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Epigenetic insights into mastitis in Mediterranean Italian River buffalo using nanopore sequencing

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ABSTRACT

Mastitis represents one of the main challenges in dairy buffalo farming, with significant implications for animal health, milk quality, and farm profitability. Among its different forms, subclinical mastitis is difficult to detect due to the absence of standardised diagnostic tools. Epigenetic mechanisms, such as DNA methylation, have been associated with immune response and disease susceptibility in cattle, but remain unexplored in buffaloes. This study explores genome-wide DNA methylation profiles in Mediterranean Italian River Buffaloes, using nanopore sequencing technology, to identify potential epigenetic signatures associated with mastitis resistance. CpG sites showed a unimodal distribution, with most sites exhibiting high methylation levels. A total of 22 differentially methylated cytosines (DMCs) were identified, with 68% showing hypomethylation in the control group and 32% showing hypermethylation. Genomic annotation revealed that hypermethylated DMCs were predominantly located in intronic regions, while hypomethylated DMCs were largely enriched in distal intergenic regions. This study is the first to investigate DNA methylation changes associated with mastitis in Mediterranean Italian River Buffalo using nanopore sequencing. Distinct epigenetic patterns were identified between healthy and mastitic animals. This study provides a first epigenetic overview of mastitis in buffaloes and lays the groundwork for future investigations with larger cohorts to validate and extend these observations. Given the small sample size, these findings should be considered exploratory, but offer insights into the molecular basis of mastitis and may support the development of new diagnostic tools based on validated epigenetic signatures.

HIGHLIGHTS

- Genome-wide DNA methylation profiling in buffalo using nanopore sequencing
- Identification of 22 DMCs associated with mastitis in buffalo
- Detection of different epigenetic patterns between groups

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Introduction

Selective breeding in livestock plays a pivotal role in improving productivity, sustainability, and animal welfare. In buffalo farming, genetic selection increasingly targets traits such as disease resistance, reproductive efficiency, milk and meat production (Albarella et al. 2017; Abdel-Shafy et al. 2021). In particular, milk quality and quantity are key traits under selection in the Italian dairy buffalo sector, as they directly influence the technological characteristics of milk and the production of high-value products like Mozzarella di Bufala Campana (MBC), a Protected Designation of Origin (PDO) cheese renowned worldwide.

However, this traits can be severely compromised by mastitis, an intramammary infection that triggers a strong inflammatory response in the mammary gland. It is caused predominantly by microorganisms, and results in significant economic losses, compromises

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animal welfare, and poses potential risks to public health if inadequately managed.

Mastitis manifests in two main forms: clinical, with visible symptoms, and subclinical, which often goes undetected due to the lack of overt signs but increases somatic cell count (Ramuada et al. 2024).

Both forms affect milk production and quality, require veterinary intervention, and may sometimes lead to culling, increasing farm costs due to replacement (Fagiolo and Lai 2007).

Subclinical mastitis (SCM) is particularly insidious, serving as a reservoir of intramammary infections and causing substantial milk production losses. Elevated somatic cell count (SCC) in milk, a hallmark of mastitis, degrade milk proteins and reduce the shelf life of pasteurised milk, ultimately compromising the organoleptic characteristics of mozzarella cheese. Guidelines for diagnosing mastitis include both microbial culture and SCC to quantify udder inflammation (Bansal et al. 2007).

Recently, differential somatic cell count (DSCC) has been proposed as a novel indicator for assessing udder health status (Bobbo et al. 2023). However, the lack of standardised SCC thresholds for buffalo milk and insufficient surveillance practices further complicates the management of mastitis in this species, leading to delayed diagnosis and treatment. Currently, SCC limits are established only for bovine bulk tank milk (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32004R0853), whereas the MBC PDO protocol does not specify any SCC threshold, highlighting the absence of a defined maximum for buffalo milk.

In cattle, an SCC thresholds of $\geq$200,000 cells/mL is commonly used as an indicator of udder infections and is widely recognised as a key diagnostic marker for mastitis (IDF, 2013). However, while similar thresholds have been proposed for buffaloes (Singh and Ludri 2001; Dang et al. 2010), precise values remain a topic of ongoing investigation, and no consensus has yet been reached on an exact cut-off for diagnosing mastitis in this species.

Epigenetic regulation has attracted growing attention in understanding mastitis infections in cattle, particularly regarding the role of DNA methylation in responding to pathogen invasion and its impact on mammary gland health (Zhou et al. 2020; Wang M et al. 2024). However, comparable studies in buffaloes remain scarce (Song et al. 2016; Varij et al. 2022). DNA methylation is one of the major epigenetic marks responsible for gene regulation. It involves the transfer of a methyl group to the cytosine of the DNA sequence by the catalysis of DNA methyltransferases producing 5–methyl cytosine (5mC), primarily appearing in Cytosine-phosphate-Guanine (CpG) dinucleotides. DNA methylation may be altered at adjacent CpG sites, which is recognised as a differentially methylated region (DMR). Gene expression and transcriptional activation are generally associated with lack of methylation, while transcriptional repression is indicated by DNA methylation in gene promoter regions.

The ability to monitor these alterations offers a valuable opportunity for the early detection and diagnosis of mastitis. In addition, methylation of specific sites can also serve as a molecular marker in livestock breeding programmes (Gonzalez-Recio et al. 2015). Nanopore sequencing (ONT) enables detailed analysis of these epigenetic modifications and the detection of CpG sites across the entire genome, including intergenic and regulatory regions. This is valuable in buffalo breeding, where epigenetic influences on health and disease resistance remain underexplored. This study aims to investigate DNA methylation changes in Mediterranean Italian River Buffalo with mastitis using nanopore technology, to uncover new insights into disease resistance and support breeding strategies for sustainable buffalo farming.

Methods

Sample collection

Peripheral blood and milk samples were collected from 100 female buffaloes reared on a dairy farm located in southern Italy. All animals were sampled in autumn and measured at the same lactation stage of 150 days postpartum. To minimise variation related to parity, only animals in their 3rd or 4th lactation were included. Data recorded during monthly functional controls, included information about animals (ID number, date of calving, stage of lactation), sampling date, daily milk yield (kg/d), milk composition (fat and protein percentages), SCC (cells/mL), DSCC (%).

Milk analyses

Before sample collection, teats and teat ends were cleaned and disinfected using one-way towels embedded with chlorhexidine. First streams of foremilk were discarded, and approximately 70 mL of milk was collected from each buffalo udder into sterile plastic tubes and immediately refrigerated (4 °C). The milk samples were analysed for SCC, DSCC and mastitis-causing agents through cultural examination within 12 h from collection by laboratories of Istituto

Zooprofilattico Sperimentale del Lazio e della Toscana 'M. Aleandri', Latina, Italy.

SCC and DSCC, in individual milk samples, were determinated using fluoro opto elecronic flow cell Fossomatic 7 DC instrument (Foss Elctric, Hillerød, Denmark) according ISO UNI EN ISO 13366-2: 2007. The instruments were operated according to the manufacture's recommendations, such as DSCC results in the range $<50 \times 10^3$ cells/mL or $>1{,}500 \times 10^3$ cells/mL and the SCC results that presented a good separation (GOSE) equal to 0 were excluded from the study.

The bacteriological examination was carried out following standard procedures (Adkins and Middleton, 2018). Ten microliters of each milk sample were spread onto blood agar plates and incubated aerobically at 37 °C for 48 h. The identification of the isolated colonies was performed using biochemical microplates API galleries (bioMerieux, Marcy, France). The isolation of at least ten colonies of the same type was considered significant to confirm the positivity of the analysed sample, while the isolation of three or more types of colonies was considered environmental contamination, and the samples were discarded from the study. Therefore, sixteen animals were classified according to these criteria: 8 negative animals, characterised by a negative bacteriological culture, somatic cell count (SCC) $\leq$200,000 cells/mL, and differential somatic cell count (DSCC) $\leq$60%; 8 positive animals, defined by a positive bacteriological culture, SCC $>$400,000 cells/mL, and DSCC $>$60% (Moroni et al. 2006; Costa et al. 2020).

DNA extraction

Genomic DNA was extracted from whole blood using Wizard DNA extraction kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The concentration and quality were measured using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany) to ensure yield $>$100 ng/μL, 260/280 ratio $>$1.7, and 260/230 ratio $>$1.8.

Library preparation and nanopore sequencing

Nanopore sequencing libraries were prepared using an initial input of 2 μg of DNA. Barcoded libraries were generated using the Native Barcoding Kit 24 V14 for gDNA (SQK-NBD114.24), following the manufacturer's protocol. Samples from positive and negative groups were mixed during library preparation ensuring a balanced representation of both conditions within each sequencing run. Libraries were loaded onto an R10.4.1 flow cell, multiplexing 3 samples per flow cell. Sequencing was performed using the PromethION 2 (P2) Solo device (Oxford Nanopore Technologies, Oxford, UK).

Raw sequencing data (pod5 files) were basecalled using the Dorado basecaller (https://github.com/nanoporetech/dorado) v0.7.3.1.

Methylation calling

The analyses were performed in CESGA supercomputing centre in Galicia, Spain. Pod5 files were indexed and aligned against the reference genome of water buffalo (*Bubalus bubalis*; NDDB_SH_1). Methylation calling was conducted using the dna_r10.4.1_e8.2_400bps_sup@v5.0.0 super-accurate model in Dorado, with the argument *–modified_bases 5mCG-5hmCG*. Base-named datasets were classified and demultiplexed into per-barcode BAMs using the *demux* command in Dorado. This process generated multiple BAM files stored in the output directory.

Methylation information was extracted using modbam2bed (https://github.com/epi2me-labs/modbam2bed) v0.10.0, with the *–extended* argument to include counts of canonical, modified, and filtered bases.

Differentially methylation analysis

Data were processed in R (version 4.3.2) using the Bioconductor package DSS v2.50.1 (Park and Wu 2016). Input files were formatted to include columns for chromosome names, genomic coordinates, total read counts and methylated read counts. CpG sites were filtered with a coverage threshold of $>$7x (sum of methylated and unmethylated read counts), and only common sites present across all samples were retained for analysis. This resulted in a total of 110,450 CpG sites included in the analysis.

Using the *makeBSseqData* function, a BSseq object was created, and two group labels (negative/positive) were assigned to define experimental conditions.

The *DMLtest* function was implemented imposing the smoothing option = 'True' to estimate mean methylation level. The obtained results were further processed for Differentially Methylated Cytosine/Regions (DMC/DMRs) identification. DMC were considered significant based on a posterior probability threshold using a delta set at 0.1, followed by filtering remaining sites with an FDR of 0.05. The same criteria were set up to apply the Call DMRs function that merge nearby loci into regions to form DMR. The

same criteria was applied to DMRs using callDMR function, which identifies statistically significant CpG regions and merges positions if at least 3 CpGs are within 50 base pairs. The DMCs and DMRs were classified into hypomethylated or hypermethylated features, using negative animals as control group and positive animals as mastitic group. In this context, we referred to a hypomethylated DMC/DMR when the methylation frequency was lower in the genome of the control group, and hypermethylated when the position exhibited a higher methylation in control group than in the mastitic group.

Annotation

Differentially methylated cytosine or regions were linked to the closest genes using the annotatePeak function in the default mode of the R package ChIPseeker version v1.40.0 (Yu et al. 2015).

The annotation considered regions spanning ±3 kb around the transcription start site (TSS). Depending on the location relative to the closest gene, each annotated site or region was categorised as 'Promoter,' Exonic,' Intronic' or 'Distal intergenic.' For these analyses, the reference genome used was *Bubalus bubalis* NDDB_SH_1, with supplementary genomic annotations provided by the associated GFF3 file (GenBank assembly accession: GCF_019923935.1), obtained from the NCBI Genome database.

Results

Sequencing results

The sequencing results are shown in Table 1. Quality assessment of the samples was performed using NanoPlot (v1.36.2) (De Coster and Rademakers, 2023). In particular the mean length of the samples were ~6 kb and the mean quality (Q score) was >13 for each sample, according to Oxford Nanopore sequencing metrics. Samples with a sequencing depth greater than 4X were retained for analysis ($n = 15$), excluding 1 sample (mastitic) for not passing the quality check. Table 1 also reports additional sequencing metrics, including the total number of reads, total bases generated, sequencing depth, and the number of modified bases detected at ≥7× coverage in the two groups.

The distribution of CpG site methylation percentages across all analysed samples follows a unimodal pattern, with most values concentrated at higher methylation levels (80–100%) (Supplementary file 1).

The CpG site distribution suggests a majority of CpG sites exhibiting high methylation levels, regardless of mastitis status.

The methylation profiles within both the positive and negative groups, exhibited a Pearson correlation coefficient, ranging from 0.91 to 0.95. The methylation frequency distributions for each sample appeared consistent and exhibited a left-skewed pattern. By comparison, inter-group correlations (between positive and negative samples) displayed slightly lower correlation values, ranging from 0.86 to 0.89 (Supplementary file 2).

Differential methylation analyses

A total of 22 differentially methylated cytosines (DMCs) were identified using a delta of 0.1, representing the minimum difference between the estimated mean methylation levels of the two groups (Supplementary file 3). A negative delta value corresponds to lower methylation in the control group compared to animals affected by mastitis, while a positive delta indicates higher methylation in the control group. Among the identified DMCs, 68% exhibited a hypomethylation pattern in the control group, while 32% displayed hypermethylation. In terms of DMRs, only one hypomethylated region was detected, located on chromosome 8.

These findings are represented by the violin plot (Figure 1), which shows the distribution of methylation differences between hypomethylated and hypermethylated sites. The central boxplot within each violin shows the interquartile range and median methylation difference, while the surrounding shape outlines the distribution pattern, highlighting regions where methylation differences are more concentrated. Wider sections of the violin indicate a higher concentration of DMCs with similar methylation differences.

The genomic annotation reveals distinct patterns for hypermethylated and hypomethylated cytosines. Among the hypermethylated DMCs, 15% were located in promoter regions, 57% were within intronic regions, and the remaining fraction resides in distal intergenic regions. In contrast, hypomethylated DMCs were overwhelmingly enriched in distal intergenic regions (93%), with only 7% found in the first intron (Figure 2a).

The distance of differentially methylated cytosines (DMCs) from the closest transcription start site (TSS) was also evaluated.

Hypermethylated DMCs are primarily enriched at distances of 10–100 kb from the TSS, accounting for most of their distribution. A smaller fraction is observed within 3–10 kb of the TSS, suggesting a

Table 1. Summary statistics of sequencing performance.

Samples	Mean lenght (kb)	Mean quality	Number of reads	Total bases	Sequencing depth (X)	Number of modified bases at 7x
B01_POS	9092.4	16.1	1,278,339	11,623,138,336	4.21	211,686
B02_POS	5172.8	17.4	8,639,370	44,689,659,004	16.15	21,722,125
B03_NEG	6495.0	17.2	3,511,399	22,806,436,254	8.09	2,395,421
B04_POS	6172.0	14.2	5,000,397	30,862,650,434	10.68	4,535,510
B05_POS	7002.0	13.6	3,062,092	21,440,891,484	7.37	1,040,494
B06_NEG	5313.1	14.5	5,084,849	27,016,546,262	9.41	3,054,790
B07_POS	6485.6	19.2	6,184,855	40,112,761,079	14.26	16,711,558
B08_NEG	6491.0	19.4	4,330,732	28,110,639,743	10.04	6,046,569
B09_NEG	6655.4	19.4	4,906,611	27,748,954,665	9.91	5,628,560
B10_POS	4202.7	19.6	4,257,668	17,893,811,745	6.99	1,161,118
B11_NEG	7554.8	19.5	24,069,059	37,422,415,922	13.07	10,689,933
B12_NEG	4278.0	19.7	4,576,061	19,576,256,845	7.06	1,584,369
B13_POS	4973.0	20.0	7,327,599	29,112,620,540	11.46	8,966,298
B14_NEG	5042.3	20.2	18,200,476	55,371,244,087	21.58	33,538,750
B15_NEG	5188.1	20.3	3,430,437	10,936,516,289	6.46	298,062
B16_POS	4531.1	20.6	1,423,800	6,451,441,763	2.72	114,430

POS = positive animals defined by a positive bacteriological culture, somatic cell count (SCC) >400,000 cells/mL, and differential somatic cell count (DSCC) >60%. NEG = negative animals, characterised by a negative bacteriological culture, SCC ≤200,000 cells/mL, and DSCC ≤60%. Mean length- = average read length in kilobases; Mean quality (Q score) = average Phred-like quality score; N. of reads = total number of reads generated; Total bases = total number of bases sequenced (bp); Sequencing depth (X) = coverage calculated as total bases divided by the reference genome size; N. of modified bases at ≥7× coverage = number of bases identified as modified with a minimum coverage greater than 7× (sum of methylated and unmethylated read counts).

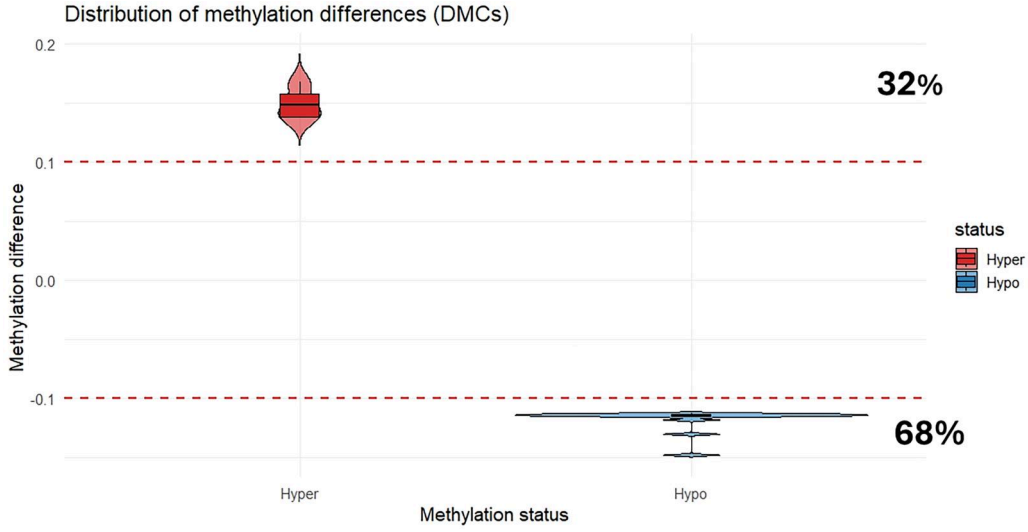

Figure 1. Distribution of the difference in methylation frequency for the DMCs between the two groups. The red lines indicate the delta threshold of 0.1, which was used to define the minimum difference between the estimated mean methylation levels. The violin plots display the overall distribution of methylation differences, with wider sections indicating a higher density of DMCs. The central boxplot within each violin shows the interquartile range and the median methylation difference, thus highlighting the relative proportions of hypo and hypermethylated sites in the control group compared to mastitis-affected animals.

preference for regulatory elements located further away from promoter-proximal regions. Hypomethylated DMCs also showed predominant enrichment at 10–100 kb from the TSS. However, a notable fraction (approximately 20%) was observed at distances greater than 100 kb, potentially reflecting long-range enhancer activity or other distal regulatory elements (Figure 2b).

Discussion

Mastitis is among the most important diseases affecting the global dairy industry, with substantial economic and animal welfare implications. To the best of our knowledge, this is the first study to investigate the role of DNA methylation on buffaloes affected by mastitis using nanopore sequencing technology.

We conducted a comparative analysis between a control and a mastitic group of buffaloes to explore the differential methylation patterns.

Previous reports indicate that an SCC >200,000 cells/mL in buffaloes can negatively affect milk yield and lactose percentage production (Cerón-Muñoz et al. 2002). Moreover, the European Union Directives set an SCC threshold of 400,000 cells/mL for milk intended for raw milk-based products. Based on these guidelines,

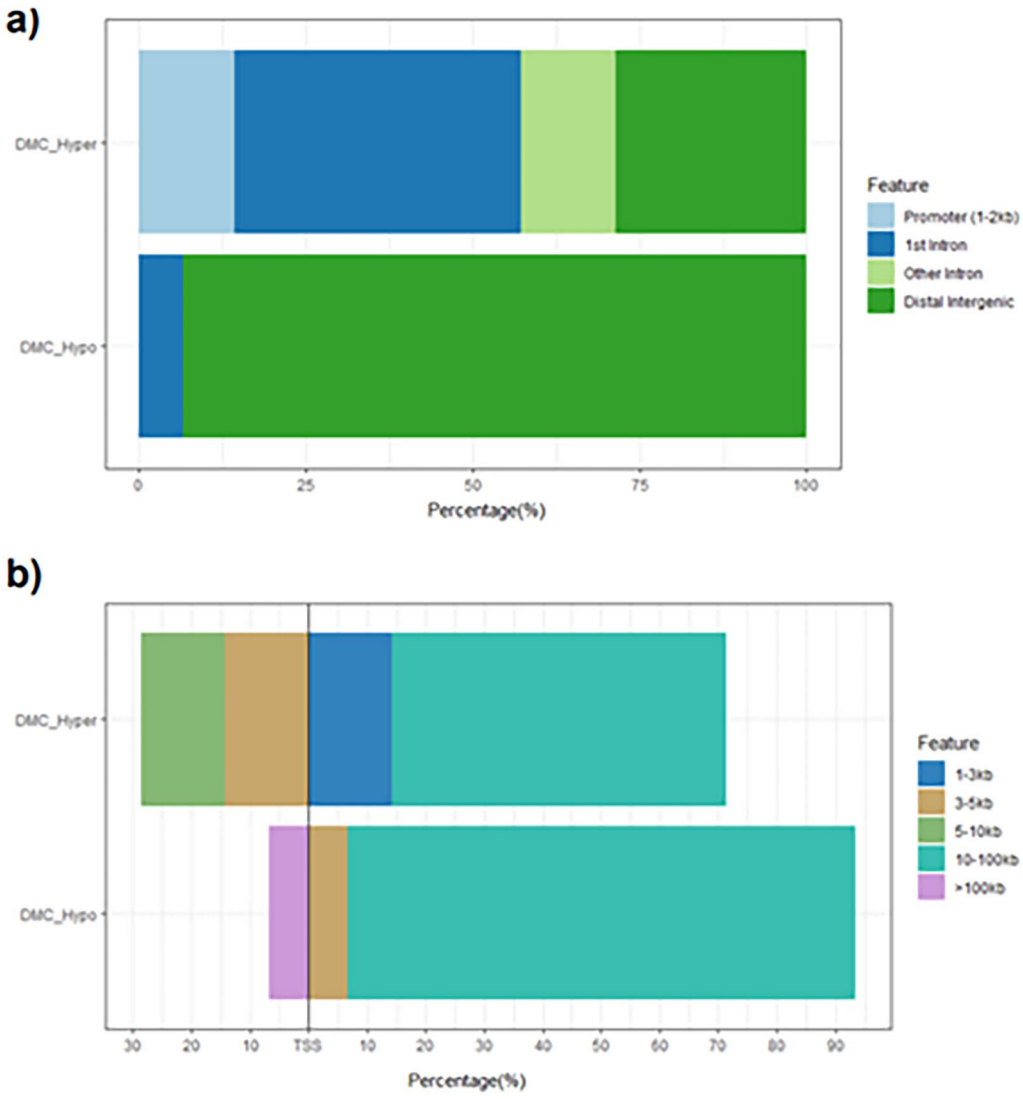

Figure 2. Distribution of genetic features associated differentially methylated cytosines (DMCs) according to their methylation status. a) illustrates the distribution of differentially methylated cytosines (DMCs) across genomic features. Hypermethylated DMCs were mainly located in intronic regions (57%), followed by promoter regions (15%), with the remaining fraction (28%) in distal intergenic regions. In contrast, hypomethylated DMCs were overwhelmingly enriched in distal intergenic regions (93%), with only a small proportion (7%) found in the first intron. b) distance of differentially methylated cytosines (DMCs) detected to the closest transcription start site (TSS). Hypermethylated DMCs were predominantly enriched at 10–100 kb from the TSS, with a smaller fraction within 3–10 kb. Hypomethylated DMCs also showed enrichment at 10–100 kb, but approximately 20% were observed at distances greater than 100 kb.

our study classified buffaloes as mastitic if they presented SCCs $\geq$ 400,000 cells/mL, DSCCs $\geq$ 60%, and positive bacteriological test results. Conversely, control group had SCCs $<$ 200,000 cells/mL, DSCCs $<$ 60%, and negative bacteriological results.

Traditional methods for detecting methylation marks typically involve bisulphite sequencing, due to their ability to accurately map DNA methylation at single-base resolution. However, these methods come with certain limitations, including the need for PCR amplification, strand synthesis, and chemical treatment, which can introduce biases, degrade DNA, and lead to incomplete methylation detection. In contrast, nanopore sequencing offers several distinct advantages that make it a powerful tool for studying DNA methylation, such as enabling simultaneous genotyping and epigenotyping in the same sequencing process even at a low sequencing depth (González-Recio et al. 2023), and no need for a PCR amplification or chemical conversion, hence preserving the native DNA structure and enabling direct detection of methylation marks. The ability to detect such modifications in real-time and at single-molecule resolution provides a more comprehensive view of the epigenome. Given these advantages, we opted for nanopore sequencing for this study.

Our results are consistent with broader studies in mammalian systems, where 70–80% of CpG sites across the genome are typically methylated (Li and Zhang, 2014).

The left-skewed (negatively skewed) distribution of methylation percentages suggests that the analysed tissue is globally hypermethylated, a characteristic typical of differentiated or quiescent tissues where most of the genome is transcriptionally silenced. Interestingly, this pattern differs from the canonical bimodal distribution typically reported in DNA methylation profiles.

We observed patterns of DNA methylation predominantly intercepting intergenic and intronic regions, in agreement with previous studies (Fang et al. 2017; Wang T et al. 2020).

It has been demonstrated that the methylation in intergenic regions has been implicated in transcriptional regulation through alternative start sites or microRNA expression, contributing to genome stability (Pheiffer et al. 2016).

Further, methylation within the first intron, a gene region often enriched with enhancers, is strongly associated with gene expression regulation (Anastasiadi et al. 2018).

Thus, epigenetic modifications in these regions may serve as valuable biomarkers of an animal's physiological status, as they influence gene activity beyond the commonly studied promoter-associated regulation.

While the overall pattern appears consistent across samples, this global trend could suggest the presence of site-specific variations, which could still contribute to the development of mastitis. In particular, our findings align with previous research demonstrating the average methylation frequency was higher in control groups compared to those with mastitis. This suggests may reflect the upregulation of specific genes potentially involved in the host response during the development of subclinical mastitis (Ju et al. 2020; Varij et al. 2022). Control group exhibited a higher proportion of hypomethylated DMCs, predominantly located farther from the transcription start sites (TSS), this pattern may suggest a tendency towards genomic stability in control group compared to those affected by mastitis. Mastitis was associated with alterations in DNA methylation patterns closer to the TSS, potentially impacting gene expression. We identified eight differentially methylated genes (DMGs), most of which are enriched in distal regions (4), intronic regions (3), and only one in a promoter region. The predicted genes (*LOC102394483, LOC123331926, LOC123331980, LOC112582810, LOC123331776, LOC112581131, LOC123333069*, and *LOC123331969*) show distinct functional roles (Supplementary file 4). The first four are involved in balancing immune responses, managing cellular stress, promoting tissue repair, and resolving inflammation during mastitis. The remaining genes are still uncharacterised, with no reliable information currently available.

LOC102394483 is a gene encoding a ubiquitin carboxyl-terminal hydrolase 17–like protein, a key enzyme in the ubiquitin-proteasome system (UPS). The UPS is essential for the degradation of oxidised or misfolded proteins, playing a pivotal role in cellular stress responses and immune surveillance. The activity of this gene supports more efficient protein turnover, reducing cellular stress and promoting homeostasis under non-disease conditions (Bozaykut et al. 2013; Bhat et al. 2022; Chen et al. 2024). Notably, this gene was found to be hypomethylated in the control group.

LOC123331926 is an endogenous retrovirus-derived pseudogene with sequence homology to viral envelope polyproteins, specifically those of the PABLB group. Endogenous retroviruses (ERV) are remnants of ancient viral infections that have been integrated into the host genome over evolutionary time. Although typically non-functional, some ERV sequences are hypothesised to retain roles in regulating gene expression and modulating immune responses.

LOC123331926 was found to be hypermethylated in the intronic region in the control group. This increased methylation might reflect transcriptional repression of *LOC123331926*, potentially serving as a protective mechanism to limit ERV activation and reduce the risk of immune dysregulation. In contrast, the hypomethylation observed in mastitic groups might be linked to increased ERV expression, which has been hypothesised to contribute to chronic inflammation or immune overactivation.

However, further studies are needed to elucidate the functional relevance of these observations.

Similar mechanisms have been described in humans, where ERVs are thought to trigger inflammatory processes through multiple pathways involving both the innate and adaptive arms of the immune system (Manghera and Douville, 2013; Gruchot et al. 2023).

LOC123331980 encodes a melanoma-associated antigen B4-like protein. The MAGE family is known to regulate critical cellular processes, including autophagy, apoptosis, DNA repair, and mRNA stability, while also playing a key role in stress responses. Although the activation of this gene may initially enhance cellular defences, prolonged or excessive activity could

contribute to tissue damage and chronic inflammation.

This hypothesis aligns with findings from other studies on MAGE proteins, which are often upregulated under stress conditions and play a role in cellular remodelling during pathological status (Florke et al. 2020).

LOC112582810 encodes a protein with a phosphoinositide phospholipase C X (PI-PLC X) domain, typically found in enzymes involved in cell physiology and signal transduction. PI-PLC isozymes play essential roles in cell metabolism by regulating calcium signalling and other intracellular pathways important for cell proliferation and differentiation in humans (Cocco et al. 2015).

In our study, *LOC112582810* was found to be hypermethylated in the control group compared to individuals affected by mastitis.

This study used 16 samples from a single farm. While this controls for environmental effects, the small sample size may limit statistical power and the robustness of the findings. To validate these results and gain a more accurate understanding of methylation patterns, increasing the number of animals analysed is crucial.

Additionally, while differentially methylated genes were identified, their direct impact on gene expression was not assessed. Since most of these genes were located in intergenic regions, it is crucial to investigate their transcriptional activity through RNA-seq or similar approaches. This would help determine the functional relevance of these epigenetic modifications and their potential effects on proximal regulatory regions.

Furthermore, *Bubalus bubalis* genome has not been fully characterised and annotated, limiting the availability of detailed data on genomic markers and specific biological pathways. In this context, this study provides valuable new insights, contributing to the expansion of genomic knowledge in this species and laying the groundwork for future research.

Conclusions

The present study is the first to explore DNA methylation changes associated with mastitis in the Mediterranean Italian River Buffalo using nanopore sequencing. This powerful technology enables simultaneous genotyping and epigenotyping, making it valuable for precision breeding and for understanding epigenetic contributions to economically important traits.

We identified distinct DNA methylation patterns between the control group and animals affected by mastitis.

Our findings provide valuable insights into the epigenetic regulation of mastitis and may highlight potential genetic mechanism associated with the disease. This knowledge could support buffalo breeders in implementing disease management programs, eventually enhancing strategies for early detection and prevention.

This study is based on a small number of animals, which reduces its statistical power. As such, the findings are exploratory, and future studies will aim to increase the sample size to confirm these results and ensure their reliability.

Future research should also investigate the correlation between DNA methylation patterns in blood cells and milk somatic cells to assess the potential of blood analysis as an indirect marker of mastitis-related epigenetic changes. This approach could facilitate the development of new diagnostic tools for early mastitis detection.

In addition, integrating transcriptomic and metabolomic data with DNA methylation profiles may provide a more comprehensive understanding of the molecular mechanisms underlying mastitis process.

Integrating epigenetic insights into breeding and management practices has the potential to improve buffalo health and increase dairy production efficiency.

Ethical approval

The present study was approved by the Institutional Animal Care and Use Committee, University of Pisa (N: 11/2021 of 19.03.2021). All samples were collected in compliance with the European rules (Council Reg- ulation [EC] No. 1/2005 and Council Regulation [EC] No. 1099/2009). The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Authors' contributions

SA and OGR: conceptualisation; OGR and ALC: methodology; TG and ED: sampling; IC and TG: investigation; IC and ALC data curation; IC: visualisation; SA and OGR: validation; ED and FC: supervision; GC, AS, TG, VP: project administration and funding acquisition; IC and ALC: writing-original draft; SA, FC, MB, OGR: review and editing. All the authors have read and approved the final manuscript.

Disclosure statement

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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