to process the PCR constituents. The following elements were present in each reaction container: 1.5 mL of each primer pair, 6 mL of genetic material, 66 mL of deionised water, and 75 mL of the master combination (Jena Bioscience, Jena, Germany). The PCR combinations were employed for five minutes at a starting temperature of 94 °C for unwinding. The 34 cycles were composed of 30 seconds of denaturation at 94 °C, one-minute annealing cycles based on the range of temperatures in Table 1, elongation at 72 °C for one minute, followed by a further 10-min elongation at 72 °C. PCR products were maintained at 4 °C. A gel certification method was applied, which involved evaluating PCR segment configurations under UV light and using 2% agarose (Bio-Rad) gel electrophoresis to produce demonstrable results.

2.3. Discovering Polymorphism

In advance of DNA sequence, Hamburg, Germany-based Jena Bioscience # pp-201s/Munich provided methods to purify PCR in order to eliminate primer dimmers, non-specific bands, and other impurities and produce the appropriate amplified result for the anticipated scope [34]. A Nanodrop (Waltham, MA, USA, UV-Vis spectrophotometer Q5000) was employed to evaluate PCR output since it offered sufficient quality and good concentrations [35]. Sequence analysis of the forward and reverse primer amplification data from PCR was performed to locate SNPs in normal and mastitis-affected she-camels. Using the Sanger et al. [36] outlined enzyme chain terminator technique, the PCR data

were sequenced on an ABI 3730XL DNA sequencer (Applied Biosystems, Waltham, MA, USA).

The tools for evaluating the results of DNA analysis included Chromas 1.45 and BLAST 2.0. [37]. Polymorphisms have been found when comparing the immune and antioxidant gene PCR results to the reference gene sequences published by GenBank. According to sequence matching among the investigated she-camels, the MEGA6 program can identify changes in the categorizations of amino acids among the examined genes [38].

2.4. mRNA Levels of Immune and Antioxidant Genes

The Trizol solution (RNeasy Mini Ki, 74104, Product No.) was used to completely extract the RNA from the blood samples collected from the studied she-camels in accordance with the manufacturer's instructions. We quantified and validated the amount of isolated RNA using a NanoDrop® ND-1000 spectrophotometer. Each sample was created using the producer's procedure (Thermo Fisher, Product No. EP0441, Waltham, MA, USA). The expression profiles of the immune and antioxidant genes were evaluated using the quantitative RT-PCR method and SYBR Green PCR master mix (2× SensiFast™ SYBR, Bio-line, CAT No. Bio-98002). The relative amount of mRNA that each sample possesses has been determined using the Quantitect SYBR green PCR reagent, Catalogue No. 204141 (Toronto, ON, Canada).

The genome of the GenBank *Camelus dromedarius* was used to create the sense and anti-sense primer sequences (Table 2). The constitutive normalisation reference was the *GAPDH* gene. The reaction mixture, which was released in an overall volume of 25 µL and contained all of the RNA, 3 µL, 4 µL 5×Trans Amp buffer, 0.25 µL reverse transcriptase, 0.5 µL of each primer, 12.5 µL 2× Quantitect SYBR green PCR master mix and 8.25 µL RNase-free water. After that, the prepared reaction mixture underwent the following processes inside a heater cyler: reverse transcription for 30 min at 55 °C, preparatory denaturation for 8 min at 95 °C, 40 cycles of 15 s at 95 °C, and primer binding temperatures as listed in Table 2 for 45 sec with a 1-min extension at 72 °C. A melting curve analysis was utilised to demonstrate the specificity of the amplified product after the amplification process. The $2^{-\Delta\Delta Ct}$ approach has been used to investigate the variations in the expression of each gene by comparing the expression of each gene in the studied sample to that of the *GAPDH* gene [39].

Table 2. Oligonucleotide-based real-time PCR primers for the immunological and antioxidant genes under research.

Marker of Research	Primer	Target Band (bp)	Annealing Temperature (°C)	GenBank Isolate
OTUD3	F5'-TGGTAGGTTTCATCGTGGGAC-3'	163	58	XM_045518474.1
	R5'-ATCTGCCACAAAGGGGCATT-3'			
TLR2	F5'-ACTGAAGCAGTCTGAGCCATC-3'	74	60	XM_010968523.2
	R5'-CCAAGACCCACACTGTCCAC-3'			
TLR4	F5'-ACAACATCCCCACATCGACC-3'	174	60	XM_010957186.2
	R5'-TCAAGGTGGCAAGGTGGTTT-3'			
STAB2	F5'-TTAGGAGCATGGCCAACCAG-3'	163	60	XM_045506980.1
	R5'-GGCAGGATTGGGACAATGGA-3'			
MBL2	F5'-AGTGTGAGGACCTTGTCCCT-3'	202	58	XM_010971305.1
	R5'-CTTGGCCTGGTTCTCCCTT-3'			
TRAPPC9	F5'-GTGTTGTTGGGTCTGAAAAGACA-3'	130	58	XM_045522194.1
	R5'-GCTCCACGGACATGTGGTAA-3'			
C4A	F5'-AGTGGAGACAGTAGACCGCT-3'	116	58	XM_031435000.1
	R5'-GAGTAGCCACACACAAGCCT-3'			
CAT	F5'-GATGAGAAGCCGAAGAACGC-3'	136	60	XM_011000575.2
	R5'-ATGCTTGGCCTCATAGGCAG-3'			
SOD 3	F5'-GACACCTCTCCAAAAGCCCA-3'	169	60	XM_031436563.1
	R5'-GCACATGGTTGGAGGCCTTA-3'			

<i>PRDX6</i>	F::5-CGTGATGGTCCTTCCAACCA-3 R:5-CTAATAAGCGAGCGGCTCCA-3	158	60	XM_010984536.2
<i>OXSRI</i>	F:5-AGCCTTGCTAACACCAACCA-3 R:5-ATGTTCCCCCTTTGCCACAA-3	126	59	XM_031469963.1
<i>NDUFS6</i>	F:5-GGGGAGAAGGTCACGCATAC-3 R:5-TTCACCTCTTTCTGGCGACC-3	86	60	XM_031442846.1
<i>SERP2</i>	F:5-GATTCCACGCCATGACTCTCT-3 R:5-AAAGGCCAGTGTATGCGG-3	106	59	XM_031466026.1
<i>STIP1</i>	F:5-CCGGTTGGCAGAGAGGAAAT-3 R:5-GCGAGCAGAGTCTTTGTCCT-3	93	60	XM_010994738.2
<i>GAPDH</i>	F:5-TCGATCCCCAACACACTTG-3 R:5-TGATGGTGCATGACAAGGCA-3	106	58	XM_010990867.2

OTUD3 = OTU Deubiquitinase 3; *TLR2* = Toll-like receptor 2; *TLR4* = Toll-like receptor 4; *STAB2* = Stabilin 2; *MBL2* = Mannose binding lectin 2; *TRAPPC9* = Trafficking protein particle complex 9; *C4A* = Complement C4A; *CAT* = Catalase; *SOD3* = Superoxide dismutase 3; *PRDX6* = Peroxiredoxin-6; *OXSRI* = Oxidative stress responsive kinase 1; *NDUFS6* = NADH:ubiquinone oxidoreductase subunit S6; *SERP2* = Stress associated endoplasmic reticulum protein family member 2; and *STIP1* = Stress-induced phosphoprotein 1.

2.5. Statistical Analysis

H₀: Combining nucleotide sequence variants and transcript levels of immune and antioxidant markers could not select selection and improve mastitis resistance in dromedary camels.

H_A: Combining nucleotide sequence variants and transcript levels of immune and antioxidant markers could select selection and improve mastitis resistance in dromedary camels.

The distribution of SNPs for the identified genes was shown to be significantly different between the study she-camels using Fisher's exact test analysis ($p < 0.01$). The Statistical Package for Social Science (SPSS) version 17 computer program and the t-test (SPSS Inc, Chicago, IL, USA) were used to ascertain the statistical significance of the variations between healthy and mastitis-affected she-camels. The results were presented using mean and standard error (Mean \pm SE). Differences were thought to be significant at $p < 0.05$.

3. Results

3.1. Genetic Variation of Immune and Antioxidant Genes

Both healthy and mastitic she-camels had SNP differences in amplified DNA nucleotides linked to mastitis, and these variations were detected by the findings of PCR-DNA sequencing for the *OTUD3* (452-bp), *TLR2* (540-bp), *TLR4* (405-bp), *STAB2* (498-bp), *MBL2* (389-bp), *TRAPPC9* (423-bp), *C4A* (444-bp), *CAT* (540-bp), *SOD3* (458-bp), *PRDX6* (471-bp), *OXSRI* (420-bp), *NDUFS6* (303-bp), *SERP2* (373-bp), and *STIP1* (442-bp) genes. Each identified SNP was validated by comparing the DNA sequences of reference genes obtained from GenBank with the indicators analysed in the study she-camels (Figures S1–S14).

Table 3 shows the distribution of a single base variation and a particular kind of genetic alteration for immunological and antioxidant indicators in healthy and mastitic she-camel populations. Fisher's exact test examination of the SNPs indicated significantly differing occurrences of the examined markers ($p < 0.01$). Exonic region alterations affected every immunological and antioxidant marker being studied, resulting in different DNA sequence coding in mastitic she-camels when compared to healthy ones. 25 SNPs were discovered by DNA sequencing the examined genes; 19 are non-synonymous, and six are synonymous. Utilising the differences in amino acids between the immunological and antioxidant genes in the research she-camels and the reference sequences retrieved in GenBank, all matching amino acids were verified (Figures S15–S28).

Table 3. Immune and antioxidant marker dispersion with a single base differential and a possible hereditary modification in mastitic and healthy she-camels.

Gene	SNPs	Healthy <i>n</i> = 60	Mastitis <i>n</i> = 60	Total <i>n</i> = 120	Kind of Hereditary Change	Amino Acid Order and Sort
<i>OTUD3</i>	T120C	28/60	0/60	28/120	Synonymous	40 H
	T270A	34/60	34/60	34/120	Synonymous	90 A
<i>TLR2</i>	G113A	0/60	37/60	37/120	Non-synonymous	38 R to Q
	A298C	42/60	0/60	42/120	Non-synonymous	100 P to T
	G342A	21/60	0/60	21/120	Synonymous	114 Q
<i>TLR4</i>	T41C	46/60	0/60	46/120	Non-synonymous	14 V to A
	C209G	0/60	37/60	37/120	Non-synonymous	70 A to G
<i>STAB2</i>	A112C	0/60	50/60	50/120	Non-synonymous	38 K to Q
<i>MBL2</i>	G144A	33/60	0/60	33/120	Synonymous	48 V
<i>TRAPPC9</i>	T115C	0/60	18/60	18/120	Non-synonymous	39 C to R
	G290C	0/60	23/60	23/120	Non-synonymous	97 C to S
<i>C4A</i>	A232G	0/60	52/60	52/120	Non-synonymous	78 K to E
	C367A	38/60	0/60	38/120	Non-synonymous	123 P to T
	G415C	21/60	0/60	21/120	Non-synonymous	139 R to G
<i>CAT</i>	C50T	0/60	29/60	29/120	Non-synonymous	17 A to V
<i>SOD3</i>	A41C	0/60	46/60	46/120	Non-synonymous	14 K to T
	T322G	0/60	25/60	25/120	Non-synonymous	108 S to A
	G342T	39/60	0/60	39/120	Synonymous	114 T
<i>PRDX6</i>	C32T	26/60	0/60	26/120	Non-synonymous	11 A to V
	C170G	0/60	53/60	53/120	Non-synonymous	57 P to R
	C236T	0/60	19/60	19/120	Non-synonymous	79 T to M
<i>OXSRI</i>	A189G	0/60	41/60	41/120	Synonymous	63 A
<i>NDUFS6</i>	G118A	17/60	0/60	17/120	Non-synonymous	40 E to K
<i>SERP2</i>	G263A	0/60	52/60	52/120	Non-synonymous	88 R to H
<i>STIP1</i>	G155A	0/60	24/60	24/120	Non-synonymous	52 R to H

Single base difference dispersal for immune and antioxidant markers in healthy and mastitic she-camels showed a highly significant variation ($p < 0.01$) according to Fisher's exact analysis. *OTUD3* = OTU Deubiquitinase 3; *TLR2* = Toll-like receptor 2; *TLR4* = Toll-like receptor 4; *STAB2* = Stabilin 2; *MBL2* = Mannose binding lectin 2; *TRAPPC9* = Trafficking protein particle complex 9; *C4A* = Complement C4A; *CAT* = Catalase; *SOD3* = Superoxide dismutase 3; *PRDX6* = Peroxiredoxin-6; *OXSRI* = Oxidative stress responsive kinase 1; *NDUFS6* = NADH: ubiquinone oxidoreductase subunit S6; *SERP2* = Stress associated endoplasmic reticulum protein family member 2; and *STIP1* = Stress-induced phosphoprotein 1. A = Alanine; C = Cysteine; E = Glutamic acid; G = Glycine; H = Histidine; K = Lysine; M = Methionine; P = Proline; Q = Glutamine; R = Arginine; S = Serine; T = Threonine; and V = Valine.

3.2. Immune and Antioxidant Indicator Transcript Levels Tendencies

Figures 1 and 2 show the immunological and antioxidant transcript levels in healthy and mastitic she-camels, respectively. In mastitic she-camels, expression levels of the genes *OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, *C4A*, *OXSRI*, *SERP2*, and *STIP1* were noticeably up-regulated. However, *CAT*, *SOD3*, *PRDX6*, and *NDUFS6* genes elicited down-regulation. The mastitic she-camels' highest possible level of mRNA for each gene was determined for *TLR2* (2.52 ± 0.09), and *OXSRI* was determined to have the lowest amount (0.51 ± 0.07). In the healthy she-camels' analysis of all the genes, *NDUFS6* was identified as the gene with the highest potential level of mRNA (1.87 ± 0.12), whereas *C4A* had the lowest level (0.35 ± 0.05).

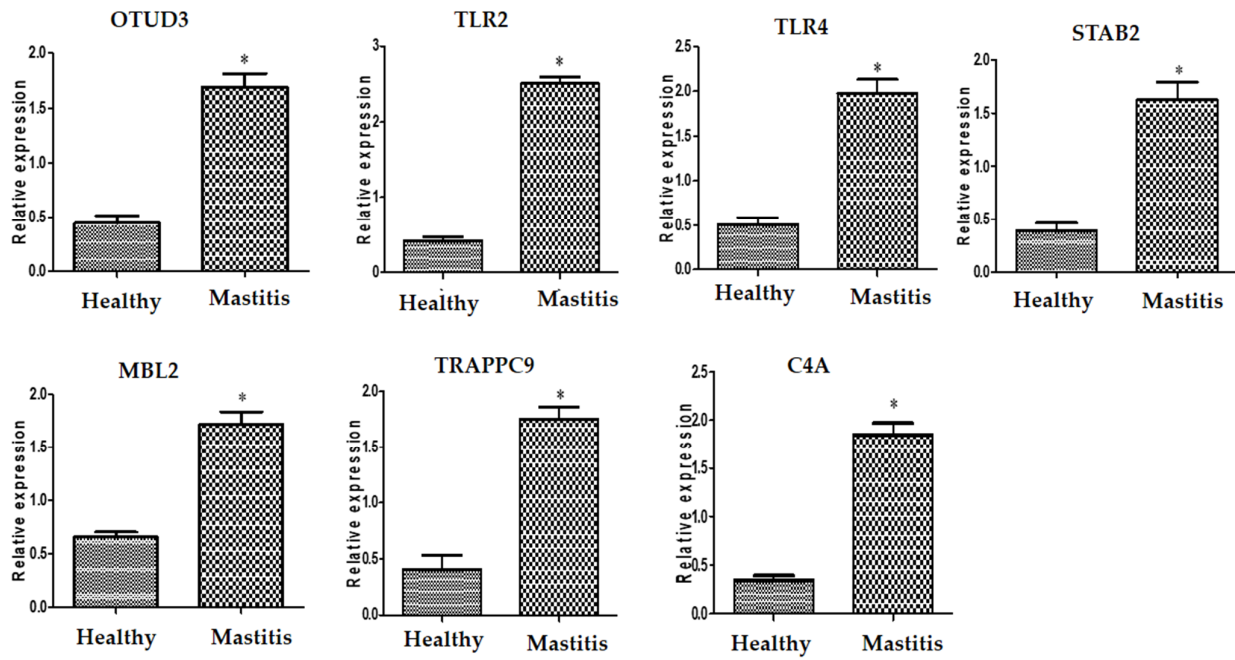


Figure 1. Immune gene transcript levels in healthy ($n = 60$) and mastitic ($n = 60$) she-camels. The symbol * denotes significance when $p < 0.05$.

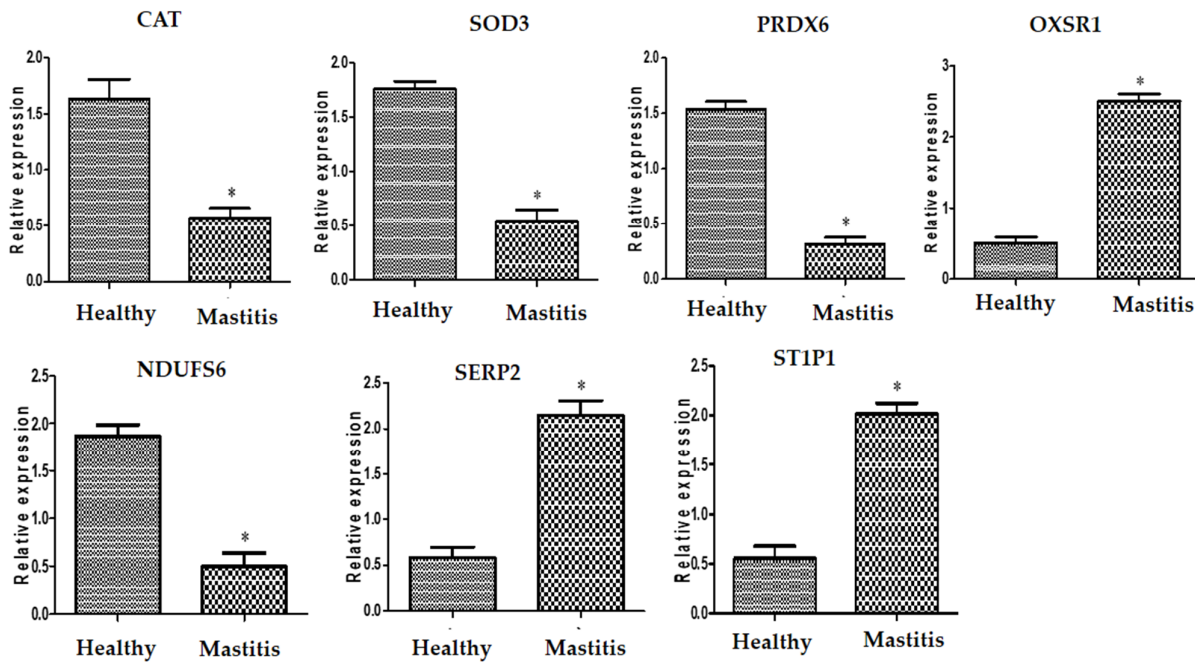


Figure 2. Antioxidant gene transcript levels in healthy ($n = 60$) and mastitic ($n = 60$) she-camels. The symbol * denotes significance when $p < 0.05$.

4. Discussion

4.1. Mastitis Susceptibility with Immune and Antioxidant Gene Polymorphisms

The need to find quantitative trait loci (QTL) and SNP in candidate genetic factors has resulted in two approaches for discovering genetic markers linked to mastitis resistance [40]. The most prevalent type of polymorphism in eukaryotic genomes that can be utilized as a superior marker type for characterizing features that are relevant economically is the SNP [41]. We hypothesize that critical shared innate immunological defense mechanisms are present in dromedary she-camels against mastitis and that differences in key genes associated with these systems can affect how resistant an animal is to developing the disease. The immune (*OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, and *C4A*) and antioxidant (*CAT*, *SOD3*, *PRDX6*, *OXSRI*, *NDUFS6*, *SERP2*, and *ST1P1*) genes in mastitis affected and healthy she-camels were discovered in this work using amplified PCR products that had been sequenced. The results demonstrate that the SNPs involving the two categories differ from one another. The Fisher's exact test revealed that the evaluated she-camels showed a significant nucleotide polymorphism dispersion ($p < 0.01$). The polymorphisms found and made available in this context give fresh data for the evaluated indicators when compared to the pertinent datasets collected from GenBank, which must be emphasized.

More data are required on the polymorphisms and expression patterns of immunological and antioxidant biomarkers for the prevalence of mastitis in dromedary camels. The gene sequences from the *Camelus dromedarius* that were employed in our research and were published in PubMed provide the first indication of this link. According to our knowledge, no previous studies have looked at the variation of the immunological (*OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, and *C4A*) and antioxidant (*CAT*, *SOD3*, *PRDX6*, *OXSRI*, *NDUFS6*, *SERP2*, and *ST1P1*) markers and how they correlate with the occurrence of mastitis in dromedary camels. Our results coincide with results using immune and antioxidant genes as candidates for assessment of the veracity of mastitis susceptibility in other livestock. For instance, it was discovered that there was a significant correlation between mastitis susceptibility and *TLR4* gene polymorphisms in sheep [42] and cattle [43].

Similarly, according to Darwish et al., SNPs in the *TLR4* and *SOD* genes were associated with mastitis susceptibility in Barki sheep [44]. Ogorevc et al. [45] found a connection between polymorphisms in the *TLR2* gene and the sensitivity or resistance of goats to mastitis. Nucleotide sequence variation and gene expression of immunological and antioxidant indicators related to mastitis risk were also investigated in the Holstein and Brown Swiss breeds [46]. According to the findings, SNPs associated with mastitis tolerance/susceptibility were discovered in the PCR-DNA *SOD1*, *CAT*, *GPX1*, and *AhpC/TSA* sequencing of the dairy cows under investigation [47].

In the same line, The substantial correlation between SNPs in the *MBL* and milk somatic cell score (SCS), a significant phenotypic marker of bovine mastitis, has also been noted by other research studies [48,49]. In Chinese Holstein cattle, the genetic variants of the *TRAPPC9* and *C4A* genes were proven to be associated with mastitis resistance [50,51]. Multiple genomic regions with possible mastitis susceptibility genes have been found in dairy cattle using genome-wide association analysis [50,52]. *OTUD3*, *STAB2*, and *NDUFS6* were found to be these indications. SNPs linked to disease vulnerability have not, however, consistently been documented in these previous analyses.

4.2. Immune and Antioxidant Markers' Gene Expression Tendencies

According to the current research, genetic differences in an individual's transcriptional response to those conditions may have an impact on the course of mastitis in she-camel. Immune (*OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, and *C4A*) and antioxidant (*CAT*, *SOD3*, *PRDX6*, *OXSRI*, *NDUFS6*, *SERP2*, and *ST1P1*) genes have been quantified utilising real-time PCR in healthy and mastitis affected she-camels. Our findings

showed that mastitic she-camels expressed *OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, *C4A*, *OXSRI*, *SERP2*, and *ST1P1* genes at greater values than healthy ones. *CAT*, *SOD3*, *PRDX6*, and *NDUFS6* genes, on the other hand, triggered a different pattern. The limitations of earlier studies were overcome by our investigation of gene polymorphism using SNP genetic markers and gene expression. In order to address this issue, key immunological and antioxidant markers were measured using real-time PCR for the first time in mastitic and healthy she-camels. The result is that both healthy and mastitic she-camels have a solid understanding of the processes governing the researched gene regulation.

In both healthy and mastitis-affected dromedary she-camels, our research is the first to show the expression tendency for immunological (*OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, and *C4A*) and antioxidant (*CAT*, *SOD3*, *PRDX6*, *OXSRI*, *NDUFS6*, *SERP2*, and *ST1P1*) genes. However, to track the occurrence of mastitis in different livestock, the transcript analysis of immunological and antioxidant indicators was examined, where gene expression rate of immune and antioxidant indicators was proven to be a reference guide for tracking animal health in case of mastitis. Our results were consistent with the transcript level of immune markers in Chinese Holstein cows suffering from mastitis, where expression changes revealed that *TLR4*, *MyD88*, *IL-6*, and *IL-10* were up-regulated [53]. In addition, the expression of the genes *SOD1*, *CAT*, *GPX1*, and *AhpC/TSA* was drastically reduced in mastitic dairy cows [46]. Mastitic Holstein and Montbéliarde dairy cows had significantly increased the expression rates of the following indicators compared to sound ones: *RASGRP1*, *EPS15L1*, *C1QTNF3*, *CD46*, *NFkB*, *CHL1*, *MARCH3*, *PDGFD*, *MAST3*, *COX18*, *NEURL1*, *PPIE*, and *PTX3* [54].

A continuation of the previous reports, in line with our study in proving the importance of immune and antioxidant genes in judging the condition of the mastitic animal, mastitic Damascus goats were shown to exhibit an up-regulated expression profile of the *LTF* gene [55]. Compared to tolerant goats, goats with *Staphylococcus aureus* mastitis had significantly higher levels of pro-inflammatory cytokines, chemokines, and their receptors [56]. In terms of the gene expression profile of immunological markers in sheep, Darwish et al. [44] found that mastitis-affected sheep displayed considerably higher levels of *IL6*, *IL1-β*, *TNF alpha*, *TLR4*, *IL5*, and *Tollip* than immune-competent ewes.

4.3. Effectiveness of Examined Genes as Potential Mastitis Susceptibility Candidates

Human inflammatory bowel illness is related to OTU deubiquitinase 3 (*OTUD3*), which plays a function in inflammation [52]. The family of recognition patterns known as toll-like receptors is linked to the control of innate immunity [57]. TLR regulates the expression of a number of chemokines and pro-inflammatory cytokines upon activation, which helps to promote neutrophil recruitment further and activates both innate and acquired immune responses [57]. The host's response to infections may differ as a result of the pattern recognition receptors (PRR) SNPs, and they affect one's susceptibility to or resistance to mastitis [58]. To expand the range of recognised ligands, TLR1 and TLR2 can form heterodimers [59]. According to Pant et al. [60], *TLR2* mutations increase the risk of mastitis in cattle and are associated with milk SCS. One of the most crucial pattern recognition receptors is TLR2, which is crucial for triggering the immunological and inflammatory response [60]. According to a study, high milk SCS and mastitis susceptibility are linked to the TLR2 gene's SNP [61].

According to studies in pigs and mice, the stables 2 (*STAB2*) gene is important in removing metabolic waste from blood circulation [62,63]. This gene's protein plays a part in the angiogenesis, lymphocyte homing, cell adhesion, and receptor scavenging processes [64]. Mannose-binding lectin, a key participant in the collectin protein family, connects to different bacteria as well as controls the complement lectin route of innate immunity [65]. The *MBL1* and *MBL2* genes code for the MBL-A and MBL-C proteins, respectively. One of the potential genes, mannose-binding lectin (*MBL*), is essential for the innate

immune system's pattern recognition molecule. [48]. The *MBL2* gene has genetic variability in buffaloes, and this variation has been linked to clinical mastitis [66].

A crucial member of the nuclear factor kappa B (NF- κ B) family that has a crucial impact on inflammation and innate immunity is the trafficking protein particle complex 9 (*TRAPPC9*) gene [67]. When mastitis develops in dairy cattle, the higher amount of the *TRAPPC9* gene promotes the NF- κ B signalling process [68]. *TRAPPC9* was identified as a potential gene for cow mastitis resistance when Wang et al. [69] conducted a genome-wide association analysis on Chinese Holstein cows. These significant gene variants were primarily found in the Chinese Holstein cows' *Bos taurus* autosome (BTA) 14. A putative gene called complement component 4 (*C4A*) represents complement activity [51]. This gene's main function in the lectin-activation and classical pathways is to guard against bacterial infections [51].

Recently, metabolic problems have been linked to oxidative stress markers, particularly in dairy cows, when the body's homeostatic mechanisms were subjected to heavy loads during the peripartum period [70]. Research has shown that the peripartum period's antioxidant capacity falls short to counter the rise in ROS [71]. Because of this, in the weeks before parturition, there is a disparity between increased ROS production and reduced fortifications with antioxidants, which encourages oxidative stress and could contribute to periparturient illnesses in dairy cows [71].

Antioxidants provide protection by removing ROS from the environment, limiting their synthesis, or securing transition metals, which are used to create free radicals [72]. These processes include the body's own enzymatic and non-enzymatic antioxidant defences, such as catalase (CAT) and superoxide dismutase (SOD), known as endogenous antioxidant indicators [73].

Hydrogen peroxide (H₂O₂) can be catalysed by the peroxiredoxin (PRDX) family of antioxidant enzyme oxidoreductase proteins thanks to a conserved ionised thiol. By detoxifying peroxides and radicals containing sulphur, thiol-specific peroxidase functions as a sensor for signalling occasions caused by hydrogen peroxide and aids in cell defence against oxidative stress [74]. The serine/threonine protein kinase (OSR1) is encoded by the oxidative stress-responsive kinase 1 (*OXSRI*) gene and regulates downstream kinases in response to environmental challenges [75]. The periparturient period's *OXSRI* expression profile showed a significant up-regulation at (14) and (+14) in comparison to at parturition, with the lowest form occurring at that time in dromedary camels [76].

The NADH ubiquinone Oxidoreductase Subunit S6 (*NDUFS6*) gene encodes the NADH: ubiquinone Oxidoreductase (Complex I), the first enzyme complex in the mitochondrial electron transport chain [77]. The respiratory chain receives electrons from NADH by the action of this complex. Mutations in this gene result in mitochondrial complex I deficiency, a condition that can lead to neonatal illness and adult-onset neurodegenerative diseases [77]. The *NDUFS6* gene in cattle, which is found on BTA20, has previously been reported to have a 900 bp deletion that completely removes exon 2 [78]. This region of the genome houses a quantitative trait locus for SCS [79].

Protein-coding genes called stress-associated endoplasmic reticulum proteins (*SERP*) are linked to the buildup of unfolded proteins in the endoplasmic reticulum (ER stress). Potentially, *SERP* could aid in correct glycosylation and stop the deterioration of unfolded target proteins [80]. The adaptor protein stress-induced phosphoprotein (*STIP1*) controls and coordinates the roles of HSP70 and HSP90 in protein folding [81]. Additionally, *STIP1* responds to cellular physiological stress by expression brought on by high temperatures or other factors [82].

The expression patterns of the genes *OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, *C4A*, *OXSRI*, *SERP2*, and *STIP1* are markedly altered in mastic she-camels compared to healthy ones. On the other side, the genes *CAT*, *SOD3*, *PRDX6*, and *NDUFS6* caused a distinct pattern. Postpartum infections in camels, such as mastitis, are primarily brought on by bacterial pathogens [2,16]. Phagocytic cells produce cytotoxic radicals and pro-inflammatory cytokines, along with severe inflammation that harms the afflicted tissue,

which may be to blame for changes in the expression tendency of immune and antioxidant markers [83]. Additionally, the immune system is compromised by the excess ROS caused by the preponderance of ROS, which occurs in the absence of an optimum total antioxidant [84].

Recently, the term "oxinflammation" was developed to describe the vicious loop that links moderate chronic inflammation to long-term, systemic oxidative stress, which may impair the ability to mount an adaptive response and increase the risk of illnesses [85]. Oxidative stress may affect the immune response at the site of an infection or lesion [86]. Neutrophils contribute significantly to oxidative stress. The actions of neutrophils should be strictly controlled to prevent unintended outcomes because they can be used to induce "collateral" damage to mammalian tissues and have a high destructive capacity against invasive diseases [86]. The host and pathogen-derived substances that neutrophils are exposed to when they reach the inflammatory site cause them to delay apoptosis and boost their functions [87]. Superoxide, which is a precursor to hydrogen peroxide and other ROS, is produced in large quantities when the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is activated [88]. When hydrogen peroxide and the enzyme myeloperoxidase (MPO) interact, they form a potent oxidant and brief intermediates that can react with halides (Cl, Br, NO) and create very microbiocidal species like hypochlorous acid (HOCl). These chemicals can also be produced to alter extracellular targets, and they have an impact on how nearby cells behave [89].

Under physiological conditions, the proper operation of T cells and the mounting of a controlled immune response are guaranteed by the balance between ROS and antioxidant mechanisms [87]. However, altered/excessive extracellular ROS accumulations may have an impact on how the immune system responds, and they may also help oxidative stress (OS), a condition that causes systemic inflammation [87]. When xanthine oxidase and TLR4 interact, neutrophils produce extracellular superoxide, which improves nuclear factor B (NF- κ B) translocation and boosts the expression of NF- κ B-dependent pro-inflammatory cytokines [88]. As a result, the prolonged or excessive generation of superoxide may also contribute to the systemic inflammatory response and oxidative stress being amplified. As was seen in cow's neutrophils, it is likely that the accumulation of oxidation products, such as oxidised proteins near an inflammatory site, may cause a progressive loss in neutrophil viability [90]. The signalling, activation, proliferation, and possibly even viability of T cells can all be adversely impacted by the superoxide that neutrophils produce [91]. Memory T cells are the least vulnerable to H₂O₂ attack, while effector T cells are more vulnerable [92]. Hydrogen hydroperoxide (H₂O₂) might have varying effects on the various T cell subsets.

The increased exposure to pathogens stimulates the she-camels' immune systems. The neutrophil recruitment cascade is initiated when macrophages and epithelial cells are exposed to lipoteichoic acid (LTA, from Gram-positive bacteria) or to lipopolysaccharide (LPS, from Gram-negative bacteria), both of which increase the release of TNF and IL1 β again [93]. When certain receptors for TNF, IL1, C5a, and histamine are activated, vascular endothelial cells react [72]. Ephrin receptor signalling, RhoA signalling, and granulocyte adhesion were the key signalling pathways emphasised in the mammary gland's response to both bacterial infections [94]. These pathways made up a network that controlled the activity of leukocytes, particularly neutrophils, during mammary gland inflammation [94].

Leukocyte migration to infection sites must be fast and carefully controlled in order to strike the right balance between eliminating the pathogen and damaging too much tissue [95]. These findings imply that variations in gene expression in these pathways may affect the capacity of she-camel to withstand mastitis. Therefore, we conclude that an infectious agent was responsible for the vast majority of the mastitis episodes in this study that affected she-camels. Furthermore, the results of our real-time PCR study provide strong evidence that the she-camel mastitis resulted in a significant inflammatory response.

The limitation of the present study should be acknowledged. First, a wide range of diverse camels should be used for obtaining a concrete conclusion. Second, different pathogens causing mastitis are not detailed. Third, other factors, such as environmental agents that influence immune responses and oxidative stress, were not studied, which could impact the results. Accordingly, such shortcomings should be considered in further investigations.

5. Conclusions

Single nucleotide variations (SNPs) were identified using PCR-DNA sequencing on healthy and mastitic she-camel samples in the genes for immunological (*OTUD3*, *TLR2*, *TLR4*, *STAB2*, *MBL2*, *TRAPPC9*, and *C4A*) and antioxidant (*CAT*, *SOD3*, *PRDX6*, *OXSRI*, *NDUFS6*, *SERP2*, and *STIP1*) indicators were found. These indicators' mRNA levels also varied across the groups of mastitis-affected and sound she-camels. These distinct functional variations present a significant opportunity to decrease the occurrence of mastitis by using genetic markers coupled with normal welfare during camel selection. Therefore, a sustainable technique of lowering the prevalence of disease and improving health in dromedary camels is genetic selection using gene polymorphisms for better innate immunity. Future treatments and selection for mastitis-resistant animals could be easier by the gene targets discovered here, therefore decreasing economic losses afforded by animal breeders. A large number of investigated markers, a wide range of diverse camels, and the study of etiological agents causing mastitis should be acknowledged by further studies.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agriculture13101909/s1> (Figure S1). GenBank gb|XM_031464240.1| and *OTUD3* marker (452-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S2). GenBank gb|MW557315.1| and *TLR2* marker (540-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S3). GenBank gb|XM_010998333.2| and *TLR4* marker (405-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S4). GenBank gb|XM_031462916.1| and *STAB2* marker (498-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S5). GenBank gb|XM_010975501.1| and *MBL2* marker (389-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S6). GenBank gb|XM_031439680.1| and *TRAPPC9* marker (423-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S7). GenBank gb|XM_031435000.1| and *C4A* marker (444-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S8). GenBank gb|XM_011000575.2| and *CAT* marker (540-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S9). GenBank gb|XM_010963047.2| and *SOD3* marker (458-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S10). GenBank gb|XM_010984536.2| and *PRDX6* marker (471-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S11). GenBank gb|XM_045509732.1| and *OXSRI* marker (420-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S12). GenBank gb|XM_031442846.1| and *NDUFS6* marker (303-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S13). GenBank gb|XM_031466025.1| and *SERP2* marker (373-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S14). GenBank gb|XM_010994738.2| and *STIP1* marker (442-bp) sequences were used to analyse nitrogenous bases matching for DNA in camels with and without mastitis (Figure S15). GenBank gb|XM_031464240.1| and *OTUD3* marker (452-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S16). GenBank gb|MW557315.1| and *TLR2* marker (540-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S17). GenBank gb|XM_010998333.2| and *TLR4* marker (405-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S18). GenBank gb|XM_031462916.1| and *STAB2* marker (498-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S19).

GenBank gb|XM_010975501.1| and *MBL2* marker (389-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S20). GenBank gb|XM_031439680.1| and *TRAPP9* marker (423-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S21). GenBank gb|XM_031435000.1| and *C4A* marker (444-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S22). GenBank gb|XM_011000575.2| and *CAT* marker (540-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S23). GenBank gb|XM_010963047.2| and *SOD3* marker (458-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S24). GenBank gb|XM_010984536.2| and *PRDX6* marker (471-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S25). GenBank gb|XM_045509732.1| and *OXSRI* marker (420-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S26). GenBank gb|XM_031442846.1| and *NDUFS6* marker (303-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S27). GenBank gb|XM_031466025.1| and *SERP2* marker (373-bp) were used to analyse amino acid matching in camels with and without mastitis (Figure S28). GenBank gb|XM_010994738.2| and *ST1P1* marker (442-bp) were used to analyse amino acid matching in camels with and without mastitis.

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Data Availability Statement: Upon justifiable demand, the supportive data for the findings of the study will be given by the relevant author.

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