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RESEARCH ARTICLE

DNA isolation, *in silico* characterization of *LALBA*, *BLG*, and *LYZ* genes in Halari donkey milk somatic cells

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Abstract: The Halari donkey, an indigenous breed from Gujarat's Saurashtra region, India is considered endangered due to a sharp population decline. Donkey milk is recognized for its hypoallergenic and cosmeceutical properties, with a composition resembling human milk. Donkey milk has garnered significant attention from the scientific community. This study examined the molecular characteristics of three important milk protein genes, namely alpha-lactalbumin (*LALBA)*, beta-lactoglobulin (*BLG)*, and lysozyme (*LYZ),* using PCR from genomic DNA extracted from somatic cells present in Halari donkey milk and subsequent sequence analysis. Additionally, phylogenetic analysis and protein-protein interaction mapping were conducted to compare these proteins with their counterparts in other species. PCR amplification and sequence analysis confirmed the presence of these genes in Halari donkey milk. Phylogenetic analysis revealed an evolutionary relationship between the Halari donkey milk proteins and other mammalian milk proteins, including cattle and humans. Protein-protein interaction networks demonstrated conserved network hub structures in donkey and human milk, associated with milk composition, nutritional and immune functions. The gene amplification and characterization of the Halari donkey milk proteins, namely alpha-lactalbumin (*LALBA)*, beta-lactoglobulin (*BLG)*, and lysozyme (*LYZ)* underscore their potential nutritional and therapeutic value. The similarities with human milk proteins suggest donkey milk's suitability as a supplement or functional food, warranting further investigation.

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Introduction

Donkey milk has long been recognized for its nutritional richness and therapeutic applications, especially in infant nutrition and in skincare as a cosmeceutical. In recent years, scientific interest has intensified due to its unique composition and emerging evidence of health-promoting effects (Li et al. 2020; Li et al. 2022; Zhang et al. 2024), setting it apart from cow, goat, and human milk. Rich in nutrients and bioactive compounds, donkey milk is especially beneficial for infants and babies with cow milk protein allergy (CMPA) due to its digestibility and hypoallergenic nature (Garhwal et al. 2022; Monti et al. 2007). Rising interest in donkey milk has also reignited explorations into donkey farming and the dairy potential of donkeys (Gupta et al. 2016; Pal et al. 2013; Pal et al. 2020; Bhardwaj et al. 2024). Garhwal et al. (2023) conducted metabolomic profiling of Halari donkey colostrum and mature milk across lactation stages using ^{1}H NMR. The biochemical, dielectric and surface characteristics of freeze-dried donkey milk powder were also elucidated (Garhwal et al. 2025). Nutritionally, donkey milk resembles human milk with lower fat and higher lactose content than cow's milk (Bhardwaj et al. 2020). Recent studies have demonstrated the inhibitory effect of donkey milk and donkey colostrum on the progression of the 4T1 tumours via apoptosis (Li et al. 2020). Also, donkey milk is involved in the scavenging of reactive oxygen species, activation of the antioxidant system, balancing intestinal flora and has played an immunomodulatory role in *in vitro* and *in vivo* models (Li et al. 2022). The intake of donkey milk was linked to improving the heart mitochondrial metabolic flexibility, lipid storage and redox status of the heart tissues and hence maintaining cardiovascular health (Trinchese et al. 2021). Huimin et al. (2025) demonstrated that donkey milk supplementation showed positive effects on the rats affected by chronic kidney disease (CKD). Moreover, it also helped in alleviating inflammation and fibrosis via miR-223-3p/IKKα/NF-κB signalling pathway.

Donkey milk contains an abundance of easily digestible whey proteins such as lysozyme, caseins, and alpha-lactalbumin, which are vital for immune system development (Guo et al. 2007;

Vincenzetti et al. 2008). Alpha-lactalbumin supports lactose synthesis and gut health (Malacarne et al. 2002). Lysozyme is an effective antimicrobial enzyme that helps protect against infection. One of the key benefits of donkey milk is its hypoallergenic properties, especially because it contains low levels of beta-lactoglobulin, which is among the major allergens found in cow's milk. The hypoallergenic properties of donkey milk make it a healthier option for children who suffer from CMPA, *i.e*., cow milk protein allergy (Polidori & Vincenzetti, 2013), and also due to their rich essential nutrients and composition closer to human milk. It makes donkey milk suitable for infant nutrition and people with special dietary needs (Salimei & Fantuz, 2012; Vincenzetti et al. 2012). Consequently, donkey milk's anti-inflammatory activities provide additional therapeutic uses, such as treating skin infections and improving gut health (Carminati & Tidona, 2017).

Alpha-lactalbumin (α-lactalbumin) is a protein found in mammalian milk whey (~41%). It contains about 20-25% of the total protein content in human milk (~2.44 g/L) and around 3-4% in bovine milk (Heine et al. 1991). It is a small single-chain calcium-binding globular protein with a low molecular weight of around 14.2 kDa (Permyakov, 2020). It plays a crucial role in lactose biosynthesis by interacting with the enzyme beta 1,4 galactosyltransferase to form the lactose synthase complex, which is essential for the production of lactose, a primary carbohydrate found in milk (Brew, 2013). Recent studies have highlighted the multifunctional nature of α-lactalbumin. In several studies, it has been shown to possess antimicrobial (Pellegrini et al. 1999), antiviral (Ng et al. 2001), anti-inflammatory (Yamaguchi et al. 2009), and antitumor activities (Yamaguchi et al. 2014), contributing to the immune defence mechanisms of infants (Pan et al. 2006). Additionally, α-lactalbumin in humans, bovine, equine and donkey consists of 123 amino acids with four disulfide bonds (Brew, 2003; Permyakov, 2020) and is rich in essential amino acids, particularly lysine, tryptophan, and cysteine, which are crucial for infant growth and development (Layman et al. 2018).

In addition to human and bovine milk, α-lactalbumin has also been studied in the milk of equines and donkeys. Research on equine milk has demonstrated its potential in promoting intestinal health and reducing inflammation due to its high content of bioactive peptides, including α-lactalbumin. Similarly, donkey milk's antimicrobial and antioxidant properties are largely attributed to its protein content, including α-lactalbumin (Vincenzetti et al. 2012).

Beta-lactoglobulin (β-lactoglobulin) is one of the major whey proteins found in ruminant species, comprising 10% of the total milk protein and approximately 50% of the whey protein content (Sawyer and Kontopidis, 2000). Although it is present in many species, it is notably absent in human milk. Naturally, it occurs as a dimer, and at neutral pH, it exists as a monomer which consists of 162 amino acid residues with two disulfide bonds and weighs approximately 18.3 kDa (Qin et al. 1998). It belongs to the lipocalin family and makes small globular proteins by folding into eight antiparallel beta strands with a 3-turn alpha-helix on the outer surface of the beta-strand (Kontopidis et al. 2004). Several studies have shown that it possesses antioxidant activity (Elias et al, 2005; Liu et al. 2007; Sharp et al. 2004) and plays a role in phosphate metabolism in the mammary gland (Farrell et al. 1987) or transfer of passive immunity to the newborn (Warme et al. 1974). It can bind to hydrophobic molecules, such as fatty acids, vitamins, and other small molecules, and enhances the bioavailability of these nutrients. This binding capacity is critical for delivering these nutrients in a stable form, making β-lactoglobulin a valuable ingredient in nutritional supplements and functional foods (Santos et al. 2020).

Lysozyme, also known as muramidase, is an enzyme with potent antimicrobial properties, prominently present in various bodily secretions, including milk, tears, and saliva. It is a basic globular protein consisting of 129 amino acids with a molecular weight of around 14.3 kDa (Cegielska-Radziejewska et al. 2008). It is particularly abundant in human milk, where it plays a crucial role in the innate immune defence system (LeBlanc et al. 2004). Lysozyme exerts its antimicrobial effects by hydro*lyz*ing the β-1,4-glycosidic bonds in the peptidoglycan layer of bacterial cell walls (Jollès & Jollès, 1984). This action is particularly effective against Gram-positive bacteria, making lysozyme a key component of the body's defence against bacterial infections (Callewaert & Michiels, 2010). Lysozyme has been used as a therapeutic agent due to its ability to enhance the body's immune response and its potential use in treating bacterial infections and promoting wound healing (Proctor et al. 1988). In human milk, lysozyme contributes significantly to the antimicrobial defence provided to infants, offering protection against infections and supporting the development of the infant's immune system. Its presence in milk is associated with the lower incidence of gastrointestinal infections in breastfed infants compared to those fed with formula milk (Levy, 2000). Additionally, lysozyme has anti-inflammatory properties that help modulate the immune response and maintain gut health.

Importantly, Milk somatic cells, comprising leukocytes and mammary epithelial cells shed during lactation, offer a non-invasive source of DNA for genetic studies, supporting ethical conservation of the endangered (Soneji, 2024) Halari donkeys. African wild ass (*Equus africanus*) is already listed as critically endangered in the IUCN red list of threatened species (Moehlman et al. 2015). A study demonstrated the viability of extracting DNA from Halari donkey milk somatic cells to amplify the κ-casein (*CSN3*) gene, establishing a methodological example for molecular characterization without invasive sampling (Singh et al. 2024). This study aimed to characterize three major milk protein genes, *i.e*. α-lactalbumin (*LALBA*), β-lactoglobulin (*BLG*), and lysozyme (*LYZ*), which are central to the nutritional, immunological, and technological properties of donkey milk.

Materials and Methods

Study design and setting

This molecular study aimed to characterize milk protein genes (*LALBA*, *BLG* and *LYZ*) in Halari Jenny's milk through DNA extraction, PCR amplification, sequencing, and *silico* analysis. The research was conducted at the Jenny Dairy Unit of the National Research Centre on Equines, Hisar, Haryana, India.

Sampling

Ten lactating Halari Jennies were selected under uniform management and feeding conditions. Approximately 10 mL of milk was collected from each animal (n = 10) by hand milking after sanitizing the udder with 70% ethanol. The samples were individually filtered using muslin cloth, transferred into a sterile 15 mL centrifuge tube, and kept at 4ºC for subsequent DNA extraction. All the procedures adhered to the ethical guidelines of the institute (IAEC).

DNA extraction

Genomic DNA was isolated from the milk somatic cells following a modified protocol of Pokorska et al. (2016). Briefly, 10 mL of whole milk was centrifuged at 400 x g for 10 min, 4ºC. The supernatant was discarded, leaving some amount behind, and the pellet was transferred into a 1.5 mL Eppendorf tube and centrifuged again. The supernatant was discarded. The pellet was washed with 500 µL wash buffer (15 mM Tris-HCl (pH 7.4–7.6), 25 mM NaCl, 5 mM $MgCl_2$, 15 mM Na_2HPO_4, 2.5 mM EDTA, 1% sucrose) and centrifuged at 400 x g, 10 min, 4ºC, and the supernatant was discarded. Washing was performed 3 times. Afterwards, the pellet was dissolved in 1 mL lysis buffer (pH 8.8; 6 % SDS, 3 mM $MgCl_2$, 15 mM Tris-HCl, 0.5% DMSO, 6% acetone) and the solution was incubated for 30 minutes at 65ºC. DNA was precipitated by mixing 200 µL cold isopropanol in the solution and centrifuged at 10000 x g for 1 min at room temperature. The supernatant was discarded. The DNA pellet was washed with 100 µL of 75% ethanol and centrifuged at 10000 x g, 1 min, RT. It was repeated two times. Pellets were air-dried and suspended in 20 µL nuclease-free water. DNA size and quality were evaluated by electrophoresis in a 1.0% agarose gel (Lonza Biosciences),

Table 1. Primers for the target genes

Sl. No.	Gene	Forward primer	Reverse primer
1	α-lactalbumin (*LALBA*)	GCCCCCATTTCAGGTTCTTG	ATTCAGGCAAAGTGACGCCT
2	β-lactoglobulin (*BLG*)	TCACAGAATCCACTGCCTTCC	GAGCCTCCTGCACTTACAGTTG
3	Lysozyme (*LYZ*)	AAGACTGAGGCTCTCGACTTG	TGGAGCATAGCATGTTGGGT

Table 2. List of organisms and their amino acid accession no. from NCBI

Sl. No.	Order	Organism	Accession No.		
			LALBA	*BLG*	*LYZ*
1	Artiodactyla	*Bubalus bubalis*	APQ30588.1	CAA06532.1	ABF20557.1
2		*Bos taurus*	AAF63624.1	CAA32835.1	AAC37311.1
3		*Bos grunnies*	AER42591.1	AFB74991.1	AGF69232.1
4		*Cervus elaphus*	XP_043734415.1	XP_043772424.1	XP_04374346
5		*Camelus dromedarius*	QEG79382.1	XP_064346964.1	XP_06434759
6		*Capra hircus*	NP_001272564.1	CAA41385.1	ACX50258.1
7		*Ovis aries*	NP_001009797.1	CAA31305.1	NP_00109111
8	Carnivora	*Lynx rufus*	XP_046923253.1	XP_046949665.1	XP_04692257
9		*Felis catus*	XP_003988664.1	XP_019672359.3	XP_02311299
10	Lagomorpha	*Lepus europaeus*	XP_062058222.1	XP_062062831.1	XP_06205923
11	Perissodactyla	*Equus asinus*	XP_014705618.2	ADG21873.1	XP_01471407
12		*Equus caballus*	XP_001915824.1	AAC95385.1	XP_00149418
13		*Equus quagga*	XP_046494183.1	XP_046534388.1	XP_04651842
14	Primate	*Homo sapiens*	NP_001371279.1	---	NP_000230.1
15		*Pan paniscus*	XP_003825848.1	XP_034785676.1	AAB41214.1
16	Rodentia	*Rattus rattus*	XP_032746860.1	---	XP_03276866
17		*Rattus norvegicus*	---	XP_063140881.1	---
18		*Cavia porcellus*	NP_001166360.1	XP_063100159.1	XP_00347613

including ethidium bromide. Electrophoresis was carried out in 1 x TAE solution, at 80V for 45 minutes, and then the gel was observed under UV light image ana*lyz*er (Syngene, G: BOX)

Polymerase Chain Reaction

A PCR reaction mix of total volume of 25/ µL or a reaction mixture contained 12.5 µL of PCR master mix (Thermo Scientific), 1µL of each forward and reverse primer (Table 1), and 2 µL of DNA sample. The PCR conditions for these genes were as follows:

LALBA - 4/ min at 95/ °C, 35 cycles of 95/ °C for 1/ min, 56/ °C annealing for 1 min, 72/ °C for 1/ min, with a final extension at 72/ °C for 10/ min.

BLG - 4/ min at 95/ °C, 35 cycles of 95/ °C for 1/ min, 60/ °C annealing for 1 min, 72/ °C for 1/ min, with a final extension at 72/ °C for 10/ min.

LYZ - 5/ min at 94/ °C, 35 cycles of 94/ °C for 1/ min, 60/ °C annealing for 1 min, 72/ °C for 1/ min, with a final extension at 72/ °C for 10/ min.

Gel electrophoresis

The PCR products were ana*lyz*ed by electrophoresis in a 1.5% agarose gel (Lonza Biosciences) stained with ethidium bromide in a 1x TAE buffer at 80 V for 45 minutes. A 100 bp DNA ladder was run to ensure the size of the amplification product. A 171 bp, 116 bp, and 216 bp DNA fragment indicated the presence of donkey *LALBA*, *BLG*, and *LYZ* genes, respectively.

***In silico* analysis of *LALBA, BLG, and LYZ* amino acid sequence**

Amino acid sequences of *LALBA, BLG*, and *LYZ* proteins of different species from different orders of the mammalian class were retrieved from NCBI GenBank for the phylogenetic analysis (Table 2). Multiple sequence alignment of the amino acid sequences was done by the MEGA 11.0 program using the MUSCLE function. Further, molecular phylogenetic analysis was performed from the data provided by multiple sequence alignment patterns of amino acids following the Maximum-Likelihood method based on 1,000 bootstrap replications and Jones-Taylor-Thornton (JTT) matrix-based model by the MEGA 11.0 program (Nayan et al. 2013). Since *BLG* is absent in humans, it was not used in the phylogenetic analysis of *BLG* protein. The amino acid sequence of *LYZ* for *Rattus rattus* was not available; hence, the sequence of *Rattus norvegicus* was taken. The interaction of *LALBA*, *LYZ*, and *BLG* proteins with other proteins present in donkey and human milk was ana*lyz*ed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software with default parameters.

Sanger Sequencing

Sanger sequencing of *LALBA*, *LYZ,* and *BLG* amplicons was conducted by M/S Agrigenome using the respective forward and reverse primers. Sequences were curated using the BioEdit sequence alignment editor software tool, and a consensus sequence was constructed and submitted to GenBank. The sequences showed 99-100% identity to the sequences available in GenBank.

Results and Discussion

Sequence analysis

Genomic DNA was extracted from donkey milk somatic cells visualized via gel electrophoresis, revealing a distinct single band indicative of high-quality, uncontaminated DNA. PCR amplification of the target genes, i.e. *LALBA*, *BLG*, and *LYZ*, produced clear, specific bands under UV light. No evidence of nonspecific amplification or primer-dimer formation was found (Figure 1). The Sanger sequencing confirmed the identity of the amplified products (Figure 2), which showed high sequence similarity when BLAST and Clustal W alignment were performed. The sequence has been submitted to the NCBI with the accession

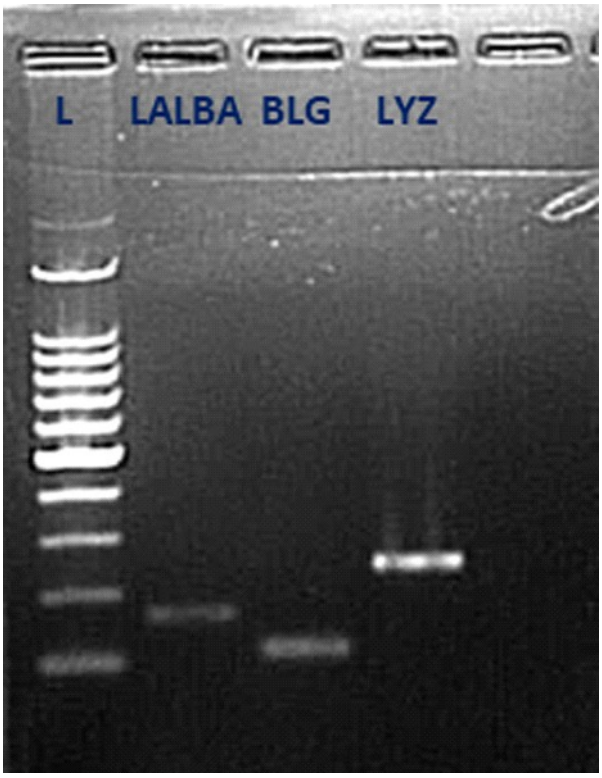

Fig. 1 Gel electrophoresis of PCR products: a 165 bp, 114 bp, and 239 bp PCR product of *LALBA* (lane 2), *BLG* (lane 3), and *LYZ* (lane 4) genes, respectively, were amplified, indicating the presence of these genes in the Halari donkey milk

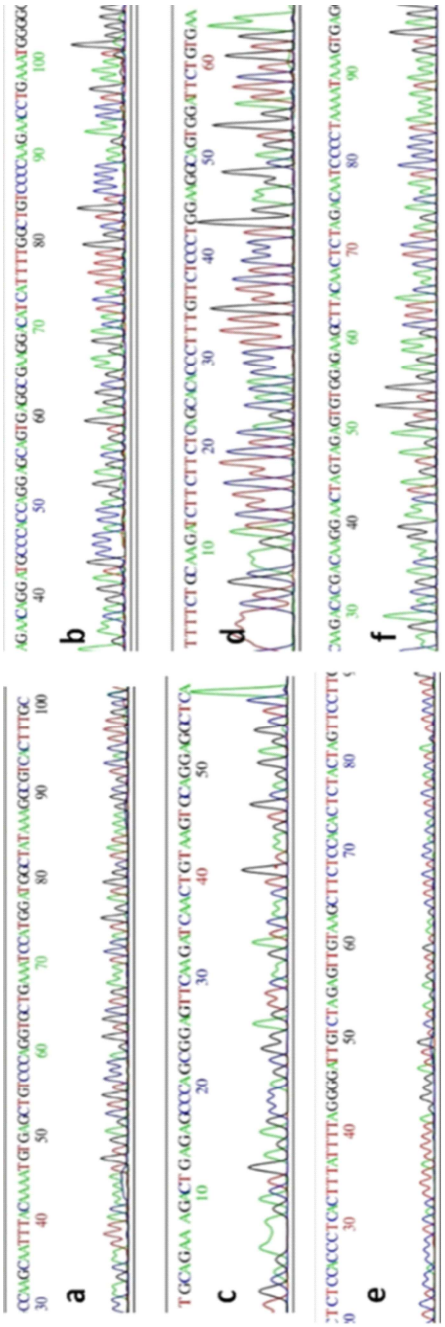

Fig. 2 DNA sequence chromatogram of forward and reverse DNA strands of *LALBA* (a, b), *BLG* (c, d), and *LYZ* (e, f) of the Halari donkey breed

numbers PP889738.1 for *LALBA*, PP918978.1 for *BLG* and PP436445.1 for *LYZ*.

Phylogenetic analysis of *LALBA, BLG* and *LYZ* proteins

To investigate the phylogenetic relationships of the *LALBA*, *BLG,* and *LYZ* proteins, the amino acid sequences from the donkey and diverse mammalian species were analysed. These included the representatives from various orders, *i.e.* donkey, horse, zebra, cattle, buffalo, goat, sheep, red deer, oryx, yak, camel, Bactrian camel, hare, rat, guinea pig, lynx, cat, human and chimpanzee. The phylogenetic trees generated (Figure 3(A-C)) revealed notable patterns. *LALBA* sequences showed high similarity among donkey, horse, camel, human, and zebra compared to other species, suggesting a closer evolutionary relationship. The *BLG* sequences of donkey and horse clustered with the artiodactyls, whereas the zebra appeared to diverge independently. Similarly, the *LYZ* gene in donkeys also showed its common ancestry with the artiodactyls, though it remained distinct from the human lineage.

Protein-protein interactions

The protein-protein interaction (PPI) network revealed a central cluster comprising the key milk proteins like *LALBA*, *LYZ*, *CSN2* (casein), and *LPO* (lactoperoxidase) (Figure 4 (A-B)), highlighting their coordinated roles in milk composition and innate immunity. The *LALBA* protein appeared as the principal hub protein across both networks, underscoring its critical function in lactose synthesis and mammary gland physiology. In donkey milk, the antimicrobial protein *LYZ* is linked to several lysozyme-like proteins, whereas in human milk, *LYZ* interacts with immune-regulatory proteins like *LTF* (lactoferrin). These milk proteins also associate with other milk proteins and enzymes involved in the functioning of milk constituents.

In the present study, we successfully isolated genomic DNA from the donkey milk somatic cells present in the donkey milk and confirmed the presence and amplification of important milk protein genes, α-lactalbumin (*LALBA)*, β-lactoglobulin (*BLG)*, and lysozyme (*LYZ*), through PCR and gel electrophoresis. These findings align with our previous research work (Singh et al. 2024). Phylogenetic analysis revealed that these proteins share a close evolutionary relationship with those of other equids and exhibit remarkable similarity to human milk proteins, particularly *LALBA*, in contrast to bovine counterparts. This is particularly significant given the relatively low number of somatic cells in donkey milk, ranging from 4.0-4.87 log cells/mL (Garhwal et al. 2023), which is substantially lower than the approximately 100,000 somatic cells/ mL found in bovine milk. The isolation of DNA from the milk somatic cells provides an alternative, convenient, animal-friendly, and non-invasive method for isolating genetic material compared to blood, as in our earlier study (Singh et al. 2024). Among the whey proteins in donkey milk, α-lactalbumin (*LALBA*), β-lactoglobulin (*BLG*), and lysozyme (*LYZ*) stand out, considering their antimicrobial, anti-tumour, and hypoallergenic properties. *LALBA* serves as a good source of essential amino acids in milk, and generates peptide fragments during digestion in the gastrointestinal tract that exhibit immunostimulatory and antimicrobial effects (Lönnerdal et al. 2017). In one study, over 90% of the *LALBA* from the donkey milk remained intact after

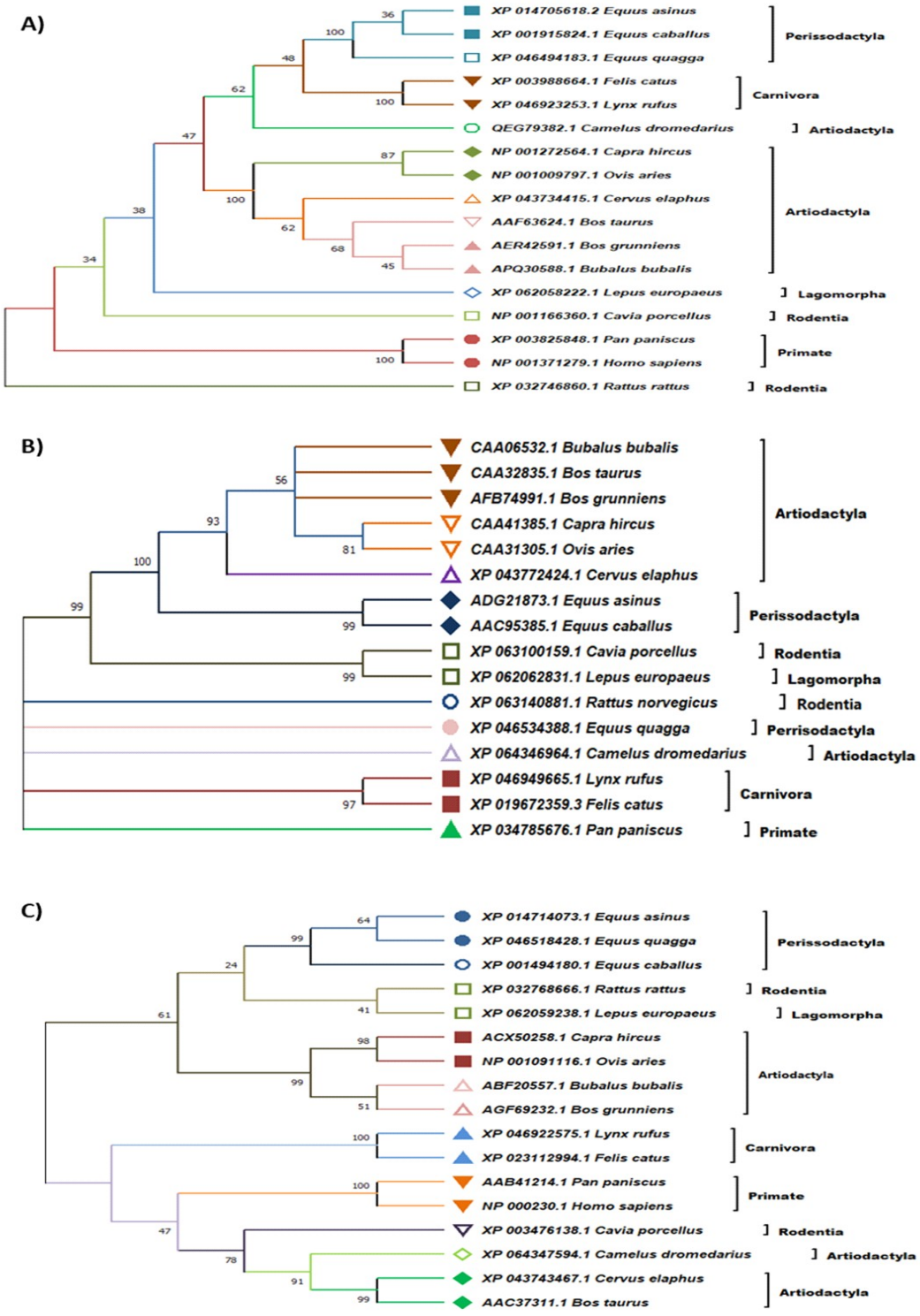

Fig. 3 Phylogenetic relationship of the peptide sequences of A) *LALBA*, B) *BLG,* and C) *LYZ* donkey milk proteins with other mammals. Molecular phylogenetic analysis was performed from the data provided by the multiple sequence alignment patterns of amino acids. The sources of all the sequence data are represented in the phylogenetic tree. The phylogeny is an un-rooted tree recovered using the maximum-likelihood (ML) method based on 1,000 bootstrap replications and Jones–Taylor–Thornton (JTT) matrix-based model by the MEGA 11.0 program. Only a bootstrap value greater than 0 % is shown on the branch.

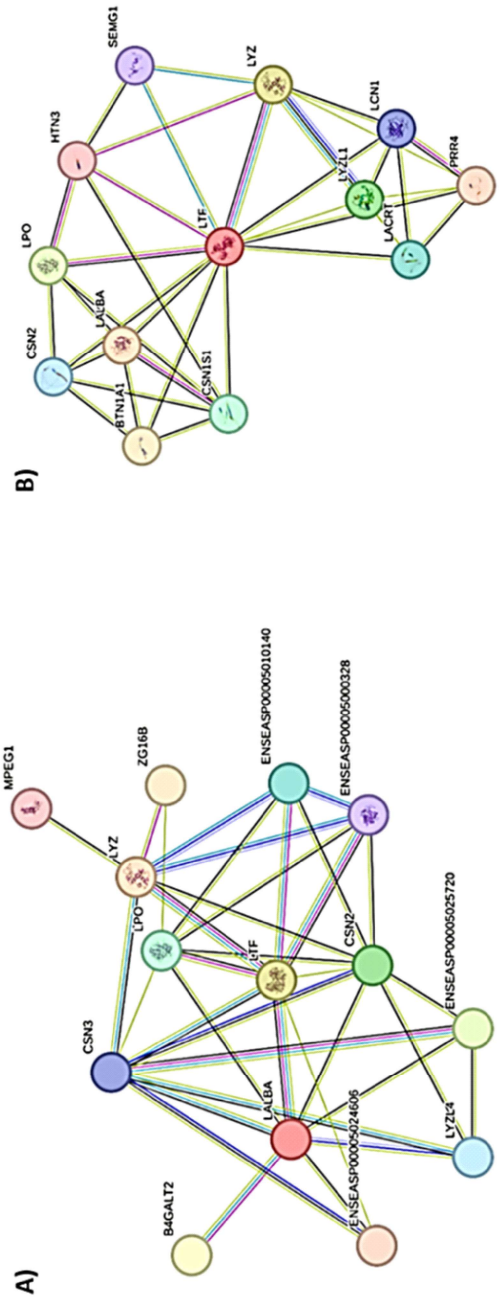

Fig. 4 Protein-protein interaction network by the STRING online tool for the major milk protein in A) donkey and B) human**.** Nodes represent proteins and edges indicate predicted interactions. Key hub proteins in both donkey and humans include *LALBA*, *LYZ*, *LTF*, and *LPO*

exposure to the gastric and duodenal enzymes, suggesting its potential to reach the intestine largely intact (Tidona et al. 2014). *LALBA* has also exhibited anti-inflammatory properties by inhibiting cyclooxygenase-2 (COX-2) and phospholipase A2 (Yamaguchi et al. 2009). Since *BLG* is present in donkey milk and hence, the use of donkey milk in subjects with a prior history of anaphylaxis to cow milk should be contemplated with considerable caution (Monti et al. 2007). Donkey milk contains two distinct isoforms, β-lactolgobulin I (*BLG* I) and β-lactolgobulin II (*BLG* II), and unlike ruminant milk, *BLG* exists in a monomeric form (Vincenzetti & Polidori, 2012). Lysozyme (*LYZ*) is present in exceptionally high concentrations in donkey milk (1.0 mg/mL), far exceeding the levels found in cow milk (in traces) and human milk (0.12 mg/mL), and closely resembling those in mare's milk (0.79 mg/mL) (Polidori & Vincenzetti, 2013). The high content of lysozyme in donkey milk may be correlated with the high gene expression of *LYZ* gene in breast tissue (Wang et al. 2021). Donkey milk's protein profile makes it a promising candidate as a human milk substitute. In fact, donkey milk is used as a breast-milk substitute in European small-scale farms that choose diversified production (Conte & Panebianco, 2019). One study reported 71% overlap between donkey and human milk proteins, which was higher than the overlap observed with bovine, sheep and swine milk (Wang et al. 2021). This underscores the further exploration of donkey milk as a human milk substitute. Protein-protein interaction studies revealed that human and donkey milk protein interactions share a common basic tenet, particularly those involved in antimicrobial activity and immune regulation. The preservation of vital hub proteins, including lactoferrin, lysozyme, α-lactalbumin, and lactoperoxidase in both species, indicates their critical roles in the sustenance of the fundamental milk functions.

Conclusions

This study extracted and characterized vital milk protein genes, namely *LALBA*, *BLG*, and *LYZ*, from the Halari donkey milk somatic cells using a non-invasive, animal-friendly approach. High-quality genomic DNA enabled clear PCR amplification, with sequences validated through Sanger sequencing and bioinformatic alignment. Phylogenetic results revealed a close evolutionary relationship between donkey, equine and human milk proteins, with *LALBA* clustering alongside horse, camel, and human sequences. This evolutionary proximity emphasizes the nutritional and immunological relevance of donkey milk relative to bovine milk for human nutrition. Protein-protein interaction mapping highlighted the central role of *LALBA* and *LYZ* in milk physiology and innate immunity, with *LYZ* showing strong antimicrobial associations. The abundance of lysozyme, monomeric *BLG*, and the intact bioactivity of *LALBA* post-digestion support the hypoallergenic and therapeutic potential of donkey milk. However, caution should be exercised for individual sensitive to cow milk allergies due to the presence of *BLG*. Collectively, these findings support a non-invasive genetic approach and delineate donkey milk as a nutraceutical, given its high protein overlaps with human milk. The conservation of hub proteins across species suggests a shared functional architecture in milk's immune and nutritional roles. Deeper inquiries are needed for the domains of food science, paediatrics, and immunology.

Ethics approval and consent to participate

The animal study was reviewed and approved by the Institute Animal Ethics Committee of the ICAR-National Research Centre on Equines (ICAR-NRCE), Hisar.

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