Investigation of polymorphic variations in the *Alpha-lactalbumin* gene and their association analysis to milk characteristics in *Bubalus bubalis*

ABSTRACT

Bubalus bubalis is a critically important livestock species in many poor countries because of its invaluable contributions to numerous agricultural fields. Finding single nucleotide polymorphisms (SNPs) in exon 1 of the alpha-lactalbumin gene was the main goal of the current investigation. Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) assay and sequencing analysis were used to find out the SNPs. Statistical testing was carried out on the records ($p \leq 0.05$) using the Duncan post hoc test. The present study confirmed four SNPs in ALA gene. The ALA gene in the present study shows that homozygous genotype AA was significantly associated with FP. At the same time, genotype LA was associated with PP, and FY. The research emphasized the necessity of screening a broader range of buffaloes to identify genetic polymorphism and its correlation with crucial milk characteristics. This approach will establish a reliable foundation for marker-assisted selection that enables the selection of animals at a young age for breeding programs.

Keywords: Bubalus bubalis, ALA, PCR-SSCP, SNPs, Genotypes association

1. INTRODUCTION

Bubalus bubalis is a valuable livestock animal in some developing countries due to its valuable contribution to different agricultural sectors [1]. As a versatile animal with many uses, the buffalo is regarded as a genetic resource of exceptional value. It is used as a useful dairy animal that yields large quantities of milk and as a draught animal that significantly facilitates a variety of agricultural tasks [2]. The river buffalo produces 2,000 kg of milk annually, but the swamp buffalo only produces 500–600 kg. Species account for around 13% of the world's milk production [3]. Buffalo is far more significant and superior to domesticated cattle due to its strong ability to climate change, good

nutritional benefits, and disease resistance [4]. Each lactating animal breed has a genetic potential to contribute to specific milk properties [5]. Particularly in developing nations, it has an economic impact as a multipurpose animal and is crucial to many agricultural aspects [6]. Despite their monetary value, buffalo milk production efficiency is low (8-10 kg/day) due to the absence of systematic breeding strategies [1]. In the dairy industry, the primary goal of animal selection is to improve milk yield and composition [7]. Milk production is a complex trait that is controlled by a wide range of genes that can function alone, in concert with other genes, or in response to environmental factors [8]. However, [9][9] report that in poor nations, the quality of water buffalo milk is solely determined by its protein and fat content. As the main source of energy in milk, milk fat is crucial to the product's overall quality and health benefits [10]. Breeds, genetic information, environmental factors, and management techniques all have an impact on milk yield and composition [11]. Many aspects should be considered to achieve rapid genetic improvement in buffaloes, such as forming nucleus herds, applying modern breeding strategies, and using molecular biology techniques such as marker-assisted selection (MAS) technology, etc. [12]. Indeed, SNPs inside the open reading frame of a gene can result from genetic variations and nucleotide deletions or insertions [13]. These differences may result in substitutions or deletions of amino acids in the finished protein due to changes in the amino acid sequence during translation [14]. The animals' genotype selection must be made according to their productivity for optimal animal output [15].

Milk protein genes are renowned for being polymorphic and showing different genetic variations. The genetic differences in milk proteins and casein haplotypes have been shown in numerous studies to have a major impact on the nutritional and technical properties of [16]. The expression of the *alpha-lactalbumin* gene has been thoroughly studied as a potential marker for lactation and is regarded as a promising candidate gene. In order to comprehend its impact on important milk characteristics during the whole lactation period, researchers have researched its involvement in milk production and composition. So, It has regularly been reported as a lactation-specific milk protein [8]. It is found on chromosome 5 (BTA5q12-13). Although the buffalo LALBA gene has not been thoroughly investigated, *Alpha-lactalbumin* (ALA) is a specific whey protein connected to ruminant lactation features [17]. The ALA gene is a potential gene for the discovery of molecular markers linked to characteristics related to milk production and growth in farm animals [18]. Buffalo-LA accounts for about 3.5% of total milk protein. According to studies, milk fat, protein, and

renneting abilities correlate with the LALBA gene [17]. ALA catalyzes lactose production in the Golgi apparatus in mammary epithelial cells, forming lactose synthase with the membrane-bound enzyme - 1, 4-galactosyltransferase and controlling milk production [19]. Milk proteins' amino-acid sequence or post-translational modifications, as well as their bioactivity, can be impacted by SNPs [20]. Researchers' interest in the genetic polymorphism of milk proteins has increased due to the identification of whey protein polymorphism and the quantitative distribution of its variants [21]. The polypeptide chain's amino acid substitutions or deletions cause these genetic variations. These heritable variations vary in frequency and prevalence based on the species and breed [22]. This study aimed to describe the ALA gene genetic polymorphism and association analysis in the *Bubalus bubalis*.

2. MATERIAL AND METHODS

2.1 Experimental Animals and Sample Collection

The research work was carried out on 240 animals Murrah, Bhadawari, Indian buffalo breeds, and Egyptian buffaloes. Murrah is found in Hisar, Bhadawari buffalo in Agra and Etawa (India), and Egyptian buffalo from Egypt. Each breed's blood was drawn in around 10 mL falcon tube using the anticoagulant ethylenediaminetetraacetic acid. The samples were kept at -20^{0}C until the isolation of genomic DNA.

2.2 DNA isolation

Genomic DNA was extracted from the collected blood samples using the standard phenol-chloroform technique described by Sambrook and Russel (2001). Extracted DNA was analyzed in a NanoDrop spectrophotometer (DeNovix DS-11) to measure the DNA concentration and purity. The DNA was examined for purity and quantity, diluted to a final concentration of 100 ng/ml in TE (Tris-EDTA) buffer, and kept at 4°C.

2.3 Pimers Designing and Amplification of genes

Primers were designed to amplify the buffalo ALA gene exon using IDT oligo analyzer, OligoCalc, and NCBI primer blast online software (Table 1)

Table 1. Primers information for polymorphism identification

Primer Name	Exon No.	Primer sequence	Amplicate size	Annealing temp.
ALA F ALA R	1	5' AAAGGAGGTGAGCAGTGTG 3' 5' AAGAGGATGAAGAGAATGGAG 3'	262 bp	53.5°C

Using a controlled thermal cycler (Bio-Rad), Alpha-lactalbumin gene was both amplified in 0.2 ml PCR reaction tubes. The volume of the PCR reaction was 25 µL, and the template used was roughly 100 ng of genomic DNA. The reaction mixture contained 25 µL of nuclease-free water, 5 µL of 5X Phusion HF buffer, 0.5 µL of 10 mM dNTPs, 1.25 µL of 10 mM of each primer, and 0.25 µL of Phusion DNA polymerase. The ALA gene was amplified using a PCR cycling profile that included a 30 second initial denaturation phase at 98 °C, followed by 30 cycles of 10 second denaturation steps at 98 °C, 25 second annealing steps at 53.5 °C, and 30 second extension steps at 72 °C. To determine their length, the PCR amplicons were electrophoresed on a 1.5% (w/v) agarose gel in 1 TAE buffer. A molecular weight marker of 100 bp ladder was employed to gauge the size of the PCR products. Ethidium bromide (1 mg/ml) was used to stain the agarose gel in order to see and detect the DNA bands.

2.4 Single-strand conformation polymorphism analysis and sequencing

SSCP analysis was used to resolve the PCR products and the amplicons were diluted in a denaturing solution made up of 95% formamide, 10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA in order to prepare the ALA gene amplicons for SSCP analysis. The mixture was denatured by heating it to 95°C for 5 minutes, then cooling on ice. The resulting solution was then subjected to resolution on a 15% acrylamide (29:1) gel for 18 hours at 150 volts and 4°C. Using 1 Trisborate-EDTA (TBE) buffer, the electrophoresis was performed in vertical electrophoresis equipment (ATTO KOREA Model no. AE-6530). Gels were dried on cellophane using a BioRad gel dryer after being silver-stained (Sambrook and Russell, 2001). The PCR-SSCP allele frequency was determined by manually identifying and scoring the gel phenotypes. Single nucleotide polymorphisms (SNPs) of various SSCP bands were discovered by direct sequencing using purified PCR products from ALA gene with different SSCP bands. The sequences were further examined using multiple online tools to find similarities between diverse genotype sequences and nucleotide alterations.

2.4 In silico DNA and protein sequences analysis

Sequencing results of protein-encoding ALA gene were then examined by different software. Observed nucleotide sequences were confirmed with a chromatogram. Furthermore, using EMBL transseq (http://www.ebi.ac.uk/Tools/st/), all DNA sequences were converted into protein sequences. These protein sequences were then aligned using SnapGene, a program for the multiple alignments of sequences. A phylogram was generated using Clustal W2 and tree viewer TreeDyn to analyze minor variations in the ALA gene.

2.5 Statistical analysis

The genotype frequency and allele frequency for the ALA gene were determined using the acquired results. In a general linear model (GLM) framework, a multivariate analysis of variance with fixed effects was used to investigate the relationships between various genotypes and milk qualities. Connections between numerous independent variables (genotypes) and numerous dependent variables (milk qualities) concurrently using this statistical method, which is carried out using the SPSS software (version 26.0). Statistical testing was carried out on the records ($p \leq 0.05$). The mean values were compared using the Duncan post hoc test.

3. RESULTS

The isolated DNA was quantified and checked for quality in Nanodrop. A 100 bp ladder in agarose gel electrophoresis verified the PCR products generated from the ALA gene exonic region. The analysis was carried out as minimal information on SNPs was available on the exonic regions included in the present study. Results of the ALA gene PCR: ALA genes were confirmed to be present in a fragment that was approximately 262 bp in size. Every sample that was examined exhibited this gene (Fig. 1).

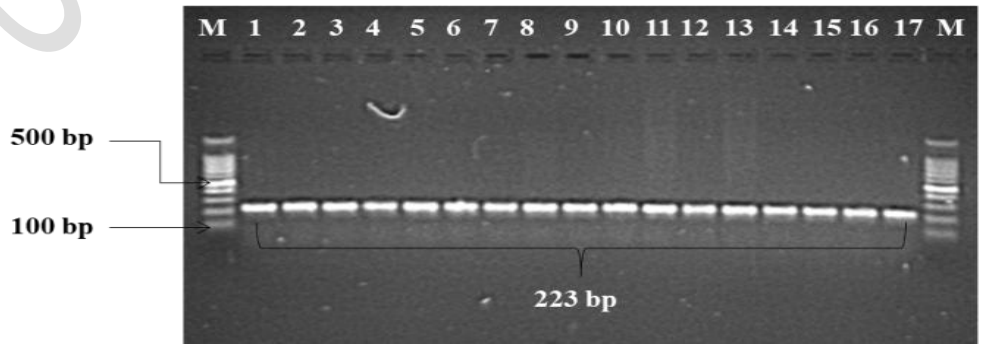

Fig.1. Electrophoresis of PCR amplifications of ALA gene (223 bp lane 1-17, M-100 bp ladder)

3.1 Single-strand conformational polymorphism analysis

The Single-Strand Conformation Polymorphism (SSCP) method was used in the current study to look at the genetic diversity of both genes. This technique works well for identifying single base alterations that alter the nucleotide sequence of a PCR result. Coding region exon 1 of the ALA gene (Fig. 2) revealed three different gel phenotypes or conformations during PCR-SSCP. LA contains four bands, LL has only two bands, and AA shows four bands but at a different position from LA.

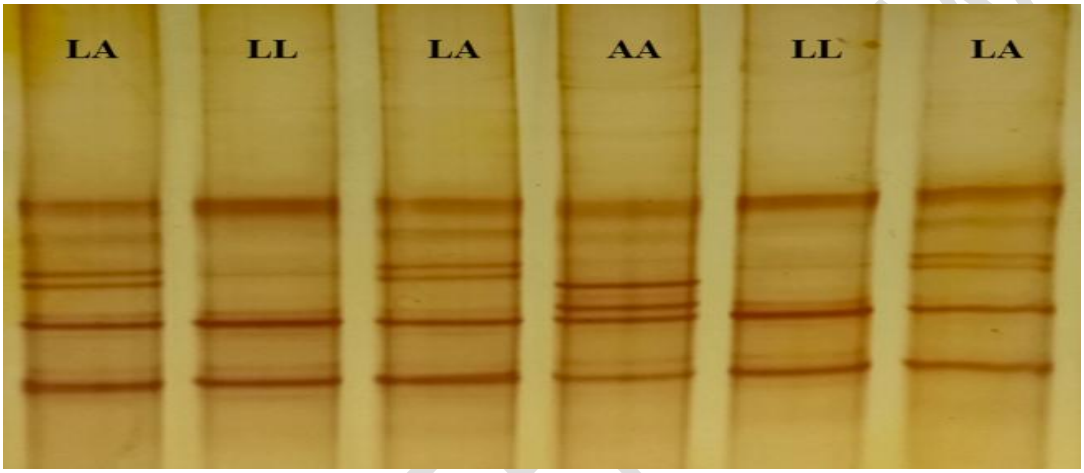

Fig. 2. PCR-SSCP conformation patterns of ALA gene exhibiting the genotypes

The genotype and allele frequencies of the ALA gene were presented in Table 2. The results of the present study confirmed that genotype LA occurs with the highest frequency in the Bhadawari breed and the LL genotype with the lowest frequency in the Murrah breed of the ALA gene.

Table 2. Genotypes and allele frequencies of ALA gene in three buffalo breeds.

Genes	Breed	n	Genotype frequency			Allele frequency	
			LL	LA	AA	L	A
ALA	EB	140	0.38	0.31	0.31	0.53	0.47
	MB	60	0.17	0.40	0.43	0.37	0.63
	BB	40	0.425	0.25	0.325	0.55	0.45

EB: Egyptian buffalo; MB: Murrah buffalo; BB: Bhadawari buffalo; n: Number of buffaloes

3.2 Sequence analysis and mutation detection

To identify the underlying SNP, eight samples from the ALA gene displaying distinct PCR-SSCP patterns were subjected to sequencing using the ABI PRISM 3730XL Analyzer (Macrogen Korea). Comparative sequence analysis between the examined samples and the reference sequence (AF194373.1) available on NCBI revealed the presence of four SNPs (Fig. 3). Specifically, SNP1-g.791G>A (Fig. 3a) and SNP2-g.848C>A (Fig. 3b) were identified in the Egyptian buffalo, SNP3-g.848C>T (Fig. 3c) was found in the Bhadawari buffalo, and SNP4-g.914T>C (Fig. 3d) was observed across all three buffalo breeds. The variant data of gene for this study have been deposited in the European Variation Archive (EVA) at EMBL-EBI under project PRJEB59596 with accession no. ERZ16351570.

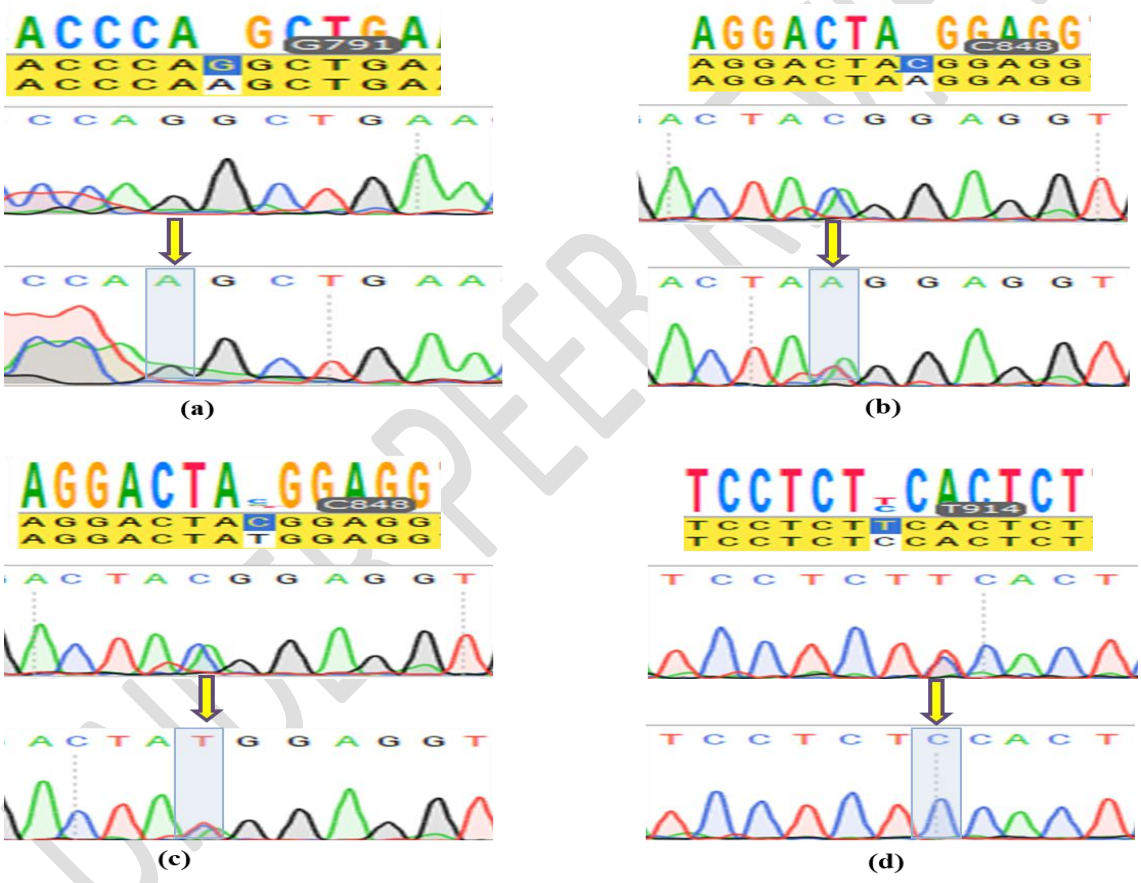

Fig. 3. (a) SNP1 G791A in ALA gene (b) SNP2 C848A in ALA gene (c) SNP3 C848T in ALA gene (d) SNP4 T914C in ALA gene

A phylogram was generated using Clustal W2 to analyze minor variations in the ALA gene. The tree viewer TreeDyn was used to confirm these variations The phylogram (Fig. 4) supported the presence of SNP1-g.791G>A and SNP2-g.848C>A in the Egyptian buffalo variants LA2E and LA1E. Additionally, SNP3-g.848C>T was found in the Bhadawari breed variants, while SNP4-g.914T>C was

observed in LA1E, LA2E, LA3B, and LA8M across all three buffalo breed variants. Moreover, the tree analysis revealed a high similarity between the reference sequence and Murrah buffalo variants (LA5M, LA6M, and LA7M).

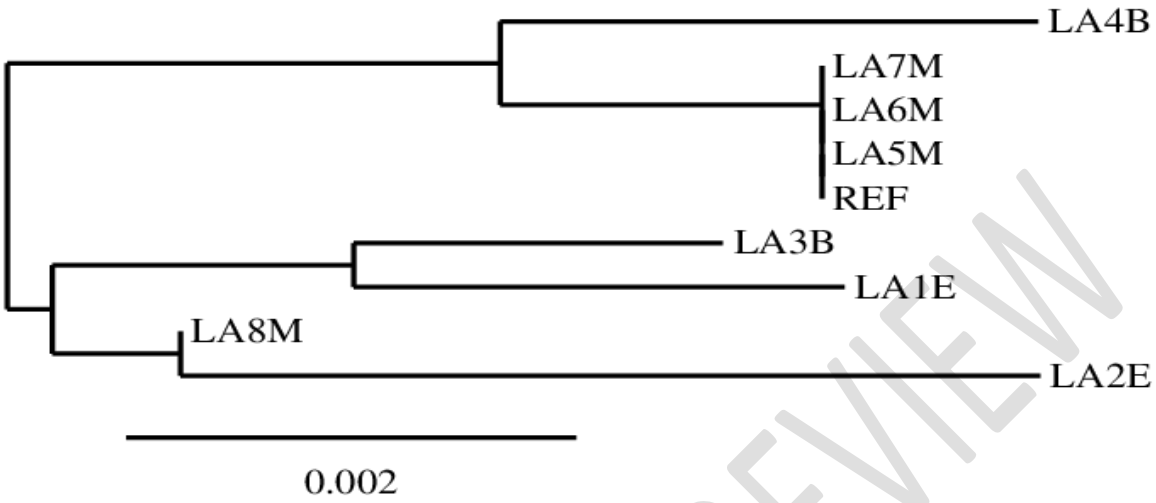

Fig. 4. Phylogram demonstrating the similarities of ALA exon-1 gene sequences from different buffalo groups

3.3 Sequence translation and amino acid substitution

Nucleotide sequences were translated to amino acids to detect the amino acid substitution. Results in Fig. 5 of the present study confirmed the four non-synonymous amino acid substitutions in the ALA gene. The first substitution involved the conversion of arginine (R) to lysine (K) due to the SNP at position 791G>A, second substitution shows the SNP at 848C>A cause transformation of amino acid from threonine (T) to methionine (M), and the third substitution threonine (T) to lysine (K) due to SNP at 848C>T. Lastly fourth amino acid substitution from phenylalanine (F) to serine (S) cause by SNP at 914T>C.

Fig. 5. Multiple sequence alignment of peptide sequences of ALA gene with reference shows amino acid substitutions

3.4 Association analysis of genotypes with milk components

The associations of the genotypes with different milk characteristics in the ALA genes were presented in Table 3. The results of this study showed that homozygous genotype AA of BB significantly associated with the highest fat percent (9.94±0.37) and protein percent higher amount (4.12±0.13) association was observed in BB heterozygous genotype LA. The highest fat yield (18.34±0.14) shows significant association in heterozygous genotype LA of BB, the highest protein percent (11.31±0.11) association was also observed in BB. FP in Egyptian buffalo, PP in both Egyptian and Murrah buffaloes, FY in Murrah buffalo and PY in Murrah and Bhadawari buffaloes shows a non-significant association.

Table 3. Summary of association analysis of genotypes in ALA gene

Buffalo breeds	Milk characteristics	Observed genotypes (Mean ± SE)			*P* Value
		CC	CA	AA	
E.B	FP (%)	8.39±0.19[a]	7.98±0.20[a]	8.04±0.17[a]	.25
	PP (%)	3.32±0.08[a]	3.34±0.08[a]	3.40±0.08[a]	.76
	FY (kg)	16.68±0.19[a]	18.34±0.14[b]	16.65±0.17[a]	.02

	PY (kg)	10.35±0.15[a]	10.66±0.17[a]	11.31±0.11[b]	.03
		(n=53)	(n=43)	(n=44)	
M.B	FP (%)	7.77±0.30[ab]	8.18±0.11[b]	7.44±0.18[a]	.004
	PP (%)	3.64±0.18[a]	3.77±0.10[a]	3.71±0.09[a]	.56
	FY (kg)	16.90±0.52[a]	16.86±0.26[a]	17.54±0.29[a]	.70
	PY (kg)	10.50±0.17[a]	10.91±0.22[a]	10.68±0.23[a]	.66
		(n=10)	(n=24)	(n=26)	
B.B	FP (%)	8.78±0.31[a]	8.73±0.36[a]	9.94±0.37[b]	.03
	PP (%)	3.16±0.16[a]	4.12±0.13[b]	3.62±0.22[ab]	.004
	FY (kg)	16.71±0.25[a]	17.85±0.44[b]	16.89±0.39[ab]	.08
	PY (kg)	10.64±0.28[a]	10.36±0.31[a]	10.47±0.27[a]	.79
		(n=17)	(n=10)	(n=13)	

Within row, means carrying distinct superscripts differ significantly. n: number of animals; SE: standard error; EB: Egyptian breed; MB: Murrah breed; BB: Bhadawari breed; FP: Fat percentage; PP: Protein percentage; FP: Fat yield; PY: Protein yield.

4. DISCUSSION

A strategic method for improving animal attributes and total production is to periodically choose animals based on their genotypes, which are an indication of their productivity and economic value. According to reports, mammalian species' *Alpha-lactalbumin* coding gene is highly variable. The allelic frequencies for A and B alleles were 0.5 and 0.5 in Marathwadi buffalo for the studied locus of the ALA gene [23]. Present study show highest allelic frequencies for L and A allele was 0.55 and 0.63 respectively. The correlations of the ALA SNPs with milk production features have been investigated in several previous findings. The LALBA gene was found to have a total of 26 single nucleotide polymorphisms (SNPs) in two different buffalo species river and swamp-type buffalo. Three of these SNPs were discovered in the 3' UTR, 18 were in the coding sequence (CDS), and five were in the 5' untranslated region (UTR) [17]. Nili Ravi buffaloes study at their second lactation found that the identified polymorphisms in ALA gene at the chromosomal position 34310940 [8]. Eight samples were sequenced for the ALA gene in the current study, and sequence analysis showed the four nucleotide substitutions (SNP1-g.791G>A and SNP2-g.848C>T were identified in the Egyptian buffalo, SNP3-g.848C>A was found in the Bhadawari buffalo. And SNP4-g.914T>C was observed across all three buffalo breeds.

At location 1264 (A–G), according to one South Kanara buffalo study, there is a replacement. The detected polymorphism at position 1264 was a silent mutation in the gene's coding region,

whereas the polymorphism at position 864 (CT) resulted in substituting the amino acid PS [24]. Out of the 10 non-synonymous SNPs in river type and swamp type buffaloes CDS, amino acid changes resulting from c.95A>G, c.218A>G, c.286T>G, and c.308A>G may affect the function of buffalo α-LA [17]. The current study's findings supported the four amino acid substitutions in the ALA gene (R-K, T-M, T-K, and F-S). Study discovered through association analysis that animals with the TT genotype displayed a number of beneficial features in comparison to those with other genotypes. The TT genotype was specifically linked to greater daily milk yield, dry matter yield, lactose yield, and lactose concentration [25]. The ALA gene in the present study shows that homozygous genotype AA was significantly associated with highest fat percent. At the same time, genotype LA was associated with higher protein percent, and fat yield. According to a study on cattle, the TT genotype was found to be associated with higher daily milk yield, dry matter yield, lactose yield, and lactose concentration. Additionally, it was observed that this genotype was linked to lower somatic cell counts, suggesting its desirability for breeders [25]. In a separate study a novel SNP at the 1847th position (T/C) in the non-coding region near CDS4 was identified. This SNP showed a significant association with milk lactose composition in Chinese Holstein dairy cows. The study also revealed a correlation between lactose content and milk protein content, suggesting the potential of this SNP as a molecular marker for lactation traits [26]. In contrast, a previous study conducted on Eastern Azarbaijan native buffaloes reported no significant effect of genotypes in the ALA gene on milk production, lactose, fat percentage, and protein percentage [27].

5. CONCLUSION

The optimization of dairy populations has extensively used polymorphism and milk quality studies, and the relationship between milk composition and genetic diversity in milk production significantly affects dairy productivity. This study sought to identify novel SNPs in the ALA gene and evaluate the associations between this gene polymorphisms and milk characteristics. The present study confirmed four nucleotide substitution (SNP1-g.791G>A, SNP2-g.848C>A, SNP3-g.848C>T, and SNP4-g.914T>C) in the ALA gene. ALA gene genotypes association analysis represents that FY and PY show significant association in Egyptian buffaloes; FP was significantly associated in Murrah breed and in Bhadawari breed FP, PP, and FY. The study emphasised the significance of identifying genetic polymorphisms and understanding their relationship with economically significant milk qualities in a wider population of buffaloes. This thorough screening will lay a solid platform for marker-assisted

selection, making it possible to choose animals for breeding programmes when they are still young. This method enables breeders to quickly recognise and breed buffaloes with desired milk-producing traits, thus increasing the productivity and efficiency of dairy farming.

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