



Methylome-wide analysis of milk somatic cells upon subclinical mastitis in dairy cattle

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ABSTRACT

Better understanding of the molecular mechanisms behind bovine mastitis is fundamental for improving the management of this disease, which continues to be of major concern for the dairy industry, especially in its subclinical form. Disease severity and progression depend on numerous aspects, such as livestock genetics, and the interaction between the causative agent, the host, and the environment. In this context, epigenetic mechanisms have proven to have a role in controlling the response of the animal to inflammation. Therefore, in this study we aimed to explore genome-wide DNA methylation of milk somatic cells (SC) in healthy cows ($n = 15$) and cows affected by naturally occurring subclinical mastitis by *Streptococcus agalactiae* ($n = 12$) and *Prototheca* spp. ($n = 11$), to better understand the role of SC methylome in the host response to disease. Differentially methylated regions (DMR) were evaluated comparing: (1) *Strep. agalactiae*-infected versus healthy; (2) *Prototheca*-infected versus healthy, and (3) mastitis versus healthy and (4) *Strep. agalactiae*-infected versus *Prototheca*-infected. The functional analysis was performed at 2 levels. To begin with, we extracted differentially methylated genes (DMG) from promoter DMR, which were analyzed using the Cytoscape ClueGO plug-in. Coupled with this DMG-driven approach, all the genes associated with promoter-methylated regions were fed to the Pathifier algorithm. From the DMR analysis, we identified 1,081 hypermethylated and 361 hypomethylated promoter regions in *Strep. agalactiae*-infected animals, while 1,514 hypermethylated and 358 hypomethylated promoter regions were identified in *Prototheca*-infected animals, when compared with the healthy controls. When con-

sidering infected animals as a whole group (regardless of the pathogen), we found 1,576 hypermethylated and 460 hypomethylated promoter regions. Both pathogens were associated with methylation differences in genes involved in pathways related to meiosis, reproduction and tissue remodeling. Exploring the whole methylome, in subclinically infected cows we observed a strong deregulation of immune-related pathways, such as nuclear factor kB and toll-like receptors signaling pathways, and of energy-related pathways such as the tricarboxylic acid cycle and unsaturated fatty acid biosynthesis. In conclusion, no evident pathogen-specific SC methylome signature was detected in the present study. Overall, we observed a clear regulation of host immune response driven by DNA methylation upon subclinical mastitis. Further studies on a larger cohort of animals are needed to validate our results and to possibly identify a unique SC methylome that signifies pathogen-specific alterations.

Key words: methyl-binding domain sequencing, milk somatic cells, subclinical mastitis, *Prototheca*

INTRODUCTION

Mastitis is a major concern for the dairy industry with consequent animal welfare and economic losses due to the adverse effects on milk production, quality and composition (Seegers et al., 2003). Bovine mastitis is mainly caused by infection with a wide range of microorganisms, among which the most common are *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis* (Ranjan et al., 2006). *Streptococcus agalactiae* is a gram-positive bacterium mostly responsible for inducing subclinical or chronic forms of the disease (Lewandowska-Sabat et al., 2019), which has recently reemerged as a significant causative agent of mastitis, despite the implementation of eradication programs (Barsi et al., 2022).

In contrast, algae of the genus *Prototheca*, which a few years ago were labeled as uncommon mastitis

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agents, have rapidly emerged as non-negligible pathogens (Shave et al., 2021). Due to their capacity to infect and survive within macrophages and invade mammary tissue, they cause persistent infection with intermittent shedding (Roesler and Hensel, 2003). In addition, as *Prototheca* spp. does not respond to antibiotic mastitis therapy, the only effective control method to date has been the elimination of the infected animals (Libisch et al., 2022), which represents a serious challenge for the dairy sector.

The mastitis-causing pathogens induce an inflammatory response in the mammary gland. In response to the inflammation the host secretes cytokines causing changes in the regulation of gene expression (Oviedo-Boyso et al., 2007). There is growing evidence on the epigenetic regulation of innate immunity genes expression (Chen et al., 2014). Epigenetic mechanisms seem to play a role in the trained immunity, as they establish a transcriptional profile that modifies the signaling and metabolism of innate immune cells, which onsets a long-lasting adaptation (Netea et al., 2011). DNA methylation, the most studied form of epigenetic modifications, is not only associated with gene repression, but also with gene activation (Bahar Halpern et al., 2014), splicing regulation (Shukla et al., 2011), nucleosomes positioning (Chodavarapu et al., 2010), and the recruitment of transcription factors (Fujimoto et al., 2005).

DNA methylation is involved in the response to pathogen challenge and ultimately in the occurrence and development of bovine mastitis (Vanselow et al., 2006; Chang et al., 2015; Zhang et al., 2018). Differences in the DNA methylation of peripheral blood lymphocytes have been observed between mastitis affected and healthy cows (Song et al., 2016; Ju et al., 2020).

Given the fact that epimutations can be transmitted to future generations, there is interest in studying the associations between the epigenome and the development of desired phenotypes (Ibeagha-Awemu and Zhao, 2015) as they could help understanding the outcome of complex dynamics between genotype and phenotype. Therefore, identifying epigenetic signatures associated with mastitis resistance can be useful in breeding programs aimed at improving animal welfare in dairy cattle.

To our knowledge, changes in genome-wide DNA methylation in bovine subclinical mastitis naturally induced by *Strep. agalactiae* and *Prototheca* have not yet been investigated. Hence, the present study aimed to provide the landscape of DNA methylome in bovine milk somatic cells (SC) of Holstein cows with natural subclinical mastitis induced by *Strep. agalactiae* and *Prototheca* spp. Understanding the possible regulatory roles of DNA methylation upon mastitis can lay the

groundwork for mechanistic studies on susceptibility to mastitis in dairy cattle.

METHODS

Animal Data

This research was approved by the Ethical Animal Care and Use Committee (OPBA–Organismo Preposto al Benessere degli Animali) of the Università Cattolica del Sacro Cuore and by the Italian Ministry of Health (protocol number 510/2019-PR of 19/07/2019).

Thirty-eight Holstein cows ranging from 2 to 5 parity and between 92 and 448 DIM were selected from one commercial farm of 450 lactating cows (Veneto region, Italy) regularly monitored by the Istituto Zooprofilattico delle Venezie (IZSVe) for the presence of *Strep. agalactiae* and *Prototheca* spp. between January 2020 and February 2021. The farm was selected because of previous collaborations with the dairy farm owners and their associated veterinary practices. To reduce to a minimum the sources of variation which could affect the methylome analysis, animals were selected based on the following criteria: (1) absence of any clinical sign of disease for at least 1 yr; (2) no medical treatment for at least 6 mo before enrollment; (3) being multiparous (parity ≥ 2) and in mid-late lactation (≥ 92 DIM), to exclude cows with negative energy balance condition, which could affect the proper activation of the immune cells metabolism in response to pathogens' invasion (Wathes et al., 2009; Ingvarstsen and Moyes, 2013). Moreover, we required that animals assigned to the healthy group had no previous history of clinical mastitis. Cows with clinical signs of mastitis or other diseases (e.g., metritis, hepatic lipidosis, ketosis, abscesses, laminitis) as well as animals under medical treatment were excluded from the trial. Animals' data were collected from the herd management software (Dairy Comp Sata, Alta Italia Srl, Milan, Italy). Considering these criteria, the identification of healthy individuals and cows with subclinical mastitis from either *Strep. agalactiae* or *Prototheca* spp. was based on an initial bacteriological screening (time 0, T0), which was conducted on 188 lactating cows. Then, a second bacteriological examination was conducted 2 wk after T0 (T1) to confirm the animals' infection status. Between the T0 and T1 animals underwent daily monitoring by both the farmers and the local veterinarian to ensure they remained free from any visible signs of mastitis, thereby maintaining their subclinical condition. Following the results of the bacteriological test at T0 and T1, 3 experimental groups were defined: (1) healthy individuals ($n = 15$) with a negative bacteriological examination in all glands at T0 and T1; (2) animals

naturally infected by *Strep. agalactiae* ($n = 12$); and (3) animals naturally infected by *Prototheca* spp. ($n = 11$), having a positive bacteriological result for either *Strep. agalactiae* or *Prototheca* spp., respectively. Animals with co-infections with either environmental or other microorganisms responsible of mastitis were excluded from the experiment. Cows were fed TMR formulated to meet or exceed the requirements of mid-lactation dairy cattle, mainly based on corn silage, sorghum silage, and concentrate (Pegolo et al., 2023). Drinking water was available in automatic water bowls, and cows were milked twice a day, from 2 a.m. to 6 a.m. and from 2 p.m. to 6 p.m. Animal welfare was managed by the farmers and local veterinarians, who intervened when needed.

Milk Sampling

Before morning milking, ~200 mL of milk from all quarters (pool sample) was aseptically collected from each animal according to the National Mastitis Guidelines (NMC, 2017). Briefly, the teat was disinfected pre-milking and, after discarding the first stream of foremilk from each quarter, composite milk from the 4 glands was collected. For each milk sample, 3 aliquots (~50 mL) were collected and gently mixed into sterile tubes for the following analyses: (1) bacteriological test; (2) milk composition, SCC, and differential SCC (**DSCC**); and (3) DNA extraction and DNA methylation analysis. All the samples were immediately refrigerated at 4°C and transferred to the different laboratories.

Microbiological Analysis

Microbiological examination of milk samples was conducted at the IZSve laboratories (Legnaro, PD, Italy). Samples were frozen and analyzed within 3 d. Details of microbiological analysis are reported in Pegolo et al. (2022). Briefly, 10 μ L of every composite sample were inoculated in the following selective media: (1) tallium kristalviolette tossin agar (IZSve internal production), and (2) *Prototheca* isolation medium (**PIM**; IZSve internal production). After 24 h of incubation, suspected colonies of *Strep. agalactiae* were confirmed using the Christie–Atkins–Munch–Peterson test (NMC, 2017). After 24, 48, and 72 h, PIM plates were examined for the growth of *Prototheca*. Suspected colonies were confirmed by mass-spectrometry analysis (NMC, 2017). Furthermore, a screening of the most common microorganisms responsible for mastitis, such as *Staph. aureus* and *Strep. uberis*, as well as some environmental ones, such as *Streptococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., and *Enterococcus* spp. was conducted to avoid possible bias in the trial.

Milk Composition and Quality Traits

Milk composition (protein, casein, lactose, fat, and urea content) and udder health traits (lactose, milk conductivity, and pH) were assessed on fresh samples using an FT6000 Milkoscan infrared analyzer (Foss A/S, Hillerød, Denmark). Both SCC and DSCC were measured through the Fossomatic 7 DC analyzer (Foss A/S). The SCC were log-transformed to SCS according to Ali and Shook (1980).

DNA Extraction from Milk SC

A 50-mL aliquot from each sample was centrifuged at $500 \times g$ for 10 min at 4°C. The fat layer and the supernatant were discarded, and the cell pellet was washed with 50 mL of PBS containing EDTA at 0.05 mM, pH 7.2. Samples were then centrifuged again at $1,500 \times g$ for 10 min at 4°C, the supernatant discarded, and the pellet stored at -80°C until the DNA extraction. Genomic DNA was isolated from SC pellet using the commercial kit NucleoSpin Tissue (Macherey-Nagel, Düren, Germany), following manufacture instruction and an overnight proteinase K digestion. The DNA quality was evaluated by agarose gel electrophoresis and DNA concentration was estimated by PicoGreen (Thermo Fisher, Waltham, MA).

Library Preparation and Sequencing

About 200 ng of genomic DNA from each sample were sonicated to produce DNA fragments between 300 and 350 bp using Covaris S220 instrument (Covaris, Woburn, MA). DNA was enriched for methyl-binding domain (**MBD**) using the MethylMiner Methylated DNA Enrichment Kit (Invitrogen, Carlsbad, CA), following manufacturer instructions. Library construction was performed using the TruSeq Nano Library Preparation Kit (Illumina, San Diego, CA). Libraries were quantified and quality checked with 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the High Sensitivity DNA Kit (Agilent Technologies). Libraries were sequenced on an Illumina Hiseq X (Macrogen, Seoul, Republic of Korea) to generate 150 base-paired end reads for high confidence mapping of captured fragments. The MBD sequencing (**MDB-Seq**) data are available at the Sequence Reads Archive, BioProject accession number, PRJNA976818 (<https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA976818>).

Bioinformatic Analysis

The MBD-Seq reads were analyzed as described in detail by Decock et al. (2016). Briefly, raw reads were

quality checked using FASTQC tool v.0.11.9 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and were aligned to the *Bos taurus* genome ARS-UCD1.2 (Ensembl release 108) using Bowtie 2 v.2.2.9 (Langmead and Salzberg, 2012). The PCR duplicates were marked with Picard v. 2.6.0 (<http://broadinstitute.github.io/picard/>) and the BAM files were sorted and indexed using SAMtools v.1.9 sort and index commands. The detection of enriched regions (peak calling) was performed using MACS2 v.2.1.1 (Zhang et al., 2008) using the callpeak function. BED files with location and score (linked to the *P*-value) of the identified peaks were generated with bedtools v.2.17.0 (<https://bedtools.readthedocs.io/en/latest/>). Due to the inconsistencies of the number of peaks called (low average number of peaks compared with other samples), 2 samples from the group of *Strep. agalactiae*-infected were discarded from subsequent analysis. The DiffBind package (Ross-Innes et al., 2012) in the R environment (<https://www.R-project.org/>) was used to create the consensus peak set matrix, which includes merged peaks that overlap in at least 2 samples and using the option *summits = 300*, which results in 601 bp peaks (the summit point plus 300 bp in each direction) representing the consensus sites. Then, DeSeq2 package (Love et al., 2014) was called via DiffBind package to perform the differential methylation analysis. Reads were normalized using a library size normalization based on Reads in Peaks. Four comparisons were performed: (1) *Strep. agalactiae*-infected versus healthy samples; (2) *Prototheca*-infected versus healthy samples, (3) mastitis versus healthy samples and finally (4) *Strep. agalactiae*-infected versus *Prototheca*-infected samples. Genomic regions that were significantly differentially methylated ($P < 0.05$; false discovery rate [FDR] corrected) were considered as differentially methylated regions (DMR). To quantify the changes between 2 conditions, \log_2 fold change (FC) was used. Differentially methylated regions were annotated using HOMER v.4.11 (<http://homer.ucsd.edu/homer/>) annotatePeaks function to extract differentially methylated genes (DMG). The promoter-transcription start site (TSS) was defined when the distance from the gene is comprised from -1kb to $+100\text{ bp}$ and the transcription termination site (TTS) from -100 bp to $+1\text{kb}$. If a region mapped to more than one gene, a priority was assigned based on the shorter distance from the promoter region. All downstream analyses did not account for the sexual and mitochondrial chromosomes.

Exploratory and Functional Analyses

Exploratory analysis of the matrix built on the 24,916 methylated regions was conducted through

a principal component analysis (PCA) after the regularized-logarithm transformation of the counts data using the DESeq2 package. Euclidean distances between samples were calculated and visualized in a heatmap. Moreover, for each experimental comparison, the R package GenomicFeatures (v.1.46.4) was applied to display the chromosomal locations of the DMR. The *exonsBy()* function was used to extract the coordinates of the associated genes, which were then plotted using the *plotGrandlinear()* function in the *ggbio* package (v.1.42.0; Yin et al., 2012).

For functional analyses, the DMG extracted from DMR at promoters were fed to the Cytoscape (v.3.9.1, <http://cytoscape.org>) ClueGO plug-in (v.2.5.9; Bindea et al., 2009). Default parameters for the identification of relevant ($P < 0.05$, Benjamini-Hochberg corrected) biological processes, molecular function, cellular component, and immune systems related pathways were used. The “GO term fusion” option was applied for reducing pathway redundancy and preserving the more representative terms. A second complementary approach was adopted using the whole set of genes associated with promoter-methylated regions of healthy animals and infected animals using the Pathifier algorithm from the Pathifier R package (v.1.32.0; Drier et al., 2013). With this algorithm we transformed the methylated gene-level information into pathway-level information, inferring how much a specific pathway deviated from the control samples, by computation of the pathway deregulation scores (PDS). The PDS were calculated by constructing a *n*-dimensional space ($n = \text{number of genes in the path}$), where a main curve that captures the variation of a cloud of points is calculated by nonlinear regression. Each point represents each sample and its values of expression of the *n* genes of the pathway, and expressed as the distance of the projection to the main curve of each sample with respect to the projection of normal samples. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was used. A heatmap was then built using the samples Euclidean distances and the Ward.D2 clustering method.

RESULTS

Milk Composition and Quality Traits

The average milk yield was $27.13 (\pm 8.70)$ kg/d. Milk was $2.12\% \pm 0.74\%$ fat, $3.50\% \pm 0.27\%$ protein, and $2.74\% \pm 0.24\%$ casein, 22.03 ± 4.92 mg/100 mL of urea, and $4.54\% \pm 0.42\%$ lactose. Milk pH and conductivity were 6.46 ± 0.08 and 9.84 ± 1.22 mS/cm, respectively (Table 1). *Streptococcus agalactiae* and *Prototheca* spp. infections decreased milk production ($P < 0.05$, Figure 1A). In addition, milk samples from

Table 1. Milk yield and composition of Holstein cows (n = 38)¹

Item	Mean	SD	P1	P99
Milk yield, kg/d	27.13	8.70	10.44	43.90
Milk composition	2.12	0.74	1.02	3.42
Fat, %				
Protein, %	3.50	0.27	2.99	4.16
Casein, %	2.74	0.24	2.31	3.32
Urea, mg/100 g	22.03	4.92	12.64	35.39
Udder health traits	4.54	0.42	3.51	5.20
Lactose, %				
SCC, 10 ³ cells/mL	1,514	2,297	19.88	9,280
SCS	5.06	2.61	0.43	9.52
DSCC, %	63.59	13.27	33.73	83.36
pH	6.46	0.08	6.29	6.62
Milk conductivity, mS/cm	9.84	1.22	8.09	12.62

¹The SCC score is calculated as $\log_2(\text{SCC}/100,000) + 3$ (Ali and Shook, 1980); DSCC = differential SCC; P1 = first percentile; P99 = 99th percentile.

Strep. agalactiae-infected animals had a lower lactose percentage with respect to the healthy animals ($P < 0.05$, Figure 1B). Both pathogens increased SCS ($P < 0.001$, Figure 1C) and *Prototheca* also increased DSCC ($P < 0.05$, Figure 1D).

Milk SC DNA Methylation Landscape

The MBD-Seq experiment on milk SC produced an average of over 46 million reads per sample (range 30 million to 68 million). Quality control and trimming procedures retained the majority of reads obtained (97%), with a mean percentage of uniquely mapped reads to the *Bos taurus* genome of 70% (range 53%–90%; Supplemental Table S1, <https://doi.org/10.6084/m9.figshare.24442003>).

DNA Methylated Regions Distribution. After filtering steps and assigning genome annotation with HOMER, the consensus peak set gave a total of 221,910 methylated regions of which 123,835 (55.8%) intergenic, 82,969 (37.4%) intronic, 8,827 (4%) exonic, 3,413 (1.5%) in the promoter-TSS, and finally 2,866 (1.3%) in the TTS (Figure 2). In detail, the methylation autosomal maps show the genome-wide DNA methylation distribution in the studied samples (Supplemental Figure S1 and Supplemental Table S2, <https://doi.org/10.6084/m9.figshare.24442003>). The methylation sites were unevenly distributed across bovine autosomes; BTA with the highest level of methylation sites were BTA3 (5.7%), BTA5 (5.7%), and BTA7 (5.6%). Additionally, methylation sites were generally more concentrated at the telomeres.

Exploratory Analyses of DNA Whole Methylome Data. The generalized PCA (Figure 3A) built with the 24,916 genes associated with methylated regions shows that the degree of methylation varied according to the status of infection and allowed a good

separation between healthy and infected samples (except for 2 healthy animals that clustered with the infected ones; principal component 1: 60%) while no clear separation was observed between the *Strep. agalactiae* and *Prototheca* spp. infected samples. A similar finding was evidenced using a supervised clustering using the 500 highly variable methylated regions (Figure 3B), where all samples clustered perfectly between healthy and mastitic animals but failed to differentiate the type of pathogen.

DNA Methylation Changes upon IMI

Differentially Methylated Regions. The DMR analysis focused on the 3,413 promoter-TSS regions identified in the consensus peak set. In the comparison *Strep. agalactiae*-infected versus healthy cows, we found 1,442 significant DMR, of which 1,081 were hypermethylated and 361 were hypomethylated in *Strep. agalactiae*-infected cows (Supplemental Figure S2, <https://doi.org/10.6084/m9.figshare.24442003>). When annotated to genes, the most hypermethylated DMR were co-located with *ARL6* ($\log_2\text{FC} = 1.7$), *IL17REL* ($\log_2\text{FC} = 1.3$), and *AP1G1* ($\log_2\text{FC} = 1.2$), whereas the most hypomethylated DMR were co-located with *GLT8D2* ($\log_2\text{FC} = -5.1$), *ANGPTL4* ($\log_2\text{FC} = -5.1$), and *IL10RB* ($\log_2\text{FC} = -3.2$; Supplemental Table S2).

Concerning the *Prototheca*-infected versus healthy cow comparison, samples showed a higher number of DMR compared with *Strep. agalactiae*-infected samples, with 1,872 significant DMR of which 1,514 were hypermethylated and 358 were hypomethylated (Supplemental Figure S2). The most hypermethylated DMR were co-located with *ARL6* ($\log_2\text{FC} = 1.8$) and *HACD2* ($\log_2\text{FC} = 1.5$), and *PITHD1* ($\log_2\text{FC} = 1.4$), whereas the most hypomethylated DMR were co-located with *ADAM11* ($\log_2\text{FC} = -5.6$), *APAF1* ($\log_2\text{FC} = -5.1$),

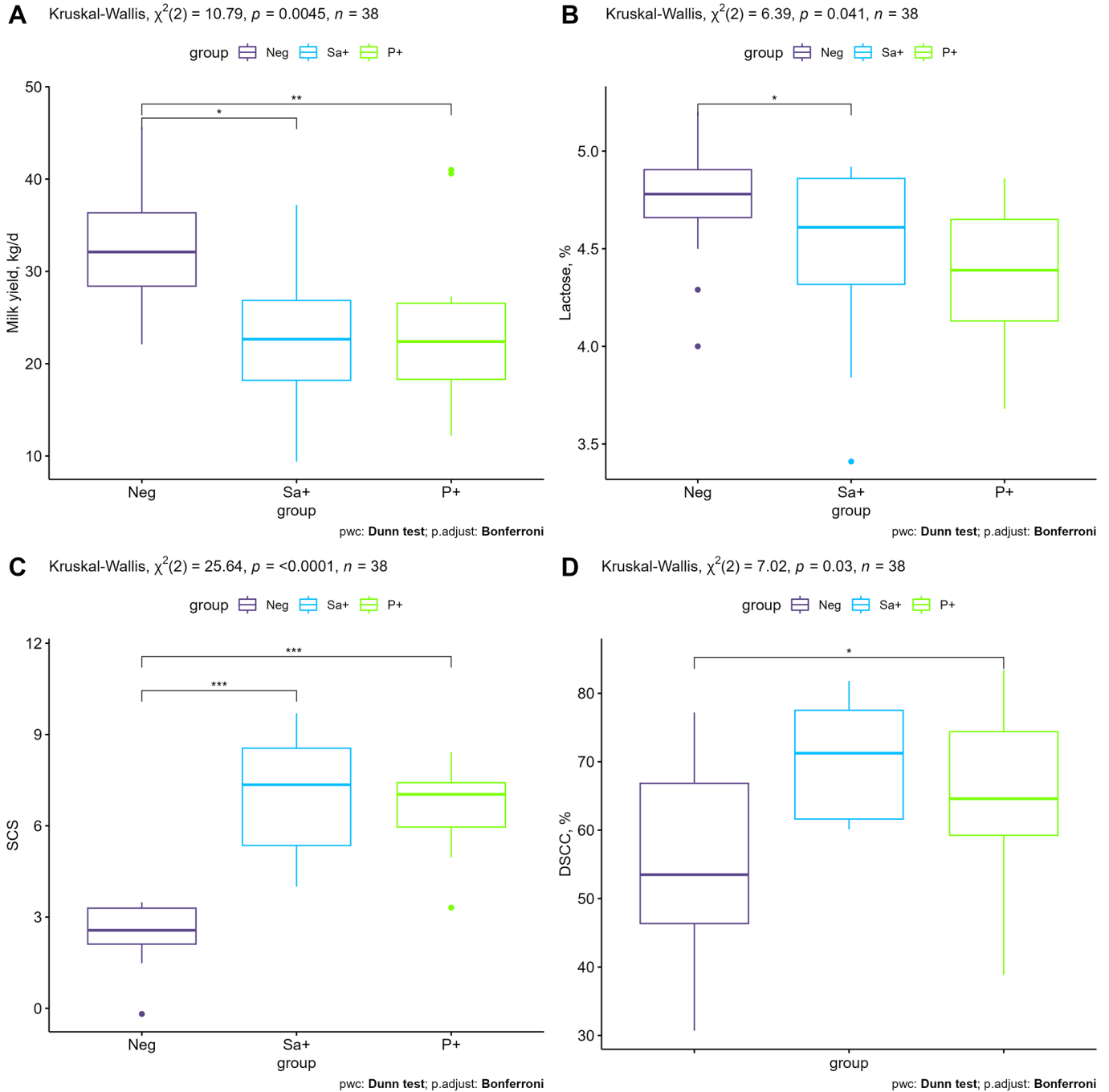


Figure 1. Boxplots of (A) milk yield, (B) milk lactose, (C) SCS, and (D) DSCC according to the 3 experimental groups of Holstein cows ($n = 38$). Only significant traits are depicted. Each box represents the interquartile range (IQR), indicating the middle 50% of the data. The horizontal line inside the box represents the median of the group. The whiskers show the variation of data, and points beyond these lines represent samples falling outside the IQR. Healthy = clinically healthy cows, negative at the bacteriological examination; Sa+ = animals infected by *Streptococcus agalactiae*; P+ = animals infected by *Prototheca* spp.; DSCC= differential SCC. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

KLRG2 ($\log_2FC = -5.1$), and *MAPKAPK2* ($\log_2FC = -4.9$; Supplemental Table S2).

When comparing subclinically infected animals, regardless of the specific pathogen, with healthy cows,

we identified 2,036 significant DMR, of which 1,576 were hypermethylated and 460 were hypomethylated (Supplemental Figure S2). Once annotated to genes, the most hypermethylated promoter regions were co-

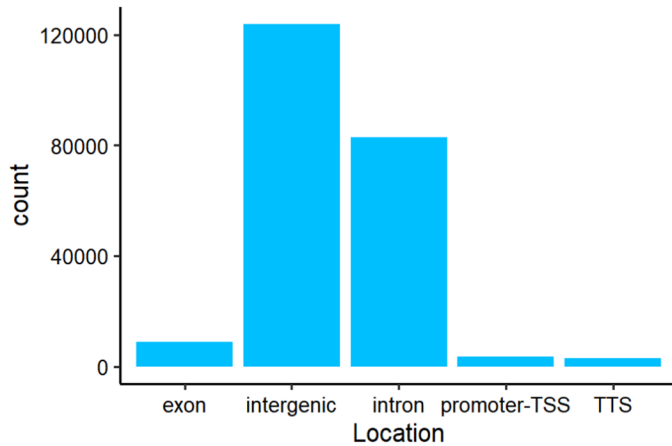
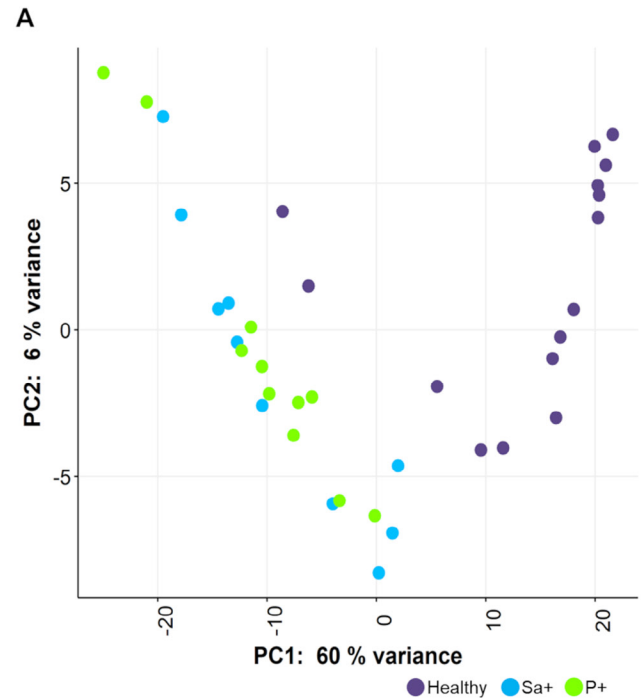


Figure 2. Distribution of DNA methylation peaks in the genome of animals with subclinical mastitis and healthy. TSS = transcription start site; TTS = transcription termination site.

located with *ARL6* ($\log_2FC = 1.8$), *PLAU* ($\log_2FC = 1.7$), *COX5B* ($\log_2FC = 1.5$), and *IL17REL* ($\log_2FC = 1.3$). Conversely, the most hypomethylated promoter regions were co-located with *TEK* ($\log_2FC = -5.1$), *LEO1* ($\log_2FC = -5.0$), *RALB* ($\log_2FC = -4.8$) and *ICAM4* ($\log_2FC = -4.8$; Supplemental Table S2).

The last comparison for evidencing DMR that might distinguish the 2 types of infection (i.e., *Strep. agalactiae* vs. *Prototheca*) resulted in only 4 significant DMR (hypomethylated in *Strep. agalactiae*-infected cows). Accordingly, we found 683 hypermethylated and 250 hypomethylated associated genes in common with the comparisons of *Prototheca* versus healthy, *Strep. agalactiae* versus healthy, and mastitis versus healthy, which did not suggest a highly divergent cow response to the 2 pathogens in the investigated population (Supplemental Figures S3 and S4, <https://doi.org/10.6084/m9.figshare.24442003>). Pathogen-specific hypermethylated genes were 2 (*Strep. agalactiae*) and 34 (*Prototheca*) and hypomethylated genes were 10 (*Strep. agalactiae*) and 4 (*Prototheca*).

Functional Analyses of DMG. Using ClueGO, we found that DMG associated with promoter DMR acted coordinately within specific biological functions (Figure 4). *Streptococcus agalactiae* infection was associated with the enrichment of several pathways related to meiosis and reproduction (e.g., meiotic cell cycle, FDR = 0.035; developmental process involved in reproduction, FDR = 0.038); nucleotide metabolism (e.g., purine nucleotide binding, FDR = 0.038; ribonucleotide binding, FDR = 0.035); fatty acid metabolism (long-chain fatty-acyl-CoA metabolic process, FDR = 0.030; very long-chain fatty acid metabolic process, FDR = 0.032); calcium transport (calcium activated cation channel activity, FDR = 0.032; calcium ion transport,



B
Top 500 most variable methylation sites across samples

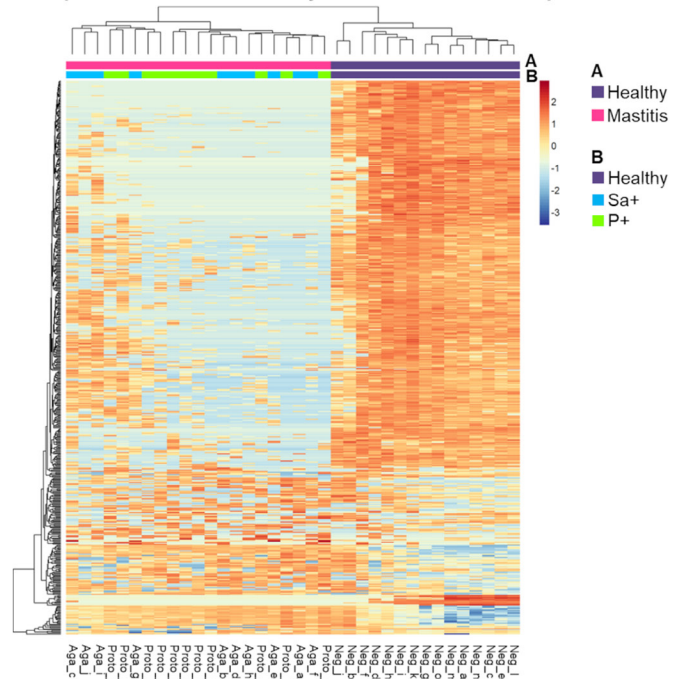


Figure 3. (A) Principal component analysis (PCA) plot of milk samples built using the entire methylome. Principal component 1 and 2 (PC1 and PC2) account for 60% and 6% of the variance between samples, respectively. (B) Heatmap with Z-score clustering based on Euclidean distance. Each row represents the gene associated with a methylated region while each column represents a sample. Bars above the plot represent the samples' categories. Healthy = clinically healthy animals, negative at the bacteriological examination; Mastitis = animals with subclinical mastitis; Sa+ = animals infected by *Streptococcus agalactiae*; P+ = animals infected by *Prototheca* spp.

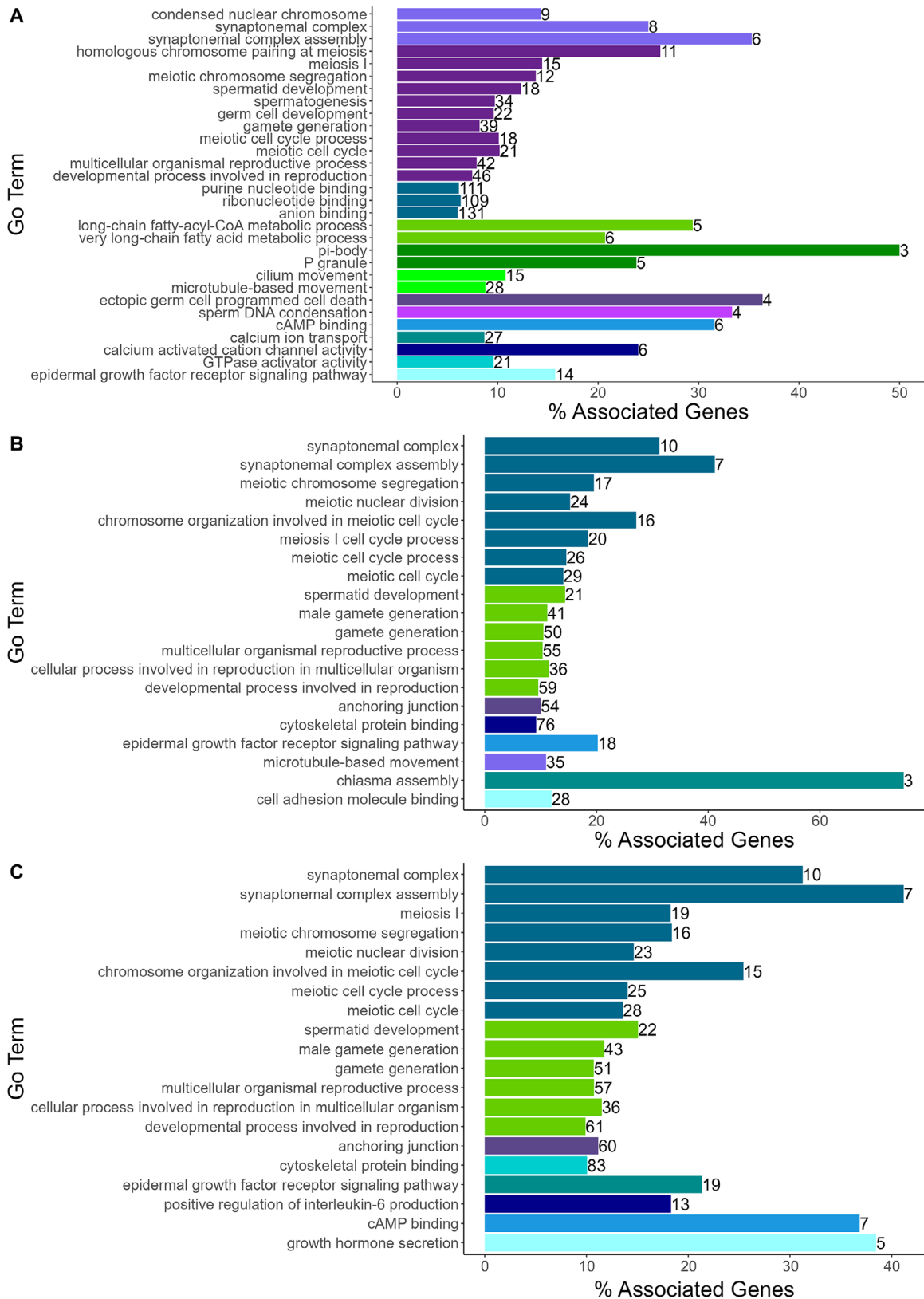


Figure 4. Results of ClueGO functional analyses (Bindea et al., 2009) for (A) *Streptococcus agalactiae* versus healthy; (B) *Prototheca* spp. versus healthy; (C) mastitis versus healthy. The number at the end of the bar corresponds to the number of differentially methylated genes for each of the enriched biological functions. cAMP = cyclic adenosine monophosphate; GTPase = guanosine triphosphatase; Go = Gene Ontology.

FDR = 0.038); regulation of actin-based cytoskeleton and tissue remodeling (e.g., GTPase activator activity, FDR = 0.035; epidermal growth factor receptor signaling pathway, FDR = 0.011; Figure 4A). Finally, we observed the enrichment of nonspecific terms related to cytokine production and specifically interleukin 1 (*IL1*; FDR = 0.035; Supplemental Table S3, <https://doi.org/10.6084/m9.figshare.24442003>). *Prototheca spp.* infection was also associated with the enrichment of several pathways related to meiosis and reproduction (e.g., meiotic nuclear division, FDR = 0.003; gamete generation, FDR = 0.012); cell adhesion, regulation of cytoskeletal dynamics and tissue remodeling (e.g., anchoring junction and cytoskeletal protein binding, FDR = 0.014; epidermal growth factor receptor signaling pathway, FDR = 0.003; Supplemental Table S3; Figure 4B). When considering subclinical mastitis, regardless of the specific pathogen investigated, growth hormone

(GH) secretion (FDR = 0.049), and positive regulation of interleukin 6 (*IL6*) production (FDR = 0.033) were enriched (Supplemental Table S3; Figure 4C).

Functional Analysis of the Whole Methylome.

Using Pathifier on the set of genes associated with the whole promoter methylome (n = 18,514), we identified 206 KEGG pathways with PDS associated with the 2 types of infection compared with healthy individuals (Supplemental Table S4, <https://doi.org/10.6084/m9.figshare.24442003>; Figure 5). The most deregulated pathway upon *Strep. agalactiae* infection was one carbon pool by folate (2.5-fold), which was one of the top deregulated pathways triggered by *Prototheca* infection (2.9-fold) as well. Overall, several pathways related to energy metabolism (e.g., tricarboxylic acid cycle, glycolysis/gluconeogenesis, biosynthesis of unsaturated fatty acids) were associated with both pathogens. Concerning the immune response, in both cases we

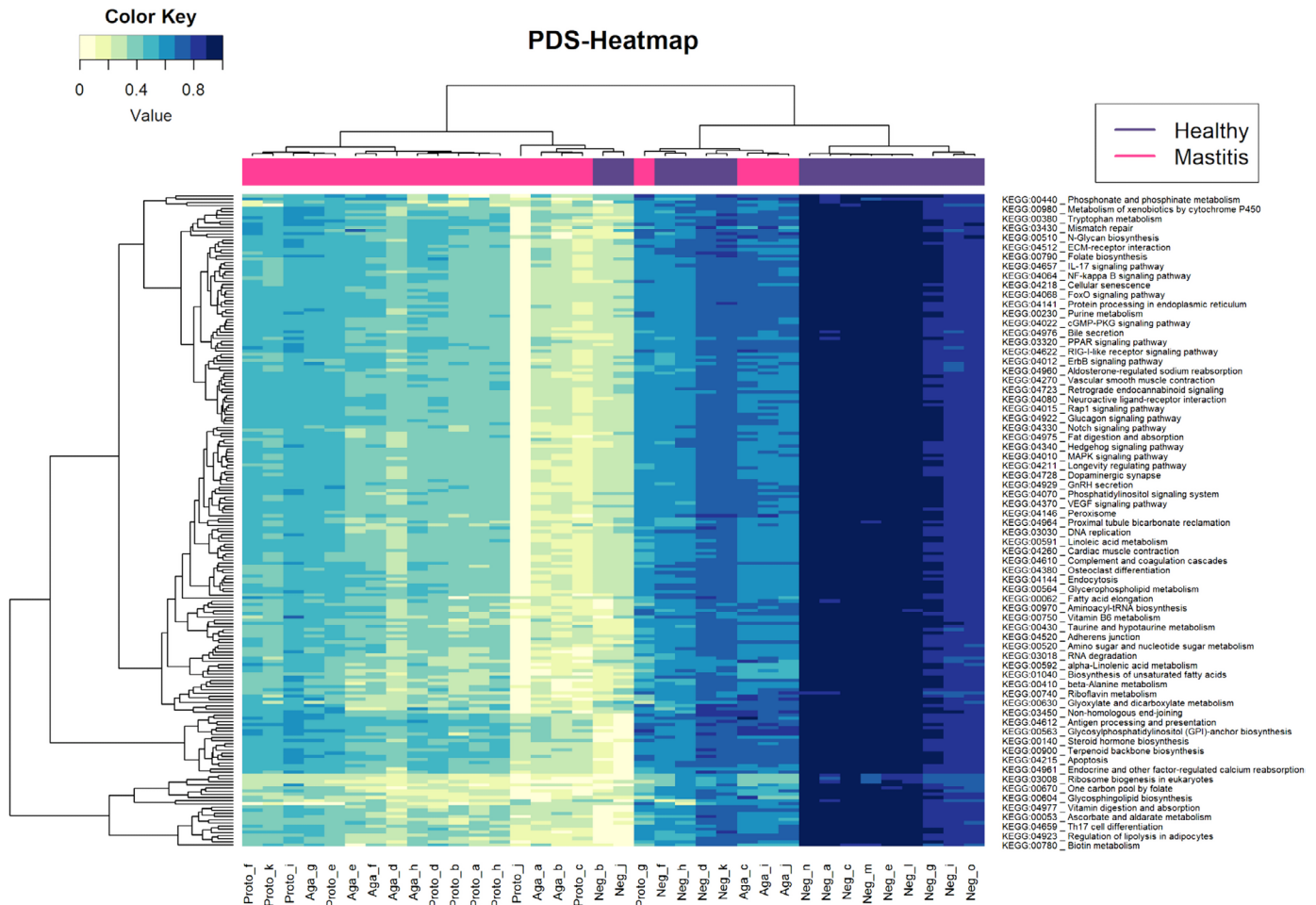


Figure 5. Heatmap built using the pathway deregulation scores (PDS) of the associated genes of the entire methylome at the promoter level of healthy animals and animals affected by subclinical mastitis, regardless the pathogen. Each row corresponds to a pathway, and each column to a sample. Blue corresponds to no deregulation, and yellow to high deregulation. KEGG = Kyoto Encyclopedia of Genes and Genomes.

observed strong deregulation in several pathways including the nuclear factor-kappa B (**NF- κ B**) signaling pathway, toll-like receptor (**TLR**) signaling pathway, retinoic acid-inducible gene I (**RIG-I**)-like signaling pathway, T-cell receptor signaling pathway, Th17 cell differentiation, IL-17 signaling pathway, and neutrophil extracellular trap formation. Overall, the most divergent results, with respect to negative animals, were observed upon *Prototheca* infection. When comparing the 2 pathogens, the only pathways with relevant deregulation scores were the fatty acid biosynthesis (2-fold) and seleno compound metabolism (1.6-fold).

DISCUSSION

Milk SC have been widely adopted as useful indicator for mastitis screening as, being the first line of defense against mammary infections, they are suitable for capturing the host immune response and possible pathogen-specific immune-molecular signatures. Furthermore, compared with mammary gland tissue or blood, they are easier to collect and more considerate of animal welfare (Wang et al., 2022). Herein, we explored the DNA methylation landscape of milk SC during naturally occurring subclinical mastitis from *Strep. agalactiae*, a gram-positive bacterium, and *Prototheca spp.*, a unicellular alga, to identify pathogen-generic and possibly pathogen-specific signatures associated with the 2 different microorganisms, which are phylogenetically very distant from each other. It is worth noting that milk SC are composed of different types of immune cells, whose relative proportion can change according not only to the infection status but also to the type of pathogen. Indeed, flow cytometry analyses conducted on the same cohort of animals enrolled in this study evidenced changes in the distribution of the milk immune cell population in response to *Strep. agalactiae* and *Prototheca spp.* infection (Pegolo et al., 2022). In particular it was shown that although *Strep. agalactiae* seemed to trigger a more innate immune response by the recruitment of macrophages and PMN, *Prototheca* seemed to drive a more adaptive response mediated by the activation of T-cells. As an outcome, the DNA methylation profile analyzed in this study is expected to originate from the immune cell population itself and, therefore, reflect their varying proportions.

DNA Methylation Data

We obtained a high depth (46 million reads per sample) and high-quality sequencing output with satisfying average alignment rate to the *Bos taurus* genome, which provided good data for subsequent analysis (Lan et al., 2011; Kim et al., 2018). Consistent with previous

studies, methylated regions were mostly concentrated in intergenic regions, followed by intragenic regions (Kim et al., 2018; de Souza et al., 2022). We focused our downstream analysis on the DNA methylation in promoter-TSS regions as, to date, they have been the most closely studied due to their association with transcriptional alterations of the involved genes, which include mostly transcriptional silencing, but also gene regulation, splicing activation, and transcription factors recruitment (Tirado-Magallanes et al., 2017). Little is known about the functional consequence and regulatory implications of DNA methylation in intergenic and intronic regions, although an indirect role in regulating gene expression is hypothesized to affect alternative splicing or noncoding RNA (Deaton and Bird, 2011; Anastasiadi et al., 2018). Moreover, a study conducted by Brenet et al. (2011) highlighted that methylation of the first exon might be critical for transcriptional silencing, therefore suggesting that we should approach the DNA methylation mechanism taking into greater consideration the different regions of the gene cassette.

Milk SC Methylome Signatures upon Subclinical Mastitis

DMG and Enrichment Analyses. When it comes to the DMR analysis, considering all the different comparisons we observed that promoter aberrant hypermethylation is more prominent than hypomethylation ($P < 0.001$) even if overall, hypomethylated DMR had higher FC. When considering immune system impairment, deregulated changes in gene expression are more attributable to aberrant hypomethylation than hypermethylation (Kushwaha et al., 2016; Upchurch et al., 2016). One of the most hypomethylated DMR found in this study in the *Strep. agalactiae*-infected group was co-located with *IL10RB* gene locus, a cytokine receptor gene which is involved in the IL-10 signal transduction (Yogev et al., 2022). The IL-10 is a key cytokine that is considered one of the master regulators of immune response, having prevalently anti-inflammatory activity and being secreted by monocytes, T-cells and macrophages (Carey et al., 2012). Notably, IL-10 signaling dysregulation has already been associated with subclinical and clinical bacterial mastitis in dairy cows (Wenz et al., 2010; Faaz and Abdullah, 2022). Another interesting finding is the hypomethylated DMR associated with *GLT8D2*. This gene encodes for a member of the glycosyltransferase family and is related to energy metabolism. The dysregulation of *GLT8D2* was already reported in clinical mastitis of cows during early lactation (Cheng et al., 2021), highlighting the potential relation between mastitis and metabolic deficit, which

may affect the immune response. Notably, we observed a strong hypomethylation in the first intron of *CIITA*, which is not only one of the master regulators in the expression of MHC complex but also involved in the transcription of several immune-related genes such as *IL-4* and *IL-10* (Devaiah and Singer, 2013). In *Prototheca*-infected cows with respect to healthy animals, one of the most hypomethylated DMR was co-located with the *ADAM11* gene locus, encoding a member of the disintegrin and metalloprotease (ADAM) protein family, which are transmembrane and secreted proteins involved in cell-cell and cell-matrix interactions. Other members of the ADAM family have been found to interact with microorganisms (e.g., *Staph. aureus*) during the pathogenesis of mastitis in dairy cows, promoting necrosis of the mammary gland tissue and cell lysis (Campos et al., 2022). Further studies are needed to explore the pathogenetic mechanisms of *Prototheca* spp. and its potential crosstalk with transmembrane receptors of the host's mammary gland epithelium. Hypomethylation was also reported in the first exon of *IFNAG*. Type I IFN not only mediate antiviral activities but also have an important influence on the adaptive immune response through the promotion of both cellular immunity and antibody responses (Baumann et al., 2017). One of the most hypermethylated DMR in the mastitis versus healthy group was co-located with *IL17REL*, which is a member of the IL-17 receptor family. The IL-17 family members are produced by Th17 cells to protect the host against bacterial and fungal infections (Kolls and Lindén, 2004), and this was also observed in dairy cows with bacterial-induced mastitis (Cebon et al., 2020). Interestingly, in humans, *IL17REL* has been shown to often be suppressed in inflammatory conditions, leading to a preponderance of pro-inflammatory factors (Franke et al., 2010).

Overall, enrichment analyses on the DMG evidenced that the 2 pathogens were associated with the regulation of the same molecular pathways. In both *Strep. agalactiae* and *Prototheca* infection functional analyses revealed that hypermethylation was associated with the upregulation of pathways related to reproduction, which might be attributable to the fact that mastitis can affect the reproduction of cows by destroying follicles, affecting oocyte growth or function and reducing ovulation ability (Boujenane et al., 2015; Eckel and Ametaj, 2020). Inflammatory cytokines and bacterial endotoxins induced by mastitis can also lead to delayed estrus, hormonal imbalances and other related problems, resulting in a decrease in fertility (Wang et al., 2021). Several genes were associated with these functions, among which *FSHR*, *CYP27B1*, and *IGF1*. Follicle stimulating hormone receptor is connected with ovarian responsiveness to FSH in ovulation induction in

humans (Perez Mayorga et al., 2000). The expression of *FSHR* has an important role in the cumulus cells' expansion and the final maturation of cumulus-oocyte complexes (Kafi et al., 2021). Pathogen-associated molecular patterns activate TLR that stimulate immune cells such as monocytes to induce expression of *CYP27B1* to convert 25-hydroxyvitamin D₃ into the more active form of vitamin D, 1,25-dihydroxyvitamin D₃ (Nelson et al., 2018) which is a direct regulator of antimicrobial innate immune responses (Liu et al., 2009). The liver modulates the reproductive function in females through secretion of IGF1. Ovarian follicles need blood derived IGF1 to complete growth and maturation before ovulation (Ginther et al., 2002). For instance, elevated blood IGF1 in Brahman heifers (Samadi et al., 2013) and postpartum Droughtmaster cows (Tena-Sempere, 2006) was associated with earlier puberty and shorter postpartum anestrus, respectively. Hypermethylation was also associated with the upregulation of pathways related to actin-based cytoskeleton and tissue remodeling, which were also in common between *Strep. agalactiae* and *Prototheca* spp. Pathogens develop different strategies to facilitate invasion of the host cell through manipulating the activity of Rho GTPases (Popoff, 2014). For instance, it has been shown that primary bovine mammary epithelial cells cultured with *Staph. aureus* have an altered, more filamentous, actin cytoskeleton, which may facilitate bacterial invasion (Günther et al., 2017). In particular, *ARHGAP26* was recently identified as a candidate gene for clinical mastitis in dairy cattle (Kour et al., 2021; Cheng et al., 2022). This is the first piece of evidence for a modulation of these pathways associated with IMI induced by an alga, i.e., *Prototheca* spp. Epidermal growth factor has been reported to have an important role in the normal development and homeostasis of the mammary gland (Xian, 2007). Among the hypermethylated genes, we found *TGFB1*, which functions as a potent proliferation inhibitor and apoptosis inducer (Ramesh et al., 2009). It is also a potent chemotactic factor, recruiting monocytes, neutrophils and lymphocytes (Bierie and Moses, 2010). Recent results showed that EGF and TGFB1 are key regulatory elements in resistance to mastitis in dairy cattle (Sharifi et al., 2020). One other study reported the overexpression of the polymeric immunoglobulin receptor (*PIGR*), which was also present among hypermethylated genes found in the current study, in a mammary transgenic model that may find application in mastitis resistance (De Groot et al., 2000).

Further to the above-mentioned pathways, in the comparison between animals affected by subclinical mastitis (regardless of the pathogen) and healthy animals we found that hypermethylation was associated

with upregulation of GH secretion which is explained by its ability to modulate neutrophil and lymphocyte function (Cirillo et al., 2017). Hypermethylation was also associated with the positive regulation of IL-6 production. Interleukin-6 is a multifunctional cytokine produced and secreted by activated macrophages, lymphocytes, and epithelial cells. In case of IMI, inflammatory and immune cells can express the *IL-6* gene and produce IL-6 (Shuster et al., 1993). Among the associated DMG, we found several IL-17 family cytokines such as *IL-17A*, *IL-17D*, *IL-17RC*. Interleukin-17A mediates the crosstalk between the immune system and different epithelial tissues, which is involved in neutrophils infiltration, activating various defense mechanisms against bacterial and fungal infections (Das and Khader, 2017). IL-17R is a heteromeric receptor comprising of IL-17RA and IL-17RC and mediates signaling of IL-17A and IL-17F, although other members of the IL-17 family such as IL-17B, IL-17C, and IL-17D can also induce the production of pro-inflammatory cytokines and chemokines (Jin and Dong, 2013). Methylation of IL-17A, as well as other interleukin genes was also observed by Wang et al., 2022, who evaluated the changes in the DNA methylome in response to *Strep. uberis* subclinical mastitis.

Functional Analyses on the Whole SC Methylome. Having analyzed the whole methylome of promoter regions, we found a clear separation between healthy and mastitic cows, highlighting that infection and the inflammatory-derived status were shown to have profound effects on the DNA methylation signature (Bayarsaihan, 2011). However, we did not observe a clear separation between the 2 groups of infected animals, consistently with the results obtained from the DMR analysis. DNA methylation in a locus can work as an inhibitor or activator for gene expression, acting on the regulatory stamping of the genome (Dhar et al., 2021). In this context, DNA methylation changes induced by environmental stimuli, including microbial infections, are targeted at the host's innate immune response rather than being pathogen-specific, which are commonly led on a transcriptional or post-transcriptional level (Bisutti et al., 2023). However, it is important to emphasize that we found a stronger pathways deregulation in the *Prototheca*-infected animals. This could be related to potential differences in the stage of infection, which we were not able to control as we dealt with naturally occurring subclinical mastitis (and that could also explain the differences in PDS within samples belonging to the same group) or possible *Prototheca*-specific DNA methylation marks.

In contrast to the DMR enrichment analyses, we found a large number of pathways associated with the immune response, which were deregulated in mastitic

animals. Overall, our results evidenced that there is a blurred line between the DNA methylation regulation of innate and adaptive host response. Deregulated pathways included genes acting in the NF- κ B signaling pathway, RIG-I-like signaling pathway, TLR signaling pathway, and T-cell receptor signaling pathway, with a specific involvement of Th17 and IL-17. Activation of Th17 differentiation and antigen processing and presentation pathways was also observed by Nayan et al. (2022), who evaluated the whole genome DNA methylation profile from peripheral blood lymphocytes in water buffaloes with subclinical mastitis. The inflammatory response requires the development of a complex and dynamic regulatory network that involves specific signaling pathways for antimicrobial defense, immune response, and tissue repair and remodeling (Medzhitov, 2008). Among those pathways, NF- κ B and IRF families are the major players acting as the most relevant effectors of innate immunity (Iwanaszko and Kimmel, 2015). Innate immunity is the host's first line of defense against invading pathogens (Vanzin et al., 2023). When microbial infection occurs, pattern recognition receptors, including TLR and RIG-I-like receptors, detect pathogen-related molecular patterns and induce a variety of downstream cascades leading to the production of pro-inflammatory cytokines and chemokines by various immune cells, such as macrophages (Akira et al., 2006; Jin et al., 2014). Moreover, TLR signaling operates an indirect regulation of T-cell differentiation and proliferation by activating innate immune cells (Iwasaki and Medzhitov, 2004), but also a direct stimulation of Th17 with the production of IL-17 which act as chemoattractant on monocytes and PMN (Bird, 2010). Consistent with our findings, the altered promoter methylation of genes pertaining to TLR, RIG-I-like, and NF- κ B signaling pathways have been shown to be associated with the crosstalk between innate and adaptive host response to microbial agents (Bayarsaihan, 2011), underlining the inextricable connection between methylation and inflammation response to pathogens.

CONCLUSIONS

Our study has contributed to bridge the gap between genotype and phenotype by providing a view of the milk SC methylome landscape involved in the occurrence and development of subclinical mastitis in dairy cattle. Although no evident pathogen-specific signature was detected, we evidenced that subclinical mastitis was associated with the methylation profile of genes involved in the regulation of meiosis, reproduction, and tissue remodeling. Furthermore, DNA methylation changes encompassed genes implicated in several immune-related pathways involving the interface between innate and

adaptive host response. DNA methylation information could complement genomic information and provide a better understanding of the factors that shape livestock phenotypes and have a directional application in breed improvement and management practices. Further studies on a bigger sample size are needed to validate our results and to possibly identify a unique SC methylome that identifies pathogen-specific alterations.

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