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The genetic polymorphisms of the *Visfatin* gene and its association with milk production, its components, and some reproductive traits in local Iraqi goats

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Abstract

Objective

Visfatin, or Nicotinamide Phosphoribosyltransferase (NAMPT), is a multifunctional adipokine. This molecule plays a role intracellularly by maintaining the metabolism of nicotinamide adenine dinucleotide (NAD^+). In addition, this molecule also functions extracellularly as an important regulator of metabolic and reproductive processes. Although this molecule has high biological importance, the genetic diversity of *Visfatin* in goats and its relationship with production traits have not been fully and accurately investigated and identified. Thus, the aim of the present study was to examine two single-nucleotide polymorphisms (SNPs) in the *Visfatin* gene of local Iraqi goat breeds and study their associations with milk yield, milk composition, and reproductive performance.

Materials and methods

Blood samples were taken from forty-six clinically healthy does, which were representative of semi-intensively managed herds. Genomic DNA was extracted and two regions of the gene were amplified, a 350 bp region from exon 2 and an 825 bp promoter region that contained position -137. These were subsequently genotyped using PCR-RFLP with HinfI for the exon-2 SNP and direct Sanger sequencing for the promoter SNP. Allele and genotype frequencies were calculated and Hardy-Weinberg equilibrium (HWE) was analyzed. General linear models were performed followed by one-way ANOVA and Tukey's test to analyze associations with productive traits.

Results

Both loci were polymorphic and met Hardy-Weinberg expectations. The exon-2 A→G mutation produced three genotypes (AA, AB, BB), with AA predominating and conferring significantly higher daily milk yield and increased milk fat and protein percentages, along with earlier age at first kidding, shorter kidding interval, and larger litter size ($P < 0.05$). The promoter -137 C→T mutation resulted in the genotypes CC, CT, and TT; the TT animals displayed similarly beneficial milk and reproductive traits, while lactose percentage was not impacted.

Conclusion

Collectively, these results indicate that both structural and regulatory variants of *Visfatin* have independent and additive effects on milk production and reproductive efficiency. The relatively high frequencies of the favorable A and T alleles underscore the *Visfatin* gene as an excellent candidate for marker-assisted selection to improve dairy and reproductive performance in indigenous Iraqi goat populations.

Keywords: genetic polymorphism, Iraqi goats, milk composition, reproductive performance

Paper Type: Research Paper.

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Introduction

One of the most basic and important pillars of livestock farming in many developing regions of the world is goat farming (Mohammadabadi et al., 2022). This is especially important in Iraq, where indigenous breeds have historically played an important role in providing milk, meat, and fiber, while also showing remarkable adaptability to harsh and variable climatic conditions. The importance of Iraqi goats is due to their high tolerance to heat stress, good resistance to endemic diseases, and high survival ability with limited food resources. However, in terms of milk production and reproductive efficiency, these goats are still less productive than improved dairy breeds available in other countries. Therefore, reducing the gap in these critical traits that improve the rural economy and sustainable food security has been a priority for researchers, breeders and policy makers (Mohammadabadi et al., 2024). One of the most relied upon methods to assist in improving traits of production and fertility, will be the identification and utilization of genetic markers (Noori et al., 2017). One genetic marker of interest for production and reproductive traits is the *Visfatin* gene (NAMPT), due to its key role in the dual role of energy metabolism and reproduction. *Visfatin* is known as a multifunctional protein because it has both intracellular and extracellular roles. The conversion of nicotinamide to nicotinamide mononucleotide is an essential step in the NAD^+ recycling pathway within the cell. This step is catalyzed by visfatin. Thus, it contributes to the maintenance of cellular redox balance and energy metabolism (Fukuhara et al., 2005). In addition to its enzymatic role, *Visfatin* can enter the circulation as an adipokine and exert hormone-like effects on multiple target tissues. *Visfatin* also acts as a key link and regulator between downstream metabolism and systemic physiology of the body. Thus, it plays a role in glucose homeostasis, regulation of immune responses, and insulin-like functions (Luk et al., 2008). In bovine mammary cells, for example, *Visfatin* is upregulated through cAMP-mediated signaling pathways supporting directly milk synthesis and the energetics of continued milk synthesis (Yonezawa et al., 2006). *Visfatin* is also related to reproductive physiology in addition to metabolic effects. Studies in cattle and buffalo demonstrate that *Visfatin* promotes IGF-1 signaling in granulosa cells, positively regulates steroid hormone secretion, and enhances follicular development which influences ovarian function and potential fertility outcomes (Reverchon et al., 2016; Thakre et al., 2021). This evidence shows *Visfatin* is involved in both metabolic regulation and reproductive physiology that support research opportunities for genetic studies in dairy animals. The extensive family of adipokines, including leptin, adiponectin, resistin, chemerin, and visfatin, are increasingly acting as mediators of the link between energy homeostasis and reproductive function. Adipokines are mainly secreted by adipose tissue. However, their receptors and downstream signaling pathways are also expressed in reproductive tissues such as the ovary, uterus, and pituitary. Therefore, these molecules can act as communication channels between the body's energy reserves and reproductive capacity. Therefore, they can ensure that the reproductive process only occurs under conditions of energy adequacy. *Visfatin* has a special place among this family. *Visfatin* simultaneously regulates metabolic homeostasis and steroidogenesis. Therefore, it can coordinate and synchronize these two key axes in livestock production. Some recent reviews have shown that expression of multiple adipokines in ovarian follicles and the corpus luteum has the ability to directly alter the secretion of estrogens and progesterones and modify follicle maturation, ovulation, and maintenance of pregnancy (Al-Thuwaini, 2024). These functional examples reinforce the emphasis on looking at genetic variants of *Visfatin* in the goat. Candidate genes with genetic polymorphisms have a long history of investigating as a means of improving productivity in livestock species (Askari et al., 2011). For example, casein genes are widely accepted genetic markers that affect milk protein content and cheese-making properties in goats and sheep (Dettori et al., 2023). Likewise, polymorphisms in genes related to adipokines have been associated with production traits in cattle, pigs, and small ruminants. For instance, the *Visfatin* gene has been correlated with meat quality traits in pigs, providing early evidence that polymorphisms in this gene are likely to affect economically relevant traits (Zrustova et al., 2009). Recently, investigations into *Visfatin* in ruminant species have begun, with some evidence of relationships with reproductive efficiency as it relates to variations in gene expression, with studies focusing on goats limited (Shokrollahi, 2024). Other names for *Visfatin* include nicotinamide phosphoribosyltransferase (NAMPT) and pre-B-cell colony-enhancing factor (PBEF). *Visfatin* is a key molecule linking cellular energy metabolism with endocrine and reproductive regulation. *Visfatin* acts as a rate-limiting enzyme in the NAD^+ recycling pathway, catalyzing the conversion of nicotinamide to nicotinamide mononucleotide. In this way, *Visfatin* directly affects the level of NAD^+ in tissues. This pathway plays a pivotal role in cellular redox balance and, as a result, regulates mitochondrial function and sirtuin activity (Fukuhara et al., 2005; Revollo et al., 2007). *Visfatin*'s lenses of such biochemical function with hormone-like potential positions the molecule at the crossroads of metabolism, immunity, and reproduction. In productive ruminants, where lactation and fertility depend on energy status, *Visfatin* has been increasingly researched as candidate genes to influence economically important phenotypes (Luk et al., 2008). Studies utilizing bovine models have provided some of the earliest preliminary evidence of the physiological role for *Visfatin* in lactation. *Visfatin* has been shown to be localized to mammary epithelial cells and detected in milk, indicating that its pattern of expression is related to cAMP-dependent pathways which suggest a direct role in udder secretory and metabolic programs (Yonezawa et al., 2006). In cultured bovine granulosa cells, *Visfatin* promoted steroidogenesis in the presence or absence of IGF-1, enhanced signaling through the IGF-1 receptor, and was seemingly responsible for some SIRT1-mediated effects, a particular NAD^+-dependent deacetylase with connections to metabolic status (Reverchon et al., 2016). These mechanistic concepts offer a biological rational to expect associations between *Visfatin* polymorphisms and reproductive traits including age at first kidding, inter-kidding intervals, and litter size in caprine systems. Reviews of ruminant physiology show that adipokines are expressed in the ovary, testes, and hypothalamic-pituitary axis, where they modulate steroidogenesis and influence oocyte maturation, follicular dynamics, and luteal function (Al-Thuwaini, 2024). Reproduction proceeds efficiently only when energy status is adequate and correctly interpreted by endocrine tissues, and visfatin, by virtue of its combined enzymatic and hormonal roles, is ideally placed to act at this interface. Casein genes such as *CSN1S1*, *CSN2*, *CSN1S2*, and *CSN3* are well-established determinants of milk protein fractions and have been repeatedly associated with milk yield, fat and protein content, total solids, and milk energy value across dairy breeds (Dettori et al., 2023). Recent genomic surveys of goat milk composition continue to highlight pathway genes and regulatory regions, underlining the potential of integrating mechanistic candidates like *Visfatin* into broader selection indices (Yang et al., 2024).

Within goats, literature directly addressing *Visfatin* genotypes remains sparse compared with cattle and buffalo, but the broader adipokine field provides clear precedents. Studies in Egyptian Zaraibi goats, for example, link polymorphisms in *PRLR*, *IGF1*, and *LEP* to milk production and litter size, demonstrating that endocrine and metabolic genes can indeed influence both dairy and reproductive outcomes in caprine populations (El-Shorbagy et al., 2022). More generally, candidate-gene studies conducted in goats have involved the characterization of lipid-metabolism genes such as *ACACA*, *LPL*, and *SCD*, as well as components of secretory pathways such as BTN1A1 in association with yield, fat, protein and somatic cell traits, and as models for the current work (Brzáková et al., 2021). Evidence from non-ruminant livestock adds to this rationale. In pigs, early mapping and association studies identified NAMPT polymorphisms and explored correlations with carcass and meat-quality traits, demonstrating the feasibility of reliably genotyping this locus and implicating it in growth and tissue metabolism (Cepica et al., 2010). Although the production traits differ between pigs and dairy goats, such cross-species convergence supports the hypothesis that functional variation in NAMPT can shape phenotypes controlled by metabolic pathways. For goat breeding programs, this type of "portable plausibility" matters in contexts where local genomic data are still emerging. *Visfatin* is ultimately mechanistically connected to phenotypes along multiple pathways. A way *Visfatin* acts is through the maintenance of NAD^+ pools, as *Visfatin* supports sirtuin-dependent changes in gene expression and metabolism; in the ovary, SIRT1 has been associated with steroidogenesis and follicular competency, with data from bovine granulosa cells (Reverchon et al., 2016). *Visfatin* is involved with immune metabolism, as an adipocytokine that acts in an inflammatory tone, and is therefore relevant to udder health and indirectly on milk quality and productivity under the stressors of pasture (Luk et al., 2008). The ecological context discusses the reason candidate genes such as visfatin, leptin, or IGF1-pathway components have sometimes had stronger associations to performance in indigenous breeds than when evaluated in dairy lines under intensive selection in dairy lines (El-Shorbagy et al., 2022; Avondo et al., 2019). More recent studies of goats used higher-throughput genotyping and targeted sequencing approaches to finely map variability of casein genes and other candidates, identifying intronic and promoter variants that regulate gene expression (Dettori et al., 2023). As functional genomics techniques propel discover in caprines, it has been possible to interrogate NAMPT promoter and enhancer regions, splice variants, and 3′ UTRs for polymorphisms that influence level of tissue-specific expression to some degree. Reviews of the genetics of milk composition in goats has argued that integrated functional annotations of polymorphic sites with association signals greatly enhance the biological meaning and practical application (Yang et al., 2024). Recent syntheses on participants of the adipokine biological systems, such as visfatin, apelin, and irisin, in farm animals, indicate that these peptides affect insulin sensitivity, vascularization, and steroid hormone production when subjected to nutritional state (Shokrollahi, 2024). In ruminants, the expression of these peptides and their respective receptors in granulosa, theca, and luteal cells establishes a systemic relationship where metabolic status regulates reproductive timing and quality. Within this system, visfatin's enzymatic control over NAD^+ demonstrates how *Visfatin* provides a 'lever' over sirtuin-dependent transcriptional programs, which may account for synchronized improvements to metrics of lactation and fertility (El-Shorbagy et al., 2022; Brzáková et al., 2021; Shokrollahi, 2024). Increasingly, recent goat genetics literature documents parity, age, season, and diet carefully, using appropriate statistical adjustment for the association analysis and include them in the discussion as a potential confounding factor (Dettori et al., 2023; Brzáková et al., 2021). Mechanistically, *Visfatin* regulates NAD^+ metabolism and in turn affects sirtuin modulation of energy and steroidogenic pathways; biochemically, *Visfatin* is produced in membranes of mammary epithelium and compartments of the ovary, signaling both autocrine, paracrine, and endocrine modes of action (Dakroub et al., 2020). In domesticated livestock, porcine studies have shown that NAMPT polymorphisms exist and can be correlated with performance traits (Suzuki et al., 2022); bovine research indicates, in major dairy breeds in particular, that functional effects can be found in mammary and ovarian cells; and goat candidate-gene studies suggest that endocrine and metabolic loci, including *Visfatin* (or NAMPT), can potentially impact both milk and reproduction. In conclusion, the next step is to fully document *Visfatin* polymorphisms, define allele and genotype frequencies at coding and promoter loci, and look for associations with milk yield, milk composition, and reproductive trait metrics in population comprising of under-researched indigenous livestock (e.g., local Iraqi goats), as dairy composite breeds are similarly under-researched (Yonezawa et al., 2006; Reverchon et al., 2016; El-Shorbagy et al., 2022; Dettori et al., 2023; Yang et al., 2024). Thus, the aim of the present study was to examine two single-nucleotide polymorphisms (SNPs) in the *Visfatin* gene of local Iraqi goat breeds and study their associations with milk yield, milk composition, and reproductive performance.

Materials and methods

This study was conducted on a total of 46 clinically healthy local Iraqi goats, representing a cross-section of smallholder herds maintained in rural areas under semi-intensive management conditions. Ethical approval for animal handling and sample collection was obtained from the institutional animal ethics committee prior to commencement. The study was designed to characterize two *Visfatin* (NAMPT) polymorphisms, a coding SNP in exon-2 (A→G) and a regulatory SNP in the promoter at -137 (C→T) and to examine, for each mutation independently, their associations with milk production traits and reproductive performance.

Animals and management: The goats were adult females aged 2 to 5, with production status ranging from first to third kidding. They were managed as semi-intensively as would be typical for central and southern Iraq, with daytime grazing and evening supplementation with barley, wheat bran, and hay. Animals had ad libitum access to clean water and mineral blocks. The health of goats was monitored by veterinary inspection, and only non-pregnant, clinically healthy animals and free from systemic disease were included. The body condition score was assessed to ensure sampled goats were representative of herd condition.

Sample size determination: The sample size (n = 46) reflected logistical feasibility and availability of animals and follows prior caprine candidate-gene studies where comparable numbers were sufficient to detect genotype-phenotype associations under strong biological priors. The present cohort provides foundational estimates of allele/genotype frequencies and effect directions for both loci, supporting future, larger multi-herd validations.

Milk data collection: Milk characteristics were assessed over a standardized lactation period. Daily milk yields were taken at morning milking with calibrated graduated cylinders and following the same methods each day; weekly average milk yield per animal was calculated to minimize day-to-day variability. Milk composition was determined bi-weekly from frozen milk samples transported at 4 °C; fat (%) was determined on bi-weekly samples using the Gerber method; protein (%) was determined using the Kjeldahl method; and lactose (%) was assessed with a spectrophotometric assay.

Reproductive trait recording: Age at first calving (AFK, in months), intercalation interval (KI, in days), and litter size (LS, number of kids per calving) were the reproductive traits investigated in this study. Information on these traits was collected from farm records and direct observations. These standard indicators comprised the reproductive trait panel for genotype-trait analyses at each locus.

Blood sampling and DNA extraction: From each goat, 5 mL of jugular blood were collected aseptically into EDTA vacutainers. These samples transported on ice to laboratory and stored at −20 °C until analysis. A commercial kit (e.g., Qiagen, Germany) following the manufacturer's protocol was used to extract genomic DNA. DNA quality was assessed by 1% agarose gel electrophoresis and A260/280 spectrophotometry (NanoDrop). High-molecular-weight bands confirmed integrity. After that working solutions were prepared for subsequent analysis.

PCR amplification of *Visfatin* gene: Two genomic regions were amplified. The first region was exon 2 fragment (~350 bp; coding SNP A→G). Primers (designed from caprine NAMPT) forward 5′-AGCCTTCTGATGGTGACTGC-3′, and reverse 5′-TCCAGGACTTGGGTTTCTGA-3′ were used. Reactions (25 µL) contained ~50 ng DNA, 10 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1 U Taq, and 1X buffer. Condition for amplifying were: Initial denaturation with 1 cycle performed at 95 °C for 5 min, step 2 with 35 cycles; denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, synthesis at 72 °C for 45 s; and final synthesis at 72 °C for 10 min. The second region was promoter fragment (~420 bp spanning -137; regulatory SNP C→T). Primers were designed from the 5′ upstream NAMPT region flanking -137 (amplicon ~825 bp). PCR reactions were performed for this region as described above for exon 2. The annealing temperature of the primers was adjusted based on their melting temperature (Tm) in the range of 60-58°C. PCR products were visualized on 1.5%

agarose gels stained with ethidium bromide. Then gels were imaged under UV to confirm single, specific bands of expected size (Figure 1).

Genotyping of *Visfatin* gene: The Exon-2 A→G (*Hinf*I PCR-RFLP with Sanger confirmation). Ten microliters of exon-2 amplicon were digested with *Hinf*I (1 U; 1X buffer; 37 °C, 4 h). Digests were separated on 3% agarose and visualized under UV. Distinct banding patterns defined AA, AB, BB genotypes according to the presence/absence of the HinfI restriction site created by the A→G substitution. To verify the causal SNP and RFLP patterns, a subset of samples across genotypes was Sanger-sequenced bidirectionally. Promoter -137 C→T (direct Sanger sequencing). The ~825 bp promoter amplicon was purified and Sanger-sequenced. Chromatograms were inspected in Chromas, and sequences aligned to the caprine NAMPT reference to score the -137 site as CC, CT, or TT. Where needed, ambiguous traces were re-sequenced from the opposite strand. Quality control and population checks. For each locus, genotype and allele frequencies were estimated by direct counting. Hardy-Weinberg equilibrium (HWE) was evaluated using χ^2 tests (biallelic, codominant model). Loci were analyzed independently (no haplotype phase inference) given the sample size.

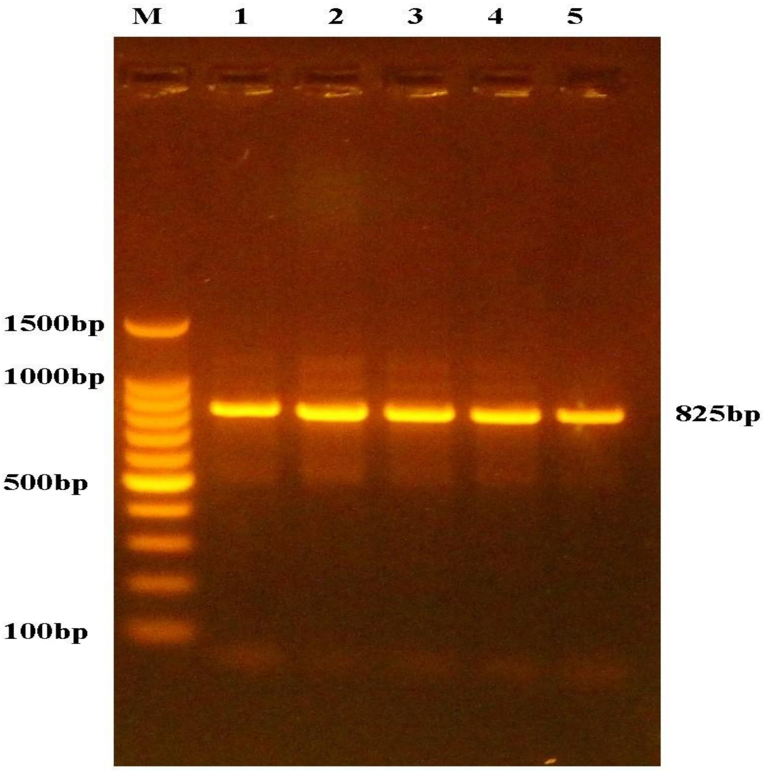

Figure 1. The results of PCR amplification for *Visfatin* exon-2 on agarose gel local Iraqi goat. Lanes 1-5 are amplified fragments (825bp). Lane M is a 100 bp DNA ladder

Statistical analyses: Data analyses were performed in SPSS v25 (IBM, USA). Two predefined panels were used for each trait separately to examine and analyze each mutation (exon-2 A→G; promoter -137 C→T). The first panel was milk panel and contained daily milk yield (L/day), fat (%), protein (%), and lactose (%). The second panel was reproductive panel and included AFK (months), KI (days), and LS (kids). For milk traits, a general linear model (GLM) was fitted:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where Yij is the trait value for individual with genotype ij, μ is the overall mean, Gi is the fixed effect of genotype (AA/AB/BB for exon-2 and CC/CT/TT for promoter), and eij is the residual error. For reproductive traits (AFK, KI, LS), one-way ANOVA was applied with genotype as the fixed factor. When main effects were significant, Tukey's HSD was used for pairwise comparisons. Significance was declared at $P<0.05$ (significant) and $P < 0.01$ (highly significant). Results are reported as mean ± SE.

Results

Two independent single nucleotide polymorphisms (SNPs) with functional significance were identified in 46 native Iraqi goats studied for the *Visfatin* gene. The first SNP was an A→G substitution in exon 2 of the coding region of the gene, and the second SNP was a C→T substitution at position −137 of the promoter region. The association between the genotypes of the two genetic polymorphisms with milk production traits, namely daily production, fat, protein, and lactose, was investigated. In addition, the association of these genotypes with reproductive performance indicators including age at first calving, intercalation interval, and litter size was also analyzed.

Allele and genotype frequencies for exon-2 (A→G polymorphism): The PCR product for this region was a 350 bp fragment that was observed in all studied samples. This result indicated that the desired fragment was successfully amplified. Digestion of the PCR products with HinfI enzyme led to the identification of three genotypes AA, AB, and BB. Sequencing by the Sanger method also confirmed that the A→G substitution had occurred. As shown in Table 1, the frequency of the AA genotype was the highest of all genotypes (65.2%) and the BB genotype had the lowest frequency (8.7%). The allelic frequencies were calculated as A = 0.78 and B = 0.22, respectively. The chi-square test showed no significant deviation from Hardy-Weinberg equilibrium ($P > 0.05$). This could indicate the absence of forces disrupting the equilibrium, such as non-random mating.

Milk production traits and exon-2: Associations between A→G genotypes and milk performance are summarized in Table 2. Goats carrying the AA genotype produced the highest mean daily milk yield (3.8 ± 0.40 L/day), significantly exceeding AB (3.1 ± 0.35 L/day) and BB (2.7 ± 0.30 L/day) animals ($P < 0.05$). The AA group also exhibited significantly greater milk fat (4.1 ± 0.08 %) and protein (3.5 ± 0.06 %), while lactose content remained relatively constant across genotypes (4.68 ± 0.05 %, 4.66 ± 0.04 %, and 4.64 ± 0.05 % for AA, AB, and BB, respectively) (P > 0.05). These findings indicate that the A allele has a positive effect on both the quantity and the protein-fat quality of milk without altering lactose concentration (Figure 2).

Table 1. Allele and genotype frequencies of the *Visfatin* exon-2 A→G locus (n = 46)

Genotype	Frequency (%)	Number of Animals (n)
AA	65.2	30
AB	26.1	12
BB	8.7	4
Allele	Frequency	
A	0.78	
B	0.22	

Table 2. Association between *Visfatin* genotypes and milk production traits in local Iraqi goats

Genotype	N	Milk Yield (L/day)	Fat (%)	Protein (%)	Lactose (%)
AA	30	3.8 ± 0.40a	4.1 ± 0.08a	3.5 ± 0.06a	4.68 ± 0.05
AB	12	3.1 ± 0.35b	3.8 ± 0.07b	3.1 ± 0.05b	4.66 ± 0.04
BB	4	2.7 ± 0.30c	3.6 ± 0.06c	2.8 ± 0.04c	4.64 ± 0.05

Values are means ± SE. Different superscripts within a column differ significantly at P < 0.05.

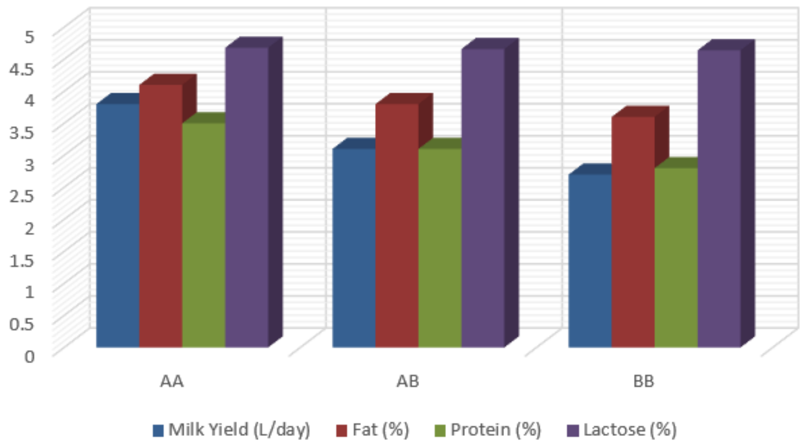

Figure 2. Mean (±SE) milk production and composition by *Visfatin* exon-2 genotype. Left to right within each group; milk yield (L/day), fat (%), protein (%), and lactose (%)

Reproductive traits and for exon-2: Genotypic variation at the A→G locus was likewise associated with reproductive performance (Table 3). AA goats reached first kidding earliest (14.5 ± 0.6 months), had the shortest kidding interval (285 ± 9 days), and produced the largest litter size (2.1 ± 0.2 kids). All significantly superior to the AB and BB genotypes (P < 0.05). These results demonstrate that the AA genotype confers advantages not only for lactation but also for reproductive efficiency. It underscores its potential value as a selection target (Figure 3).

Table 3. Association between exon-2 A→G genotypes and reproductive traits

Genotype	Age at First Kidding (months)	Kidding Interval (days)	Litter Size (kids)
AA	14.5 ± 0.6a	285 ± 9a	2.1 ± 0.2a
AB	15.9 ± 0.7b	298 ± 11b	1.8 ± 0.2b
BB	16.8 ± 0.8b	310 ± 12b	1.6± 0.1b

Values are means ± SE. Different superscripts within a column differ significantly at $P < 0.05$.

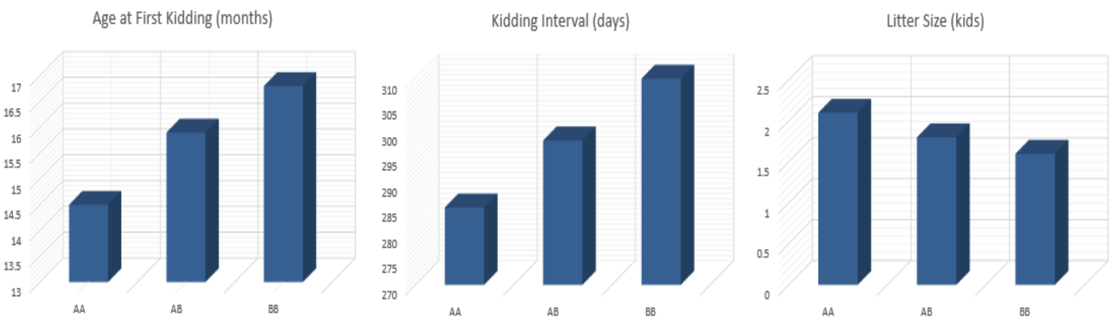

Figure 3. Mean (±SE) reproductive traits by *Visfatin* exon 2 A/G genotype. Left to right: age at first kidding (months), kidding interval (days), and litter size (kids). AA goats show earlier first kidding, shorter interval, and larger litters compared to AB and BB

Allele and genotype frequencies for promoter-137 (C→T substitution): The PCR product for this region was an 825 bp promoter fragment that was observed in all studied samples. This fragment was also sequenced and revealed a C→T substitution at position -137 (Figure 4). Three genotypes CC, CT, and TT were detected (Table 4). CT genotype was the most frequent (43.5 %) and the frequency of TT was the lowest (17.4 %). Corresponding allele frequencies were C = 0.61 and T = 0.39. The population for this SNP was in Hardy-Weinberg equilibrium ($\chi^2 = 0.41$, df = 1, $P = 0.52$). This could indicate the absence of forces disrupting the equilibrium, such as non-random mating.

Table 4. Allele and genotype frequencies of the *Visfatin* promoter -137 C→T locus (n = 46)

Genotype	Frequency (%)	Number of goats (n)
CC	39.1	18
CT	43.5	20
TT	17.4	8
Allele	**Frequency**	
C	0.61	
T	0.39	

Milk production traits and promoter-137 (C→T substitution): The promoter polymorphism significantly affected milk traits (Table 5). Goats carrying the TT genotype achieved the highest mean daily milk yield (3.65 ± 0.15 L/day), which was significantly greater than CC animals and slightly higher than CT animals ($P < 0.05$). TT goats also exhibited the highest milk fat (4.14 ± 0.07 %) and protein (3.47 ± 0.05 %) contents, while lactose levels remained consistent across genotypes (Figure 5). These findings indicate that the T allele in the promoter region enhances both yield and key quality parameters of milk.

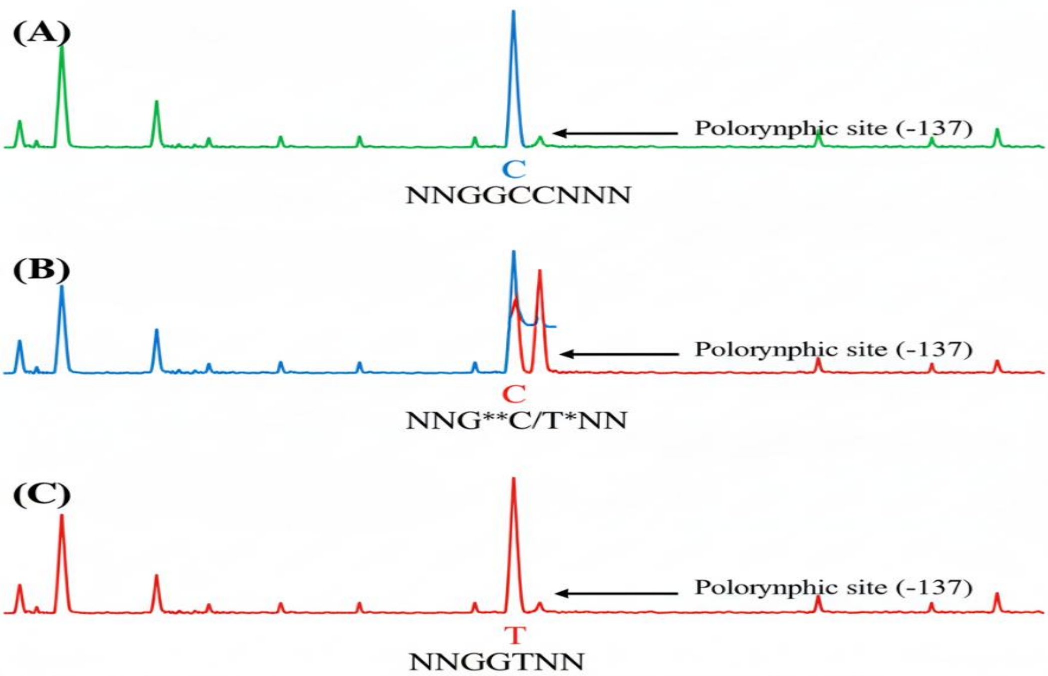

Figure 4. Sequencing results for promoter -137 of *Visfatin* gene using Sanger sequencing chromatograms. The arrow in each panel points to the polymorphic site at position -137. (A) The homozygous CC genotype, indicated by a single blue peak for cytosine. (B) The heterozygous CT genotype, showing overlapping blue (cytosine) and red (thymine) peaks. (C) The homozygous TT genotype, indicated by a single red peak for thymine

Table 5. Association between promoter -137 C→T genotypes and milk production traits

Genotype	Milk Yield (L/day)	Fat (%)	Protein (%)	Lactose (%)
CC	3.20 ± 0.12^{b}	3.75 ± 0.06^{b}	3.08 ± 0.05^{b}	4.70 ± 0.04
CT	3.46 ± 0.11^{ab}	3.98 ± 0.05^{a}	3.32 ± 0.04^{a}	4.71 ± 0.04
TT	3.65 ± 0.15^{a}	4.14 ± 0.07^{a}	3.47 ± 0.05^{a}	4.72 ± 0.05

Values are means ± SE. Different superscripts within a column differ significantly at $P < 0.05$.

Reproductive traits and promoter-137 (C→T substitution): Promoter genotypes were likewise associated with reproductive performance (Table 6). TT goats reached first kidding earliest (14.7 ± 0.6 months), had the shortest kidding interval (287 ± 10 days), and produced the largest litter size (2.03 ± 0.13 kids). CT animals displayed intermediate values, while CC goats consistently exhibited the least favorable reproductive outcomes (P<0.05). These results highlight a beneficial role of the T allele in reproductive efficiency (Figure 6).

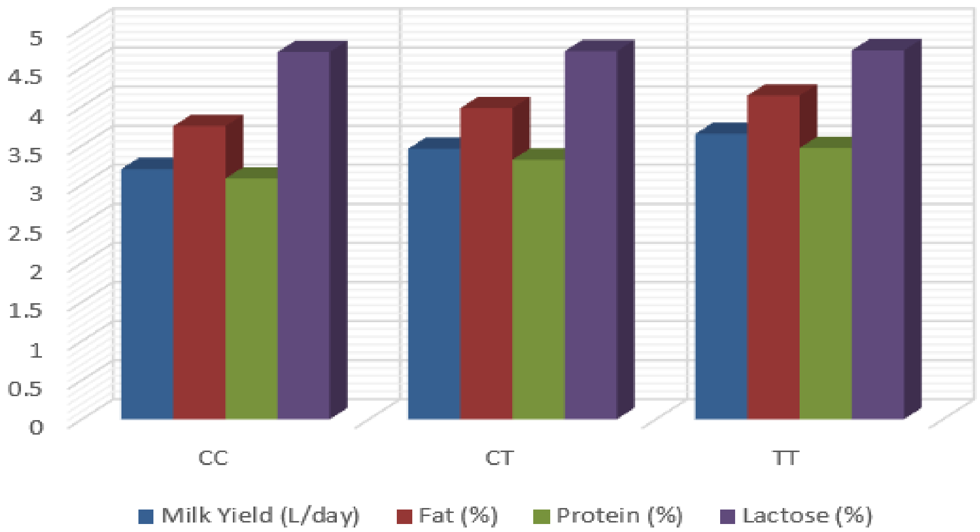

Figure 5. Mean (±SE) milk production and composition by *Visfatin* −137 C/T promoter genotype: left to right within each group - milk yield (L/day), fat (%), protein (%), and lactose (%). TT goats exhibit the highest milk yield and nutrient percentages compared to CC and CT

Table 6. Association between promoter −137 C→T genotypes and reproductive traits

Genotype	Age at First Kidding (months)	Kidding Interval (days)	Litter Size (kids)
CC	15.9 ± 0.6^{b}	302 ± 10^{b}	1.74 ± 0.10^{b}
CT	15.2 ± 0.5^{ab}	293 ± 9^{ab}	1.92 ± 0.11^{ab}
TT	14.7 ± 0.6^{a}	287 ± 10^{a}	2.03 ± 0.13^{a}

Values are means ± SE. Different superscripts within a column differ significantly at P < 0.05.

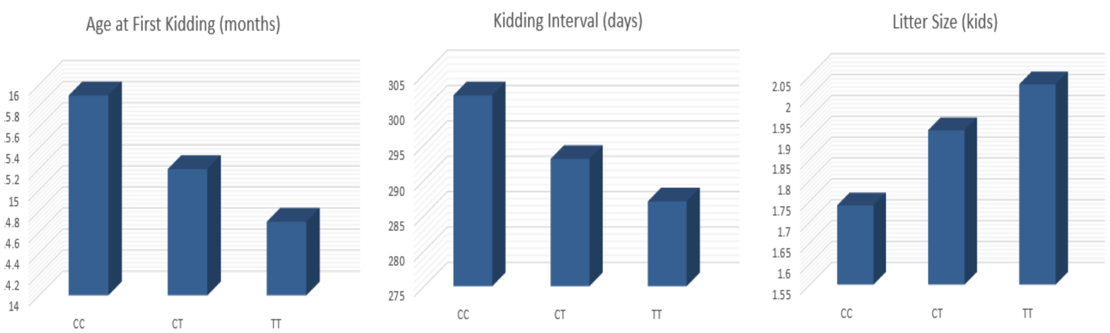

Figure 6. Mean (±SE) reproductive traits by *Visfatin* −137 C/T genotype: left - age at first kidding (months), middle - kidding interval (days), right - litter size (kids). TT goats show earlier first kidding, shorter interval, and larger litters than CC and CT

Discussion

The present study provides the first targeted evidence that the *Visfatin* gene in local Iraqi goats' harbors at least two functional polymorphisms, an exon-2 A→G substitution and a promoter −137 C→T substitution. Both of them are significantly associated with milk production and reproductive performance. The combined data indicate that coding and regulatory variation within *Visfatin* contributes to economically important phenotypes. It confirms its value as a candidate gene for marker-assisted selection. For mutation 1 (exon-2 A→G), three genotypes (AA, AB, BB) were identified, with AA predominating (65.2 %) and the A allele showing a frequency of 0.78. Goats carrying the AA genotype expressed superior lactational traits, including a mean daily milk yield of 3.8 ± 0.40 L, as well as higher milk fat (4.1 ± 0.08 %) and protein (3.5 ± 0.06 %) compared with AB and BB animals. The lactose percentage (~4.7 %) did not change, suggesting that *Visfatin* mainly affects protein-fat metabolism and its secretory ability rather than carbohydrate synthesis. There were more similarities with reproductive parameters. AA goats were younger at first kidding, had smaller kidding intervals, and had larger litters. These results are in line with mechanistic studies showing that *Visfatin* enhances granulosa-cell steroidogenesis, estradiol and progesterone secretion, and follicle formation (Reverchon et al., 2016; Thakre et al., 2021). Moreover, these results agree with the other adipokine paradigm, in which the endocrine metabolic hormones govern energy balance and reproduction (Al-Thuwaini, 2024). The second mutation (C→T at position −137 of the promoter region) adds an important regulatory dimension to the findings. The genotype frequencies were CT (43.5%), CC (39.1%) and TT (17.4%), respectively. Allele frequencies of C = 0.61 and T = 0.39 were obtained. This indicates that the population is in Hardy-Weinberg equilibrium and could indicate stable genetic segregation at this locus. At this locus, goats with the TT genotype consistently showed the most favorable phenotypes. This genotype had the highest daily milk production (3.65 ± 0.15 liters per day) and their fat (4.14 ± 0.07%) and protein (3.47 ± 0.05%) contents were significantly higher. Reproductively, TT animals reached first kidding earliest and maintained the shortest inter-kidding intervals and largest litter sizes. These data imply that regulatory variation at the promoter enhances *Visfatin* gene expression, strengthening metabolic-reproductive crosstalk and complementing the structural effects of the exon-2 mutation. The combined analyses of these two loci demonstrate how both cis-acting regulatory modifications and coding substitutions can impact productivity in a concerted fashion. They maintain a separate Hardy-Weinberg equilibrium, indicating that the favorable alleles (A and T) are not tightly linked and can each be selected for independently or in concert with the goal of increasing the respective genotype frequency. Moreover, both polymorphisms act in polygenic, environmental response traits (milk yield and reproductive efficiency, respectively). Therefore, while *Visfatin* is clearly contributing to phenotypic variance, it is likely acting in concert with other candidate genes such as the casein cluster (milk composition) or leptin and adiponectin (Dettori et al., 2023; Avondo et al., 2019)), as it relates to their respective traits. Subsequent breeding programs will utilize multi-gene selection panels that incorporate *Visfatin* along with these previously described markers as discussed in this review. These results have tangible implications for goat production in Iraq. Integrating marker-assisted selection (MAS) will help breeding programs identify better breeding stock earlier stages of the breeding program, decrease generation intervals, and ultimately, increase herd performance beyond improvement purely based on phenotypic selection. Additionally, the relatively high frequency of the alleles associated with improved performance (A and T) implies that a traditional farmer-based introduction of these favorable mutations into the population may have already occurred. Incorporating *Visfatin* genotyping formally into our breeding programs has the potential to accelerate genetic gain while still making selection for local breeds that are adapted to the challenging local environment. However, there are some limitations to mention. The current sample size(n=46) provided sufficient power to report initial associations, but it is doubtful that the sample fully represents the genetic variation of Iraqi goat populations. To confirm these associations and also to evaluate gene-environment interactions, larger multi-region studies should be conducted in various dimensions. In this study, only two regions of the genome, namely an exon and a promoter region, were investigated. While the use of whole genome sequencing, haplotype analysis, and genome-wide association studies (GWAS) can lead to the identification of other functionally effective variants. Finally, functional assays to assess expression of *Visfatin* in mammary and ovarian tissue would strengthen causality. Despite these caveats, the present research extends the *Visfatin* literature beyond cattle, buffalo, and pigs to indigenous goats, underscoring visfatin's cross-species relevance as a metabolic-reproductive regulator. The evidence that two distinct mutations each exert significant but complementary effects provides a solid molecular foundation for next-generation goat breeding strategies aimed at both dairy and reproductive improvement.

Conclusion: This study characterized two polymorphic sites in the *Visfatin* gene, an exon 2 A→G substitution and a promoter −137 C→T substitution in 46 local Iraqi goats. It showed that these sites were significantly associated with milk yield, milk composition and reproductive efficiency. The AA genotype at the exon-2 A→G locus was the predominate and consistently yielded the highest levels of milk production and reproductive performance, including higher percentages of milk fat and protein and earlier reproductive maturity. The TT genotype at the promoter −137 C→T locus produced a similar plan increased milk yield and composition as well as improved reproductive indices, though the effect sizes were slightly lower than the coding mutation described above. Both loci were in Hardy-Weinberg equilibrium. It can indicate stable inheritance and suitability for selection. In summary, these findings suggest that structural and regulatory polymorphisms in *Visfatin* can be used as molecular markers to improve goat production. The integration of these markers into breeding programs represents a useful and practical method for increased milk production and fertility without sacrificing the advantages of indigenous herds. This work therefore connects Iraqi goat breeding with the emerging capacity for genomic selection, and presents an important model for other small ruminant populations.

Author's contributions

Bashar Fadel expanded the abstract, results section, and key findings, assumed responsibility for clarifying the significance of the study and highlighting its main benefits, collected the technical data, conceptualized the main idea, organized the overall structure of the manuscript, prepared the figures and illustrations, and contributed to drafting the initial version of the manuscript. Buthaina Jasim expanded the abstract, results, and main key findings, thereby enhancing the clarity of the scientific presentation of the study. Ayat Sufyan further developed the main idea of the study and managed the literature review. All authors reviewed and approved the final version of the manuscript before submission.

Data availability statement

The data for the animals were obtained from their specific records, and no new data were generated or taken from other sources.

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Ethical considerations

A 3 mL blood sample was collected from the jugular vein of each experimental animal under the direct supervision of an experienced veterinarian, ensuring no harm or distress was inflicted on any animal throughout the procedure, which was conducted in a standardized and humane manner.

Conflict of interest

The authors declare no conflicts of interest.

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References

Al-Thuwaini, T. M. (2024). Adipokines and reproduction in ruminant livestock. Reviews in Agricultural Science, 12, 13-26. https://doi.org/10.7831/ras.12.0_13

Askari, N., Mohammadabadi, M., & Baghizadeh, A. (2011). ISSR markers for assessing DNA polymorphism and genetic characterization of cattle, goat and sheep populations. Iranian

Journal of Biotechnology, 9(3), 222-229. https://www.ijbiotech.com/article_7158.html

Avondo, M., Di Trana, A., Valenti, B., Criscione, A., Bordonaro, S., De Angelis, A., Giorgio, D., & Di Gregorio, P. (2019). Leptin gene polymorphism in goats fed with diet at different energy level: Effects on feed intake, milk traits, milk fatty acids composition, and metabolic state. Animals, 9(7), Article 424. https://doi.org/10.3390/ani9070424

Brzáková, M., Rychtářová, J., Čítek, J., & Sztankóová, Z. (2021). A candidate gene association study for economically important traits in Czech dairy goat breeds. Animals, 11(6), Article 1796. https://doi.org/10.3390/ani11061796

Čepica, S., Bartenschlager, H., Ovilo, C., Žrůstová, J., Masopust, M., Fernández, A., López, A., Knoll, A., Rohrer, G. A., Snelling, W. M., & Geldermann, H. (2010). Porcine NAMPT gene: Search for polymorphism, mapping and association studies. Animal Genetics, 41(6), 646-651. https://doi.org/10.1111/j.1365-2052.2010.02052.x

Dakroub, A., Nasser, S. A., Younis, N., Bhagani, H., Al-Dhaheri, Y., Pintus, G., Eid, A. A., El-Yazbi, A. F., & Eid, A. H. (2020). Visfatin: A possible role in cardiovasculo-metabolic disorders. Cells, 9(11), Article 2444. https://doi.org/10.3390/cells9112444

Dettori, M. L., Pazzola, M., Noce, A., Landi, V., & Vacca, G. M. (2023). Variations in casein genes are associated with milk protein and fat contents in Sarda goats (Capra hircus), with an important role of CSN1S2 for milk yield. Animals, 14(1), Article 56. https://doi.org/10.3390/ani14010056

El-Shorbagy, H. M., Abdel-Aal, E. S., Mohamed, S. A., & El-Ghor, A. A. (2022). Association of PRLR, IGF1, and LEP genes polymorphism with milk production and litter size in Egyptian Zaraibi goat. Tropical Animal Health and Production, 54(5), Article 321. https://doi.org/10.1007/s11250-022-03316-2

Fukuhara, A., Matsuda, M., Nishizawa, M., Segawa, K., Tanaka, M., Kishimoto, K., Matsuki, Y., Murakami, M., Ichisaka, T., Murakami, H., Watanabe, E., Takagi, T., Akiyoshi, M., Ohtsubo, T., Kihara, S., Yamashita, S., Makishima, M., Funahashi, T., Yamanaka, S., Hiramatsu, R., Matsuzawa, Y., & Shimomura, I. (2005). Visfatin: A protein secreted by visceral fat that mimics the effects of insulin. Science, 307(5708), 426-430. https://doi.org/10.1126/science.1097243

Luk, T., Malam, Z., & Marshall, J. C. (2008). Pre-B cell colony-enhancing factor (PBEF)/visfatin: A novel mediator of innate immunity. Journal of Leukocyte Biology, 83(4), 804-816. https://doi.org/10.1189/jlb.0807581

Mohammadabadi, M., Babenko, I., Borshch, O., Kalashnyk, O., Ievstafiieva, Y., & Buchkovska, V. (2024). Measuring the relative expression pattern of the UCP2 gene in different tissues of the Raini Cashmere goat. Agricultural Biotechnology Journal, 16(3), 317-332. https://doi.org/10.22103/jab.2024.24337.1627

Mohammadabadi, M., Kheyrodin, H., Latifi, A., & Babenko Ivanivna, O. (2022). mRNA expression profile of DNAH1 gene in testis tissue of Raini Cashmere goat. Agricultural

Biotechnology Journal, 14(3), 243-256. https://doi.org/10.22103/jab.2022.20199.1428

Noori, A. N., Behzadi, M. R. B., & Mohammadabadi, M. R. (2017). Expression pattern of Rheb gene in Jabal Barez Red goat. The Indian Journal of Animal Sciences, 87(11), 1375-1378. https://doi.org/10.56093/ijans.v87i11.75890

Reverchon, M., Rame, C., Bunel, A., Chen, W., Froment, P., & Dupont, J. (2016). *Visfatin* (NAMPT) improves in vitro IGF1-induced steroidogenesis and IGF1 receptor signaling through SIRT1 in bovine granulosa cells. Biology of Reproduction, 94(3), Article 54. https://doi.org/10.1095/biolreprod.115.134650

Revollo, J. R., Grimm, A. A., & Imai, S. (2007). The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/*Visfatin* in mammals. Current Opinion in Gastroenterology, 23(2), 164-170. https://doi.org/10.1097/MOG.0b013e32801b3c8f

Shokrollahi, B., Jang, S. S., Lee, H. J., Ahmad, H. I., Sesay, A. R., Ghazikhani Shad, A., Morammazi, S., & Abdelnour, S. A. (2024). Exploring the potential roles of apelin, visfatin, and irisin in energy regulation in farm animals: An overview. Frontiers in Veterinary Science, 11, Article 1435788. https://doi.org/10.3389/fvets.2024.1435788

Suzuki, K., Shinkai, H., Yoshioka, G., Matsumoto, T., Takenouchi, T., Tanaka, J., Shimizu, M., Kitazawa, H., & Uenishi, H. (2022). Polymorphisms in pattern recognition receptor genes are associated with respiratory disease severity in pig farms. Animals, 12(22), Article 3163. https://doi.org/10.3390/ani12223163

Thakre, A., Gupta, M., Magar, S. P., Bahiram, K. B., Sardar, V. M., Korde, J. P., Bonde, S. W., & Hyder, I. (2021). Transcriptional and translational abundance of *Visfatin* (NAMPT) in buffalo ovary during estrous cycle and its in vitro effect on steroidogenesis. Domestic Animal Endocrinology, 75, Article 106583. https://doi.org/10.1016/j.domaniend.2020.106583

Yang, X., Li, Q., Wang, Y., Wang, J., Hu, J., Ji, Z., & Chao, T. (2024). Research progress on genomic regions and candidate genes related to milk composition traits of dairy goats based on functional genomics: A narrative review. Genes, 15(10), Article 1341. https://doi.org/10.3390/genes15101341

Yonezawa, T., Haga, S., Kobayashi, Y., Takahashi, T., & Obara, Y. (2006). *Visfatin* is present in bovine mammary epithelial cells, lactating mammary gland and milk, and its expression is regulated by cAMP pathway. FEBS Letters, 580(28-29), 6635-6643. https://doi.org/10.1016/j.febslet.2006.11.014

Zrustova, J., Knoll, A., Urban, T., & Čepica, S. (2009). The *Visfatin* (NAMPT; PBEF1) gene polymorphisms and associations with meat performance traits in three pig breeds kept in the Czech Republic. Czech Journal of Animal Science, 54(10), 443-447. https://doi.org/10.17221/1673-CJAS

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چندریختی‌های ژنتیکی ژن ویسفاتین و ارتباط آن با تولید شیر، اجزای شیر و برخی صفات تولیدمثلی در بزهای بومی عراق

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چکیده

هدف: ویسفاتین یا نیکوتین‌آمید فسفوریبوزیل‌ترانسفراز (NAMPT) یک آدیپوکاین چندکاره است. این مولکول درون‌سلولی با حفظ متابولیسم نیکوتین‌آمید آدنین دی‌نوکلئوتید (NAD^+) نقش مهمی ایفا می‌کند. علاوه بر این، ویسفاتین در خارج از سلول نیز به‌عنوان یک تنظیم‌کننده مهم فرآیندهای متابولیکی و تولیدمثلی عمل می‌نماید. با وجود اهمیت زیستی بالای این مولکول، تنوع ژنتیکی ژن ویسفاتین در بزها و ارتباط آن با صفات تولیدی تاکنون به‌طور کامل و دقیق بررسی و شناسایی نشده است. ازاین‌رو، هدف از این مطالعه بررسی دو چندریختی تک‌نوکلئوتیدی (SNP) در ژن ویسفاتین بزهای بومی عراق و ارزیابی ارتباط آن‌ها با میزان تولید شیر، ترکیبات شیر و عملکرد تولیدمثلی بود.

مواد و روش‌ها: نمونه‌های خون از ۴۶ بز ماده سالم از نظر بالینی که نماینده گله‌هایی با سیستم پرورش نیمه‌متراکم بودند، جمع‌آوری شد. DNA ژنومی استخراج گردید و دو ناحیه از ژن شامل یک قطعه ۳۵۰ جفت‌بازی از اگزون ۲ و یک ناحیه پروموتری ۸۲۵ جفت‌بازی شامل موقعیت ‎-۱۳۷ تکثیر شد. سپس تعیین ژنوتیپ‌ها با استفاده از روش PCR-RFLP و آنزیم HinfI برای SNPاگزون ۲ و توالی‌یابی مستقیم به روش سنگر برای SNP ناحیه پروموتری انجام گرفت. فراوانی آلل‌ها و ژنوتیپ‌ها محاسبه شد و تعادل هاردی–واینبرگ (HWE) مورد بررسی قرار گرفت. برای تحلیل ارتباط با صفات تولیدی از مدل‌های خطی عمومی

استفاده شد و پس از آن آزمون تجزیه واریانس یک‌طرفه و آزمون توکی به کار رفت.

نتایج: هر دو جایگاه ژنی چندشکل بوده و در تعادل هاردی-واینبرگ بودند. جهش A→G در اگزون ۲ منجر به ایجاد سه ژنوتیپ AA، AB و BB شد که در این میان ژنوتیپ AA فراوانی بیشتری داشت و با افزایش معنی‌دار تولید روزانه شیر، درصد چربی و پروتئین شیر، سن کمتر در اولین زایش، فاصله کوتاه‌تر بین زایش‌ها و اندازه بزرگ‌تر زایش همراه بود ($P < 0.05$). جهش C→T در موقعیت -۱۳۷ ناحیه پروموتری نیز ژنوتیپ‌های CC، CT و TT را ایجاد کرد و حیوانات دارای ژنوتیپ TT صفات مطلوب مشابهی از نظر تولید شیر و عملکرد تولیدمثلی نشان دادند، در حالی که درصد لاکتوز شیر تحت تأثیر قرار نگرفت.

نتیجه‌گیری: در مجموع، نتایج این مطالعه نشان می‌دهد که هر دو نوع واریانت ساختاری و تنظیمی ژن ویسفاتین دارای اثرات مستقل و افزایشی بر تولید شیر و کارایی تولیدمثلی هستند. فراوانی نسبتاً بالای آلل‌های مطلوب A و T بیانگر آن است که ژن ویسفاتین می‌تواند به‌عنوان یک کاندیدای مناسب برای انتخاب به کمک نشانگرهای مولکولی به‌منظور بهبود عملکرد شیری و تولیدمثلی در جمعیت‌های بومی بز عراق مورد استفاده قرار گیرد.

واژه‌های کلیدی: بزهای عراقی، ترکیبات شیر، چندریختی ژنتیکی، عملکرد تولیدمثلی

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