Article

Transcriptome Profiling of Milk Somatic Cells in Holstein, Simmental, Simmental × Holstein Crossbreed and Podolica Cattle at Two Lactation Stages and Production Systems

Alessandra Crisà [1,*], Marco Milanesi [2,*], Giovanni Chillemi [3] and Cinzia Marchitelli [1]

[1] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Research Centre for Animal Production and Aquaculture, Via Salaria 31, 00015 Monterotondo, Italy; cinzia.marchitelli@crea.gov.it
[2] Department of Animal Science, Food and Nutrition (DiANA), Facoltà di Scienze Agrarie, Alimentari e Ambientali, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy
[3] Department of Experimental Medicine, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy; chillemi@med.uniroma2.it
* Correspondence: alessandra.crisa@crea.gov.it (A.C.); marco.milanesi@unicatt.it (M.M.)

Simple Summary

Modern breeding minimises yield-related health declines by implementing functional traits. Crossbreeding, taking advantage of heterosis and breed complementarity, is a useful way to increase sustainable productivity and fertility. This research aims to profile the bovine milk transcriptome to understand the molecular regulation of the lactation cycle. We compared purebred (Holstein and Simmental) and crossbred (Simmental × Holstein) cows in an intensive system among them and with a local breed (Podolica) under an extensive production system. A conserved cohort of highly expressed genes associated with milk protein synthesis and fatty acid metabolism was identified across all groups. Breed-specific analysis revealed SM × HO cows had the most dynamic transcriptome, involving mitochondrial activity, metabolism, and transcriptional regulation. Milk transcriptomics results effectively indicate genetic and production system impacts.

Abstract

Lactation is a dynamic process characterised by a production peak at 6–8 weeks, followed by a steady decline. To understand the molecular drivers of these phases and the influence of production systems, this study aims to provide a transcriptomic characterisation of bovine milk somatic cells (BMSCs) in Holstein (HO), Simmental (SM), Simmental × Holstein crossbreed (SM × HO), and Podolica (POD) cows at 60 and 120 days in milk (DIM). Total RNA was sequenced at high coverage, and differential expression and functional enrichment analyses were performed. While a core set of milk protein and fatty acid genes was identified, breed-specific analysis showed SM × HO had the highest variation (677 differentially expressed genes, DEGs). Genes upregulated at 120 DIM involved mitochondrial metabolism and oxidative phosphorylation, while downregulated genes were associated with nuclear transcriptional regulation. At 60 DIM, SM × HO vs. HO showed 66 DEGs, with upregulated genes linked to chromatin remodelling and immune regulation. Comparing production systems, 28 DEGs between POD and HO/SM highlighted differences in mitochondrial activity and transcriptional regulation. This study bridges a knowledge gap by profiling the milk transcriptome of unexplored cattle breeds, providing novel insights into the molecular regulation of lactation.

Keywords: bovine milk somatic cell; BMSC; RNAseq; crossbreed; production systems

Academic Editor: Juan Vicente Delgado Bermejo

Received: 28 December 2025
Revised: 29 January 2026
Accepted: 6 February 2026
Published: 20 February 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

Lactation involves continuous physiological changes in the mammary gland, directly impacting milk yield and composition [1,2]. As animal robustness and sustainability become industry priorities, understanding the molecular mechanisms regulating these traits is vital. Recently, Next-Generation Sequencing (NGS) has transformed lactation biology by enabling detailed characterisation of mammary gene expression. This technology helps identify the complex genetic networks controlling key economic traits in dairy ruminants, providing essential insights for optimising production and improving milk quality [3].

Milk yield and milk components, two of the most important dairy economic traits, are controlled by a huge number of genes. RNA-seq has been widely used to profile the mammary transcriptome through different biological matrices, including mammary gland biopsies [4–6], fat globules [7–9], milk epithelial cells [10,11] and milk somatic cells (MSCs) [12,13]. MSCs comprise exfoliated mammary epithelial cells (MECs), which handle milk synthesis, and immune cells like macrophages, neutrophils and lymphocytes [14,15]. Their concentration varies based on parity, productivity, health status, breed, and lactation stage. MSCs offer a repeatable, non-invasive RNA source, reliably reflecting mammary gland gene expression for longitudinal studies in cattle and small ruminants [16–18], and somatic cell count (SCC) remains a key indicator of udder health and milk quality [14,19,20]. Thus, MSC transcriptomics provides insight not only into milk synthesis but also into mammary immune activity. In this study, we characterised the bovine MSC (BMSC) transcriptome of four cattle populations reared under two contrasting production systems: (i) intensively managed Holstein (HO), Italian Simmental (SM), and their F1 cross (SM × HO) and (ii) extensively reared Podolica (POD), an autochthonous southern Italian breed raised with traditional production systems.

Holstein (HO) cattle are globally selected for high milk yield in intensive systems [21,22]. Conversely, the Simmental (SM) is a dual-purpose breed adaptable to grazing [23,24]. Crossbreed SM × HO leverages heterosis to enhance fertility and efficiency [25,26]. Finally, the Podolica (POD), once a triple-purpose breed, now produces high-quality milk for traditional cheeses [27]. Its resilience in low-input grazing systems makes it vital for biodiversity conservation, sustainable livestock production, and effective ecosystem management in challenging environments [28,29]. Driven by the demand for sustainable products, this study addresses the lack of RNA-seq data for SM × HO, SM, and POD breeds. While HO transcriptome data exists for other lactation periods, this research investigates gene expression in BMSC during two critical phases: peak lactation (60 DIM) and mid-lactation (120 DIM). This study aimed to analyse transcriptional dynamics within and between these breeds to understand their specific adaptations to different production systems.

The specific objectives were to (i) profile the BMSC transcriptome of the four breeds jointly at two lactation stages; (ii) identify differentially expressed genes (DEGs) within breeds across lactation; (iii) to evaluate breed-specific expression signatures; (iv) compare the intensive and extensive production systems; and (v) perform functional enrichment analyses to elucidate the biological processes, pathways, and gene networks underlying observed differences among the comparisons. Together, these analyses provide new insights into breed-specific lactation biology, mammary immune function, and the impact of the production systems on the transcriptomic profile of milk in dairy cattle.

2. Materials and Methods

2.1. Experimental Design and Animals

All the experimental procedures used in this study were not invasive and, therefore, did not require the authorisation of the animal welfare committee.

https://doi.org/10.3390/ruminants6010016

The study was carried out in four breeds: HO, SM, SM × HO, and POD. The SM × HO herd was produced through rotational crossbreeding (SM sire × HO dam). HO, SM and SM × HO were raised on the CREA experimental farm (Monterotondo, Italy) in an intensive production system. Animals were managed uniformly and fed a total mixed ration (TMR) consisting of alfalfa hay, polyphyta hay, sorghum silage, barley, corn, triticale, and soybean. POD cows were reared on a farm situated in southern Italy (Basilicata region) under an extensive grazing system. Pasture composition and quality varied seasonally [30]. The 30 enrolled cows for each breed were both primiparous and multiparous with average lactation numbers of 1.8 (±0.7), 2.4 (±1.7), 1.9 (±0.79), and 4.1 (±2.1) for HO, SM, SM × HO, and POD, respectively, and milk samples were collected from the same animals at 60 (±7) (D60) and 120 (±7) (D120) days in milk (DIM), during morning milking. The milk samples from POD cows were taken by hand milking, while calves suckled. In this case, milk yield measurement was not possible and was estimated at approximately 3.5 L, based on the farmer's experience The samples were transferred, at 4 °C, to an external accredited laboratory and analysed for fat, protein, lactose, urea, SCC content, following the ICAR (International Committee for Animal Recording) guideline (https://www.icar.org/guidelines/—Section 12) (accessed on 27 December 2025).

2.2. Samples Collection and RNA Extraction

In total, 50 ml of milk was processed following the protocol from Boutinaud et al. (2002) [31], and the total RNA was extracted using the kit TripleXtractor direct RNA, following the manufacturer's instructions (GRiSP Research Solutions, Porto, Portugal). RNA concentration and purity were assessed with a Nano-Photometer™ Pearl (Implen GmbH, München, Germany), and RNA integrity (RIN) was evaluated using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano chip (Agilent Technologies, Santa Clara, CA, USA). A total of 60 samples with RIN > 7 were sequenced by an external service, specifically, 6 HO, 7 SM, 6 SM × HO, 6 POD, at D60 and at 10 HO, 10 SM, 6 SM × HO, 9 POD at D120 (at least the same three cows per breed per time point were used). Libraries were prepared using the Zymo-Seq RiboFree Total RNA kit (Zymo Research Corp., Irvine, CA, USA), following the manufacturer's instructions (library type: fr-first strand). All the libraries were sequenced pair-end 150 bp on NovaSeq 6000 (Illumina, San Diego, CA, USA).

2.3. RNA Sequencing and Data Analysis

Base calling and demultiplexing were performed using Bcl2Fastq (v2.20) of the Illumina pipeline, and adapters were removed using Cutadapt (v1.11). Raw quality was assessed using the FastQC software (v0.12.0) [32]. Low-quality reads and bases were trimmed using the Trimmomatic software (v0.36) (parameters: ILLUMINACLIP:adapters/TruSeq3-PE.fa:2:30:10; HEADCROP:13; TRAILING:30; SLIDINGWINDOW:4:15; AVGQUAL:20; MINLEN:36) [33]. Processed reads were aligned to the bovine reference ARS-UCD v1.2 [34] using the STAR software (v2.6.1d) [35]. For protein-coding genes, the annotation GTF file (*Bos taurus* Bos_taurus.ARS-UCD1.2.108.gtf) was downloaded from the ENSEMBL database v 108 [36]. Aligned reads were assembled and quantified using StringTie [37]. Normalisation was obtained using the R package DESeq2 [38] for 21.671 genes. Genes were ranked by expression values, reported as a percentage, and a threshold of 0.1% [39] was set to identify the most expressed ones.

2.4. Transcriptomic Analyses

RNA-seq read counts were modelled by a generalised linear model (GLM) that includes breed (HO, SM, SM × HO, POD) lactation time (D60 and D120) and their interaction, parity and season factors (see Section 2.5 for specification).

DEGs were identified using the DESeq2 software 3.22. Four different analyses were performed to evaluate the expression profile of the mammary gland: (i) across lactation (D60 vs. D120) in all the breeds jointly; (ii) across lactation (D60 vs. D120) in each breed separately; (iii) between the breeds in each lactation point; and (iv) between production systems (intensive vs. extensive—POD). In the model, parity classes (1, 2 and $\geq$3) and sampling season were included as fixed effects.

DEGs were defined using thresholds of $|\log_2 FC| \geq 0.58$ (Fold Change) and FDR $\leq$ 0.05 (False Discovery Rate). For genes lacking a gene symbol, a manual search in Ensemble, NCBI, and UCSC databases was conducted. Venn diagrams were drawn using Jvenn software [40].

Functional enrichment analyses were performed using the DAVID (v2023q1) web tool [41,42]. Enrichment was evaluated using the EASE-modified Fisher's exact test ($p < 0.05$). Protein–protein interaction (PPI) networks were investigated using STRING v9.1 [43,44]. In the present study, we considered *Bos taurus* as the reference organism and a minimum interaction score of 0.7. Terms with FDR < 0.05 were classified as significant. Significant PPI interaction networks were visualised using Cytoscape v3.10 [45] and the stringApp plugin [46]. Hub genes were identified with cytoHubba [47] using the MCC algorithm; hubs with at least 4 genes were reported. GO graphical representations were obtained using the clusterProfiler v4.16 R package [48].

2.5. Statistical Analysis

Normalised expressed values for the top-ranking genes were analysed using STATISTICA© 12.0 package (StatSoft Inc., Tulsa, OK, USA) for descriptive statistic analyses and GLM procedure, according to the following mixed linear model:

$$Y_{ijklmnopqr} = \mu + B_i + P_j + S_k + T_l + (B_i \cdot T_l) + \beta_{MY} \cdot MY_{ijklm} + \beta_{FAT} \cdot FAT_{ijkln} + \beta_{PROT} \cdot PROTEIN_{ijklo} + \beta_{LACT} \cdot Lactose_{ijklp} + \beta_{SCC} \cdot log10\left(SCC_{ijklq}\right) + \beta_{DIM} \cdot DIM_{ijklr} + e_{ijklmnopqr}$$

where $Y_{iklmnopqr}$ is the observation vector for each trait (i.e., expressed genes), μ is total average, B_i is the breed effect (4 levels: HO, SM, SM $\times$ HO, POD), P_j is the parity effect (3 levels: 1, 2, $\geq$3); S_k is the season effect (4 levels: autumn, winter, spring, summer); T_l is the sampling day class effect (2 levels: D60 and D120), MY_{ijklm} is the covariate for milk yield, FAT_{ijkln} is the covariate for fat%, $PROTEIN_{ijklo}$ is the covariate for protein %, $(Lactose)_{ijklp}$ is the covariate for lactose content, $(log_{10}SCC)ijklq$ is the covariate for somatic cell count, DIM_{ijklr} is the covariate for the effective day in milking, and $e_{ijklmnopq}$ is the casual error.

Post hoc comparisons were performed using Tukey's test ($p < 0.05$).

For the milk phenotypic traits, a simplified model including only DIM and MY was applied.

3. Results

3.1. Sequencing and Expression Data Statistics

Sequencing of the 60 libraries generated a total of approximately 5 billion reads (range: 57–146 million paired reads/sample). After quality control steps, an average of 39.01 $\pm$ 10.08 million paired reads per sample was retained. Most of the samples showed high values for uniquely mapped reads, properly paired reads percentages and mapping percentage (Table S1). A count matrix with normalised values was used for downstream analyses. A preliminary inspection identified residual rRNA, and those with ribosomal genes were excluded to focus subsequent analyses strictly on the remaining protein-coding genes.

https://doi.org/10.3390/ruminants6010016

3.2. Highly Expressed Genes

The expression level of the genes was calculated as percentages of the total expressed genes within each experimental design (different breeds, sampling times and interactions). Genes were ranked, and those with at least 0.1% expression level in every analysed condition were retained, resulting in a list of the 25 most expressed genes (Table 1).

The top-ranking genes encoded for the most abundant protein synthesised during lactation, which belonged to the casein and whey protein genes. In particular, *CSN1S1*, *CSN2*, *CSN1S2*, *PAEP*, *CSN3*, and *LALBA* accounted for 32,2%, 26,37%, 30,85% and 18.04% on the total gene expression in HO, SM, SM × HO and POD, respectively. Four genes are implicated in different steps of fatty acid (FA) biosynthesis: *FASN*, *SCD*, *XHD* and *PLIN2*. The other genes identified are: *GLYCAM1*, *EEF1A1*, *COX1*, *TPT1*, *ACTB*, *SPP1*, *NFKBIA*, *FTH1*, *SRGN*, *CTSB*, *PABPC1*, *ACTG1*, *HSPA8*, *B2M*, and *EEF2*. A strong positive correlation between milk protein genes and fat synthesis genes was observed within and among them (r = 0.55–0.95) (Table S2).

Milk protein and fatty acid genes, including *GLYCAM1* and *EEF1A1*, peaked at D120 versus D60. Conversely, other genes showed higher expression at D60. Significant sampling effects ($p < 0.05$) were confirmed for *CSN1S1*, *CSN2*, *CSN1S2*, *PAEP*, *CSN3*, *LALBA*, *GLYCAM*, *EEF1A*, *XDH*, *SCD*, and *PLIN2*. In POD cattle, milk protein and fat synthesis genes showed lower expression than in other breeds. Specifically, *CSN1S1*, *CSN1S2*, and *PAEP* were significantly higher in HO than in POD. *EEF1A1* was higher in HO and SM × HO; *TPT1*, *HSPA8*, and *XHD* were higher in HO; and *PLIN2* was higher in SM × HO compared to POD. *CTSB* and *ACTG1* were significantly higher in SM than in POD. Only *SRGN* was significantly more expressed in POD than in SM × HO.

In the intensive system, *EFF1A1* and *XDH* expressions were significantly higher in HO than in SM, whereas *CTSB* and *B2M* were significantly lower. Additionally, *FTH1*, *CTSB*, *B2M*, and *PLIN2* showed significantly lower expression levels in SM × HO compared to the SM breed.

Some statistically significant differences in expression were observed even considering the interaction of breeds and sampling time. At D60, *FTH1* and *B2M* were significantly expressed at a lower level in SM × HO than in SM. At D120 *CSN1S2*, *EEF1A1*, *TPT1*, and *HSPA8* were significantly more highly expressed in HO than in POD; *PLIN2* was significantly more highly expressed in SM × HO than in POD and in SM × HO than in SM; and *SRGN* was significantly more highly expressed in POD than in SM × HO.

https://doi.org/10.3390/ruminants6010016

Table 1. Genes with percentage expression value over 0.1% in the total of the expressed genes considering breed, sampling time, or their interactions.

	BREEDS				Sampling Time		BREEDS and Sampling Time							
GENE *	**HO**	**SM × HO**	**SM**	**POD**	**D60**	**D120**	**HO D60**	**HO D120**	**SM × HO D60**	**SM × HO D120**	**SM D60**	**SM D120**	**POD D60**	**POD D120**
CSN1S1	13.05	11.97	9.80	6.54	7.79	11.93	11.88	13.69	6.58	16.09	7.50	11.38	4.44	7.61
CSN2	3.62	4.77	4.17	3.46	3.20	4.43	4.46	3.16	3.51	5.74	2.79	5.12	1.91	4.25
CSN1S2	5.82	4.61	4.37	1.90	3.18	4.88	4.53	6.52	2.40	6.30	3.55	4.93	1.80	1.94
PAEP	5.04	4.25	3.46	2.70	2.94	4.45	4.40	5.39	2.90	5.28	2.24	4.30	2.20	2.96
CSN3	3.28	3.69	3.14	2.63	2.47	3.60	3.42	3.21	2.04	4.95	2.54	3.56	1.64	3.13
LALBA	1.39	1.56	1.43	0.81	1.08	1.43	1.53	1.31	1.00	1.98	1.03	1.71	0.69	0.87
GLYCAM1	1.02	1.12	0.88	0.88	0.81	1.06	1.10	0.98	0.82	1.35	0.61	1.07	0.75	0.95
EEF1A1	0.99	0.97	0.78	0.64	0.83	0.85	0.99	0.98	0.91	1.02	0.73	0.81	0.70	0.60
FASN	1.36	1.03	0.84	0.61	0.82	1.06	0.99	1.57	1.18	0.91	0.61	1.00	0.56	0.64
COX1	0.47	0.47	0.67	0.63	0.64	0.52	0.38	0.52	0.63	0.35	0.69	0.66	0.91	0.49
TPT1	0.64	0.60	0.54	0.47	0.58	0.55	0.58	0.67	0.58	0.62	0.58	0.51	0.58	0.42
ACTB	0.44	0.45	0.60	0.47	0.60	0.43	0.45	0.44	0.65	0.29	0.71	0.53	0.59	0.41
SPP1	0.55	0.36	0.69	0.43	0.69	0.43	0.72	0.46	0.48	0.26	0.81	0.61	0.68	0.31
XDH	0.86	0.63	0.40	0.44	0.49	0.64	0.79	0.90	0.54	0.69	0.29	0.47	0.34	0.49
NFKBIA	0.28	0.27	0.47	0.49	0.48	0.33	0.40	0.22	0.43	0.14	0.64	0.35	0.38	0.55
SCD	0.38	0.47	0.41	0.34	0.33	0.44	0.48	0.33	0.39	0.53	0.26	0.51	0.18	0.42
FTH1	0.29	0.22	0.45	0.32	0.40	0.29	0.25	0.31	0.30	0.16	0.60	0.35	0.39	0.29
SRGN	0.20	0.19	0.32	0.43	0.33	0.27	0.21	0.19	0.30	0.11	0.41	0.26	0.38	0.46
CTSB	0.23	0.21	0.42	0.26	0.36	0.24	0.23	0.22	0.28	0.16	0.54	0.34	0.35	0.21
PABPC1	0.23	0.29	0.22	0.26	0.26	0.24	0.30	0.20	0.32	0.26	0.18	0.24	0.27	0.26
ACTG1	0.23	0.23	0.24	0.18	0.25	0.20	0.22	0.23	0.30	0.17	0.27	0.22	0.22	0.16
HSPA8	0.25	0.23	0.22	0.17	0.25	0.20	0.22	0.27	0.30	0.17	0.26	0.19	0.23	0.14
B2M	0.18	0.17	0.28	0.23	0.26	0.19	0.18	0.19	0.23	0.13	0.36	0.22	0.24	0.22
PLIN2	0.22	0.31	0.19	0.18	0.21	0.23	0.19	0.24	0.28	0.33	0.18	0.19	0.18	0.18
EEF2	0.21	0.21	0.19	0.18	0.22	0.18	0.23	0.20	0.24	0.19	0.20	0.19	0.21	0.16

Numbers in the table represent the percentage of expression of the total expressed genes. * α-S1 casein (*CSN1S1*), β-casein (*CSN2*), α-S2 casein (*CSN1S2*), progestagen-associated endometrial protein (*PAEP*), κ-casein (*CSN3*), α-lactalbumin (*LALBA*), glycosylation-dependent cell adhesion molecule 1 (*GLYCAM1*), eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*), fatty acid synthase (*FASN*), Cytochrome C Oxidase subunit I (COX1), tumour protein translationally controlled (*TPT1*), actin beta (*ACTB*), secreted phosphoprotein 1 (SPP1), xanthine dehydrogenase (*XDH*), NFKB inhibitor alpha (*NFKBIA*), stearoyl-CoA desaturase (*SCD*), ferritin heavy chain 1 (*FTH1*), serglycin (*SRGN*), cathepsin B (*CTSB*), poly(A) binding protein cytoplasmic 1 (*PABPC1*), actin gamma 1 (*ACTG1*), heat shock protein family A (Hsp70) member 8 (*HSPA8*), beta-2-microglobulin (*B2M*), perilipin 2 (*PLIN2*), eukaryotic translation elongation factor 2 (*EEF2*).

3.3. Phenotypic Traits

Milk traits per breed, sampling times, and their interactions are summarised in Tables S2 and S3. No difference in milk production was found between the intensively raised breed, while a decrease in production from D60 to D120 lactation was observed ($p < 0.01$). Due to the milking procedure, it was not possible to evaluate POD milk production. In general, POD had higher values for protein and lactose, and lower values for urea, compared to intensive breeds.

3.4. Differential Gene Expression Results

A comprehensive set of comparisons was performed to examine the effects of lactation stage, breed, and production system on BMSCs gene expression. The number of significant DEGs is reported in Table 2a,b.

Table 2. (**a**) DEGs ($|\log_2 FC| \geq 0.58$ & FDR ≤ 0.05) between lactation stages comparison in the different breeds. (**b**) DEGs ($|\log_2 FC| \geq 0.58$ & FDR ≤ 0.05) between breeds in the different sampling times.

(**a**)

Breed	D60 vs. D120
ALL	12
HO	----
SM × HO	677
SM	4
POD	50

(**b**)

Sampling Time	HO vs. SM × HO	HO vs. SM	HO vs. POD	SM × HO vs. SM	SM × HO vs. POD	SM vs. POD
D60	66	40	285	1	1	177
D120	1	21	860	5	285	551

The overall comparison between the two lactation stages, beyond the breeds, identified 12 DEGs, of which 9 were upregulated (UP) and 3 downregulated (DOWN) at D120 relative to D60. The most UP genes were calbindin 1 (*CALB1*) and fructose-1,6-Bisphosphatase 2 (*FBP2*), while the most DOWN genes were killer cell lectin-like receptor subfamily G, Member 1 (*KLRG1*) and L-amino-acid oxidase (*LOC782545*) (Table S5).

Considering the lactation stages within each breed, HO did not show any DEGs, while SM × HO has the highest number (677). In SM, only four genes were found to be differentially expressed and UP at D120: regulating synaptic membrane exocytosis 4 (*RIMS4*), ankyrin repeat and SOCS box protein 11 (*ASB11*), estrogen receptor 1 (*ESR1*), and choline dehydrogenase (*CHDH*) (Table S5).

Considering the comparisons between breeds at D60, only one gene was identified DOWN in SM × HO vs. SM (HLA class II histocompatibility antigen, or DQ haplotype D alpha chain-like—*LOC100848815*). Also, in the SM × HO vs. POD at D60 comparison, only one gene was identified as DOWN, ribonuclease A family member 1, pancreatic (*RNASE1*). At D120, only one gene was found to be DOWN in HO vs. SM × HO, phosphorylase kinase catalytic subunit gamma 1 (*PHKG1*). Five genes, of which three were UP (major allergen Equ c 1-like—*LOC783399*; ankyrin repeat, SAM and basic leucine zipper domain containing 1—*ASZ1*; myomesin 1 *MYOM1*) and two were DOWN (synaptic vesicle glycoprotein 2C—*SV2C*; voltage-dependent anion-selective channel protein 2-like pseudogene—*LOC784294*), were identified in the SM × HO vs. SM comparison at D120. Of the 21 DEGs between HO and SM at D120, 18 and 8 were, respectively, UP and DOWN

in SM relative to HO. The most UP genes were the sodium voltage-gated channel alpha subunit 7 (*SCN7A*) and MHC Class I JSP.1 (*JSP.1*) genes, while the most DOWN genes were major allergen Equ c 1-like (*LOC783399*) and nicotinamide nucleotide adenylyltransferase 2 (*NMNAT2*) (Table S5).

Overall, the largest number of DEGs at the two sampling times was identified by comparing POD with breeds raised in the intensive system (Tables 2 and S5). However, taking into account both the entire experiment and the particular time points (D60 or D120), we found shared DEGs between POD and the other breeds beyond the single comparisons.

In the former, only the V-type proton ATPase subunit H pseudogene (LOC782343) gene was identified. At D60, 28 DEGs were identified in common in the analyses with HO and SM (Figure 1A); at D120, 62 DEGs were found in common in all the comparisons (Figure 1B, Table S6).

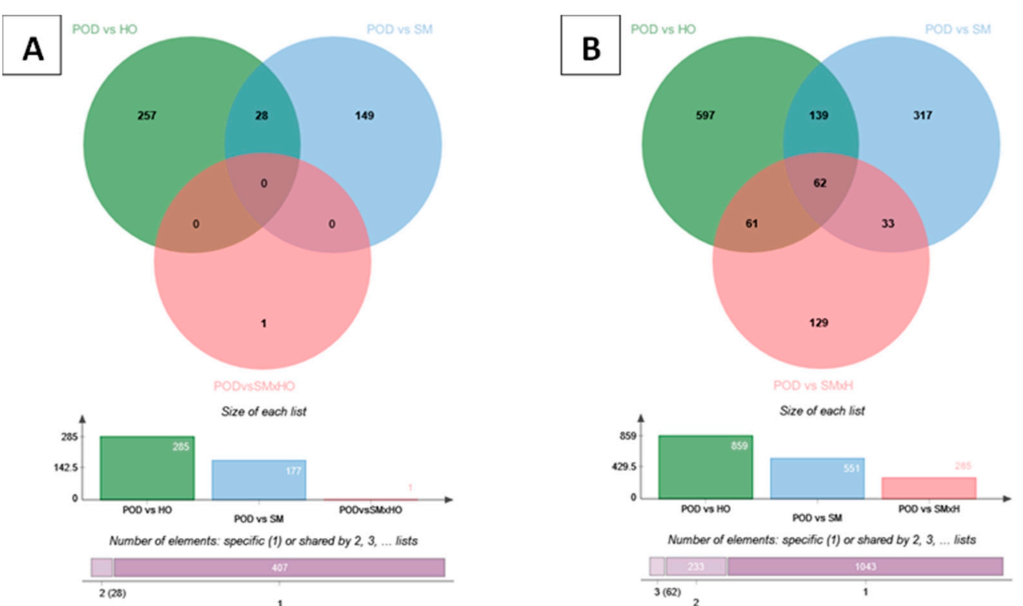

Figure 1. DEGs in common between POD and the three other breeds at 60 (**A**) and 120 (**B**) lactation days.

3.5. *Functional Enrichment Analysis of the DEGs*

3.5.1. DEGs in the Single Breeds

In the SM × HO crossbred, 677 DEGs were identified: 544 UP and 133 DOWN at D120 vs. D60. Enrichment analysis of UP genes yielded 35 BP, 46 CC, 9 MF, GO terms, and 15 KEGG terms, primarily linked to oxidative phosphorylation, fatty acid metabolism, and mitochondrial function (Tables S5 and S7, Figure S1). STRING analyses showed a significant PPI enrichment (p-value < 1.0×10^{-16}) and 93 enriched terms. The DOWN genes were associated with 80, 17, and 32 significant terms for BP, CC, and MF, relative to the nucleus compartment, active transcription process and gene transcription regulation (Tables S5 and S7, Figure S2). Several highly enriched annotation clusters and functional groups were also identified (Tables S5 and S7). STRING analyses showed a PPI enrichment (p-value < 1.0×10^{-11}) and 91 significant enriched terms. The STRING network of UP and DOWN genes was visualised in Cytoscape, revealing six subnetworks, containing more than four proteins each (Figure 2). CytoHubba identified the top 20 hub genes with the highest score, belonging to the NADH: Ubiquinone Oxidoreductase (*NDUF*) and Cytochrome C Oxidase (*COX*) gene families.

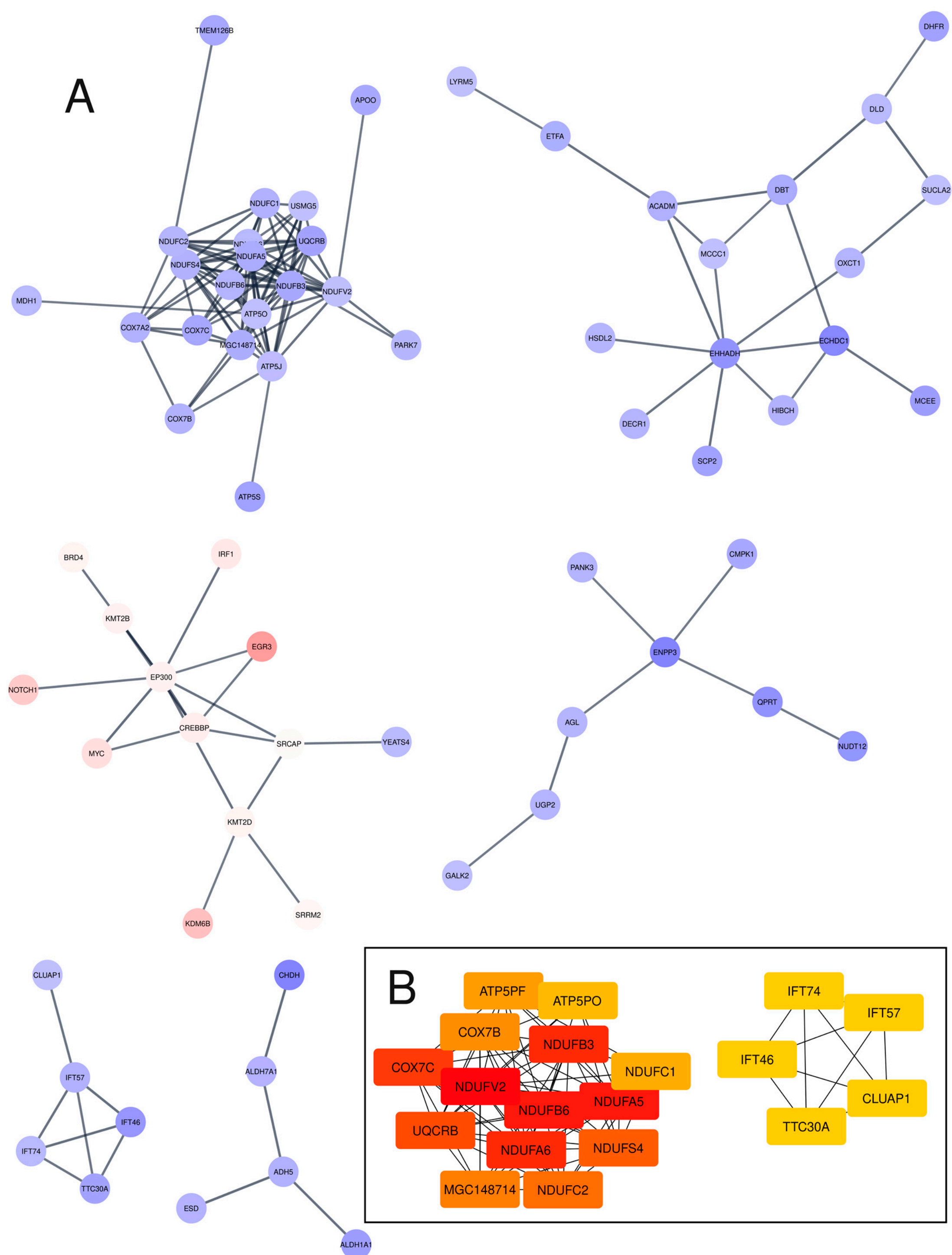

Figure 2. Cytoscape visualisation of the STRING PPI network from DEGs identified in SM × HO D120 vs. D60 analyses (**A**). The blue and red nodes indicate up- and downregulated genes, respectively, with colour intensity proportional to log2Fold change (higher values correspond to more intense colours). In the HUB gene network identified by cytoHubba, the most interactive nodes are shown in red and the least in yellow (**B**).

In POD, 50 DEGs were identified: 28 UP and 22 DOWN (D120 vs. D60). UP genes enriched 8 BP, 1 CC, GO terms, and 1 KEGG term involving cytokine and vascular endothelial growth factor production. DOWN genes enriched 1 BP, 3 CC, 1 MF, GO terms, and 1 KEGG term related to organelle organisation, nucleoplasm, and phosphatidylinositol-3,4,5-trisphosphate binding (Tables S5 and S8). No gene clusters and networks were found.

The top UP genes were calbindin 1 (*CALB1*) and echinoderm microtubule-associated protein like 1 (*EML1*), while the top DOWN genes were cytotoxic and regulatory T cell molecule (*CRTAM*) and serine protease inhibitor, Kunitz type, 3 (*SPINT3*).

3.5.2. DEGS Among the Breeds

DEGs identified between the breeds raised under the intensive system, at the two sampling times, were explored. In the SM vs. HO at D60 comparison, 40 genes were identified, with only 8 UP and 32 DOWN in SM. No significant enriched terms were identified (Tables S5 and S9). The somatomedin B and thrombospondin type 1 domain-containing (*SBSPON*) gene was the most upregulated, while the synaptic membrane exocytosis 4 (*RIMS4*) gene showed the most downregulation.

In SM × HO vs. HO at D60, 66 DEGs were found: 48 UP and 18 DOWN. UP genes enriched 32 BP, 14 CC, 8 MF, GO terms, and 4 KEGG terms; eight annotation clusters and one gene function classification group with high enrichment scores were also identified. STRING analysis showed significant PPI enrichment (p-value = 1.6×10^{-5}) and 41 significant enriched terms. The functional annotations were relative to chromatin assembly and remodelling and regulation of the immune system (Tables S5 and S10). Highly upregulated genes were the T cell receptor alpha variable 12-3-like (*LOC100298992*), natural killer cell antigen CD94 (*LOC618565*), GTPase, IMAP family member 1-like (*LOC512867*), and interleukin 2 receptor subunit beta (*IL2RB*). No significant enriched terms were found in the DOWN genes; the most downregulated genes were histatherin (*HSTN*) and Solute Carrier Family 27 Member 6 (*SLC27A6*). Figure 3 reports the STRING network for up- and downregulated genes. The top 10 hubs are related to cytokines/adhesion molecules and histone genes.

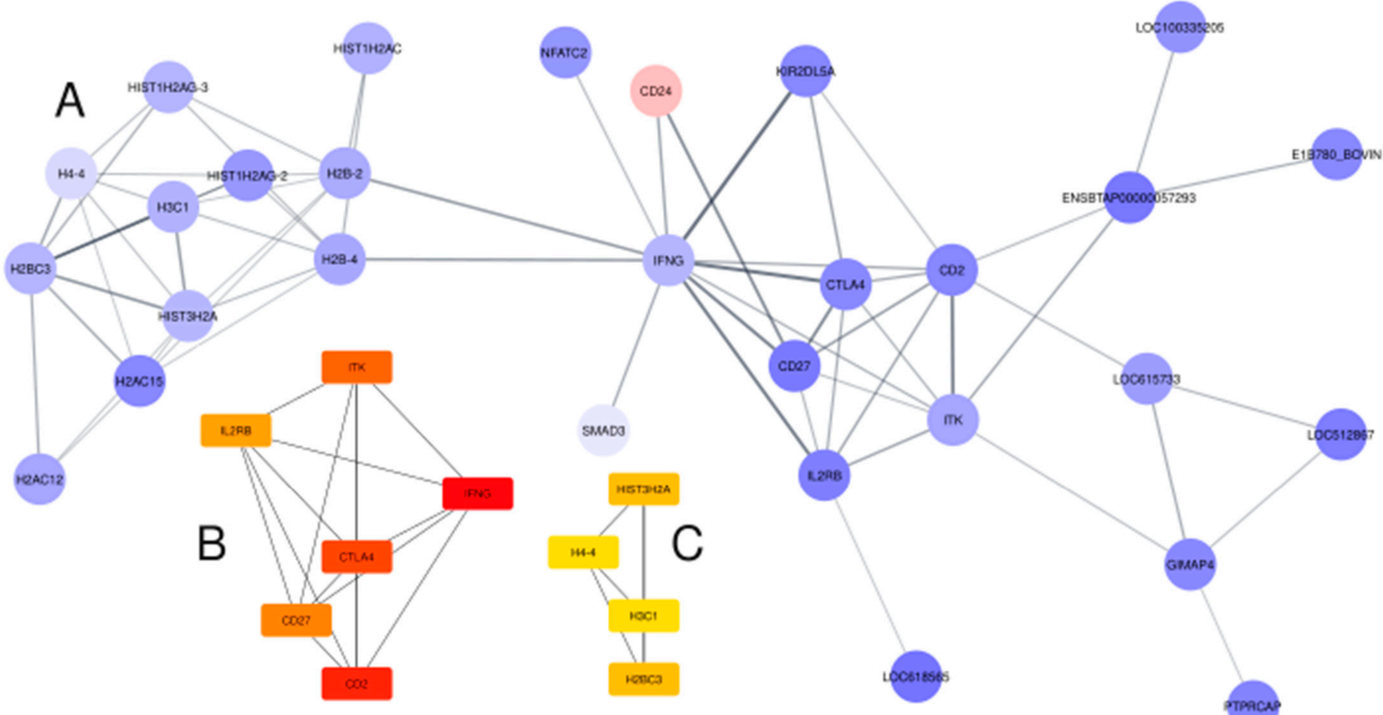

Figure 3. Cytoscape visualisation of the STRING PPI network from DEGs identified in SM × HO vs. HO at D60 analyses. The blue and red nodes indicate up- and downregulated genes, respectively, with colour intensity proportional to log2Fold change (higher values correspond to more intense colours) (**A**). In the HUB gene network identified by cytoHubba, the most interactive nodes are shown in red and the least in yellow (**B**,**C**).

At D60, POD shared 28 common genes with HO and SM (15 UP and 13 DOWN). The upregulated genes enriched 38 BP, 10 CC, and 5 MF GO terms, primarily focusing on the nucleus and positive transcription regulation. STRING analyses showed a significant PPI enrichment (p-value = 0.0265). The two most upregulated genes are the L-amino-acid oxidase (*LOC782545*) and V-type proton ATPase subunit H pseudogene (*LOC782343*). The NAD(P)HX dehydratase (*NAXD*), solute carrier family 7 member 5 (*SLC7A5*), and 3-hydroxy-3-methylglutaryl-CoA lyase (*HMGCL*) genes were the most downregulated genes. Functional enrichment analyses identified three BP and five CC significantly enriched terms, as well as one mitochondrial-related cluster (enrichment score = 1.82) (Tables S5 and S11). In Figure 4A,B, the STRING network of the up- and downregulated genes.

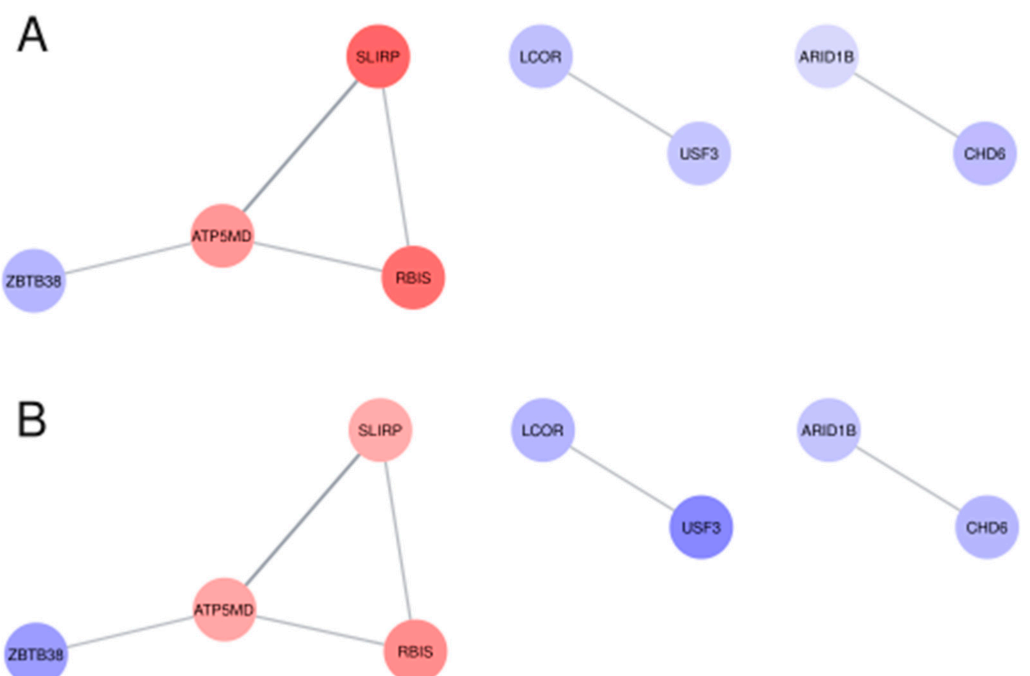

Figure 4. Cytoscape visualisation of the STRING PPI network from DEGs identified at D60 in (**A**) POD vs. HO and (**B**) POD vs. SM. The blue and red nodes indicate up- and downregulated genes, respectively, with colour intensity proportional to log2Fold change (higher values correspond to more intense colours).

Comparison at D120 revealed 62 common DEGs between POD and intensive breeds, with 26 and 36 genes significantly up- and downregulated in POD, respectively. For the upregulated genes, only 10 significantly enriched BP GO terms were identified, primarily related to the regulation of nervous system development and cell differentiation. In contrast, the downregulated genes showed 31 BP, 23 CC, 2 MF, GO terms, and 8 KEGG significantly enriched terms. The terms with the highest gene counts are related to metabolic process, intracellular organelle, catalytic activity and disease for BP, CC, MF and KEGG, respectively (Tables S5 and S12). Three clusters were obtained for the DOWN genes; however, STRING analysis did not show any PPI-enriched interaction in a network of the encoded proteins.

4. Discussion

This study investigates the milk transcriptomic profile across two production systems (intensive vs. extensive) and four cattle breeds, including a crossbreed (SM × HO) and a local breed (POD), for which no transcriptomic analyses were previously available, in two lactation stages (peak and mid-lactation), corresponding to different physiological status [1]. Podolica cattle represent an indigenous southern Italian breed characterised by high levels of rusticity and environmental plasticity, traditionally managed under extensive

grazing systems in semi-arid climates [49]. Simmental cattle are important to global farming because they are excellent for both milk and beef and tend to be healthier and more fertile than highly specialised breeds [50].

Generated within a beef-on-dairy (BoD) research program, SM × HO crossbreds enhance farm profitability by producing higher-value calves [51]. Extensive research confirms their superior reproductive, physiological, and productive performance compared to traditional dairy breeds [26,30,52–56].

Many comparisons were explored to uncover the DEGs and increase our knowledge of the biological processes associated with the lactation process. A moderate threshold for identifying DEGs was chosen to capture biologically meaningful transcriptional modulation underlying complex lactation-related traits, consistent with evidence that coordinated regulation of multiple genes with modest individual effects drives these phenotypes and that pathway-level coherence often reflects functional relevance [57,58]. In Table 3, we highlight the main findings of the study and below we discuss some of these.

Table 3. Summary of the main findings identified in this study.

Breed	Key Findings & Phenotypic Impact	Involved Genes & Pathways
All Groups (Conserved Profile)	Identification of a highly expressed cohort related to milk proteins and fatty acids.	*CSN1S1*, *CSN2*, *CSN1S2*, *CSN3*, *PAEP*, *LALBA*, *GLYCAM1*, *XDH*, *SCD*, *FASN*, *PLIN2*
SM × HO crossbreed	Most pronounced transcriptional shifts; focus on high milk fat synthesis and energy oxidation (120 DIM).	*LPL*, *LIPE*, *ACAD11*, *ACADM*, *DECR1*, *DLD*, *ETFA*, *SREBF2* (lipid uptake & FA oxidation). *MDH1*, *PDK3*, *UGP2*, *FBP2* (glycolysis & TCA cycle).
SM	Upregulation of genes critical for lactation performance and tissue structure remodelling (120 DIM).	*RIMS4*, *ASB11 & ESR1*
SM × HO vs. HO	Activation of subclinical defence and antigen recognition during early lactation (60 DIM).	Immune cell receptors, antigens, histone clusters.
SM vs. HO	Enhanced pathogen detection and MHC Class I facilitation for T lymphocyte activity (60 and 120 DIM).	*JSP.1*
POD vs. Intensive Breeds	Distinct innate modulation; adaptation to different physiological energy demands.	ROS & antimicrobial pathways; reduced mitochondrial protein synthesis.

SM × HO crossbreds exhibited the most significant transcriptional changes throughout lactation. While HO showed more DEGs than POD at D60 and D120, comparisons must account for POD's unique husbandry, including hand-milking and suckling, which complicates milk yield accuracy [59,60]. Furthermore, by 120 DIM, POD cows approach the dry period due to shorter lactation cycles, whereas intensive breeds remain at mid-lactation [2]. Consequently, 60 DIM serves as a point of physiological equivalence for crossbreed comparison despite husbandry differences. Conversely, the 120 DIM data likely reflects divergent mammary statuses—specifically the onset of involution in POD—rather than purely breed-specific or environmental effects. This distinction is crucial for accurately interpreting differences between traditional and intensive dairy systems.

4.1. Highly Expressed Genes

Our milk somatic cell RNA analysis confirmed the gene expression patterns during lactation identified in previous single-cell experiments. Single-cell RNA sequencing analyses gene expression across various mammary milk cell subsets, including immune and epithelial cells. Recent studies by Zorc and colleagues (2024) and Ren and colleagues (2025)

used this method to compare differentially expressed genes across these populations [61,62]. They showed that genes highly expressed in epithelial cells, but downregulated in immune cells, drive the synthesis and secretion of lactose, proteins, and milk fats.

4.1.1. Casein Cluster

As expected, casein and whey genes dominated, reflecting their 90% contribution to milk protein [63]. *CSN1S1* was consistently the most expressed gene across breeds and lactation stages (from 45.02% in POD to 50.6% in HO). Concerning the other *CSN*, *CSN2* (from 14.05% in HO to 23.83% in POD), *CSN1S2* (from 13.04% in POD to 22.56% in HO), and *CSN3* (from 12.74% in HO to 18.1% in POD) ranked immediately after (Table 1).

Wickramasinghe and colleagues identified *CSN1S2* as highly expressed (20.34%) at 15 and 90 DIM in BMSCs [12]. In HO mammary epithelial cells (MECs), transcript abundance follows *CSN3*, *CSN1S1*, *CSN1S2*, *CSN2*, *PAEP*, and *LALBA*, with higher values at 57 than 113 DIM [64].

Bionaz and colleagues reported higher *CSN3* expression at 60 DIM than 120 DIM in HO mammary biopsies [65]. Similarly, our results show decreased *CSN2* and *CSN3* expression in HO between D60 and D120, whereas other breeds exhibited increased transcription across all casein genes (Table 1). The higher *CSN2* expression detected in SM compared with HO could reflect the greater β-casein content, previously reported [66]. The elevated *CSN3* expression observed in HO compared to SM may suggest higher κ-casein synthesis in this breed, consistent with the high metabolic demand for milk protein production during peak lactation [67]. Total milk protein concentration increased from D60 to D120, though not significantly. While all *CSN* genes rose across lactation—aligning with established trends in milk casein proteins—no statistically significant correlation with total protein percentage was found. Considering breed differences, POD samples showed the highest concentration of milk protein, both at D60 and D120, despite the lowest expression in *CSN* genes (Tables 1, S3 and S4), likely reflecting a concentration effect due to lower milk yields and specific metabolic adaptations in this hardy breed [68,69], in contrast with the high-volume dilution typical of Holstein and Simmental breeds [70].

4.1.2. Whey Protein Cluster

Milk protein's whey fraction is dominated by highly abundant proteins: α-lactalbumin, β-lactoglobulin (encoded by the PAEP/BLG gene), serum albumin, IgG, GlyCAM-1 (PP3), and lactoferrin. Notably, β-lactoglobulin is the major whey protein in bovine, buffalo, goat, and horse milk and contributes to milk structural properties [71].

In our study, *PAEP* expression increased from D60 to D120, supporting an increase in β-lactoglobulin production during lactation [66]. *LALBA*, encoding α-lactalbumin, is among the most highly expressed genes in mammary glands of several species [12,13,72,73], and this was confirmed here (Table 1). α-lactalbumin has important biological and functional properties and plays a central role in lactose synthesis: in the mammary gland, it interacts with B4GALT1 to form the lactose synthase complex [74]. Because lactose regulates milk osmotic pressure, high *LALBA* expression in mammary epithelial cells is essential during lactation. In our dataset, *LALBA* and *B4GALT1* gene expression are strongly correlated (r = 0.84, $p \leq 0.001$), and both show a weak positive correlation with lactose concentration (r = 0.35, $p \leq 0.01$). *GLYCAM1* encodes a dominant N-linked glycoprotein in bovine whey; its glycan composition may affect interactions with immune receptors [75]. High *GLYCAM1* transcript abundance has been reported in goat and bovine mammary tissue and milk [76,77]. In single-cell RNA sequencing, *GLYCAM 1* is one of the 10 most expressed genes in bovine luminal cells [61]. Using an in vivo induction model experiment of lactation, LeProvost and colleagues [76] demonstrated that the *GLYCAM1* gene expression

in ewe mammary tissue is hormonally regulated, following a similar mechanism to that of casein genes. In our study, *GLYCAM1* and *CN* genes were significantly highly positively correlated (r = 0.79–0.92, $p \leq 0.001$).

4.1.3. Fatty Acid Cluster

Milk fat composition depends on nutrition, genetics, and lactation stage [78]. In the mammary gland, metabolism involves five processes: fatty acid uptake, de novo synthesis, desaturation, esterification, and secretion. Key genes regulating these pathways are highly expressed in BMSCs, with their expression patterns shifting significantly throughout the lactation cycle [12]. The *FASN* gene plays a central role in the de novo synthesis of many short- and medium-chain fatty acids in milk and is essential for the development, functional competence, and maintenance of the lactating mammary gland [72,79,80]. The *SCD* encodes Δ9-desaturase, converting saturated fatty acids into monounsaturated fatty acids and contributing to CLA isomer synthesis [81,82]. XDH and PLIN2 are key proteins in milk fat globule formation [83,84], and PLIN2 variants have been associated with milk FA profile [85]. We did not detect statistically significant changes in *FASN* expression across lactation, whereas *SCD*, *XDH* and *PLIN2* showed higher expression levels at D120. These four genes showed a significantly strong positive correlation (r = 0.63–0.87; $p < 0.001$, Table S2), although no significant correlation was detected between the expression of the individual gene and overall milk fat content.

4.1.4. Other Highly Expressed Genes

SPP1, encoding osteopontin, is crucial for mammary development, milk production, and protein gene modulation [86]. We found higher SPP1 expression at D60 compared to D120. *NFKBIA* regulates immune responses by inhibiting NFκB [87] and has been linked to mastitis in HO and buffalo [13,88]; in our data, *NFKBIA* expression is strongly positively correlated with log SCC (r = 0.71; $p \leq 0.001$), confirming its central role in mammary inflammation.

High *EEF1A1* expression aligns with the protein synthesis demands of lactation [89], though we observed no significant correlation with protein content.

The presence of cathepsin B (CTSB) in milk and its broad specificity against caseins has been documented [90]; its high expression here may be related to proteolysis and to differences in casein composition [91].

FTH1 encodes ferritin heavy chain, an anti-apoptotic protein found in blood and milk; ferritin levels increase during mastitis [92,93] and are considered potential infection indicators. We found a high correlation between *FTH1* expression and log SCC (r = 0.68; $p \leq 0.001$). *B2M* encodes β-2-microglobulin, part of the Fc receptor that mediates IgG transfer to milk, and is associated with MHC and mastitis traits [94–96]; here, B2M expression was positively correlated with log SCC (r = 0.72; $p \leq 0.001$). *HSPA8* encodes an ATP-dependent molecular chaperone involved in protein folding and proteostasis [96,97]; although integrated into networks regulating milk protein synthesis [65], we did not detect a significant correlation between HSPA8 expression and milk protein content.

4.2. DEGs and Functional, Annotation Enrichment

4.2.1. D60 vs. D120 in SM

In SM, *RIMS4* emerged as the most significantly upregulated gene at D120 compared to D60 and was also differentially expressed relative to HO at D60. RIMSs are scaffold proteins involved in neurotransmitter release and vesicle exocytosis [98,99]. In mammary epithelial cells, massive exocytosis of proteins and lipid droplets is required for milk secretion [100]. Although a specific role for RIMS4 in lactation has not been previously

documented, its upregulation suggests involvement in intracellular trafficking and secretion of milk components.

With the onset of lactation, the metabolic and secretory activity per cell increases as the lactation becomes established; however, mammary cell number and secretory activity change during lactation [100]. We identified two additional genes potentially implicated in regulating mammary cell dynamics: Ankyrin Repeat and SOCS Box Protein 11 (*ASB11*) and oestrogen receptor 1 (*ESR1*). ASB11, also upregulated in SM × HO at D120, has been reported as upregulated postpartum in bovine mammary gland and is a mediator of canonical Delta–Notch signalling, crucial for cell fate and tissue differentiation [101,102]. *ESR1* encodes a key regulator of mammary development and epithelial proliferation [103]. Increased oestrogen responsiveness during lactation has been linked to mammary involution and reduced persistency [100]. The increased *ESR1* expression observed in SM at mid-lactation and in POD relative to SM at D60 suggests early transcriptional shifts linked to tissue remodelling and declining efficiency; *ESR1* expression may thus serve as a potential biomarker for milk persistency. In our study, SM milk yield decreased from 16.33 to 13.62 L from D60 to D120 (Table S2).

We found the *JSP.1* (major histocompatibility complex, class I, A) gene to be upregulated in the comparison of SM vs. HO, both at 60 and 120. This gene is referred to as Bovine Lymphocyte Antigen (BoLA) and is crucial for presenting intracellular peptides to CD8+ cytotoxic T lymphocyte cells, part of the cow's immune defence against pathogens [95]. Some studies report an association between Class I alleles and mastitis traits [104]. Although all animals were free from clinical mastitis, SM showed higher SCC than HO, which may reflect differences in immune responsiveness.

4.2.2. D60 vs. D120 in SM × HO

One of the most enriched STRING PPI networks involved in upregulated genes is associated with mitochondrial oxidative phosphorylation, including the NADH: Ubiquinone Oxidoreductase, Cytochrome C Oxidase, and ATP synthase family genes. All these genes contribute to elevated ATP demand in the mammary gland to support milk production [105], as mitochondria play a decisive role in meeting the bioenergetic and substrate requirements of lactation [106].

A HUB gene network, even with fewer interactive nodes, includes some intraflagellar transport genes (ITF 46, 74, 57), whose role could be associated with morphogenesis during mammary gland development, as demonstrated for an analogous gene [107,108].

Genes directly involved in lipid uptake, intracellular trafficking, and fatty acid oxidation were identified as upregulated at D120 in SM × HO, highlighting sustained mammary capacity for milk fat synthesis (*LPL*, *LIPE*, *ACAD11*, *ACADM*, *DECR1*, *ETFA*, and *SREBF2*). We also found upregulated genes at D120 associated with glucose metabolism and energy balance, reflecting the increased metabolic demand of mid-lactation (*MDH1*, *PDK3*, *UGP2*, *FBP2*). These changes highlight coordinated transcriptional control ensuring efficient glucose utilisation and energy partitioning in mammary epithelial cells during lactation.

4.2.3. SM × HO vs. HO at D60

At D60, DEGs upregulated in SM × HO versus HO were related to immune cell receptors, antigens related and histone clusters. Our observation of histatherin (*HSTN*) and Solute Carrier Family 27 Member 6 (*SLC27A6*), among the most downregulated genes in cattle mastitic milk samples, is in accordance with Bisutti et al. (2024)'s results [109].

Functional annotation revealed enriched terms related to chromatin organisation, immune regulation, apoptosis, and Neutrophil Extracellular Trap (NET) formation. Beyond DNA packaging, histones serve antimicrobial roles within NETs—an extracellu-

lar DNA meshwork that traps pathogens but may also cause inflammatory tissue damage [83,84,110,111]. Notably, NET-like structures appear in mastitic milk and are associated with increased mastitis risk during the transition period [112]. In sheep, these structures are linked to potential milk duct blockages, suggesting that while NETs provide a vital defence mechanism, their presence reflects significant inflammatory responses within the mammary gland [113,114]. Although the cows here analysed did not show clinical mastitis, the SM × HO breed had a slightly higher, but not statistically significant, value of SCC compared with HO (173,780 vs. 144,543 cells/mL). Combined with the transcriptomic profile, this may indicate activation of a subclinical immune defence response.

Concerning milk protein gene expression, Vanselow et al. [115] have demonstrated that mastitis caused by *E. coli* infection of the udder results in an increased DNA methylation status in the far upstream promoter of the bovine aS1-casein gene and tighter DNA chromatin packing. In our experiment, SM × HO showed almost half of the *CSN1S1* expression percentage relative to HO, with small differences in SCC values (173.780 vs. 144,543), suggesting a possible effect of chromatin remodelling due to an increase in histone gene expression.

DNA methylation is associated with chromatin condensation through histone modifications, and there is emerging evidence to suggest that histone modifications are involved in modulating the milk production of MEC both in mouse and human cell-cultured studies [116,117].

4.2.4. POD Comparisons

Across POD comparisons with other breeds at D60 and D120, the only common DEG identified was ENSBTAG00000035572 (LOC782343), a V-type proton ATPase subunit H pseudogene. This gene encodes a V-ATPase component essential for acidifying intracellular organelles [118], enabling protein sorting, endocytosis, and phagosome–lysosome fusion [119]. Acidification is critical for activating microbicidal enzymes and degrading apoptotic cells [120]. Its upregulation in POD cows, alongside slightly higher average somatic cell counts (SCCs), suggests that this gene plays a key role in supporting enhanced innate immune activity in this specific breed.

4.2.5. POD vs. HO/SM at D60

By day 60, 28 common DEGs were identified between POD and the intensive breeds (HO and SM), of which 15 were UP, and 13 were DOWN in POD. Notably, functional analysis showed enrichment in the branched-chain amino acid degradation pathway. Within this pathway, the *LOC782545* gene (L-amino-acid oxidase, LAAO) was upregulated in POD. LAAOs generate hydrogen peroxide, contributing to innate immune defence [121] and antimicrobial defence in milk [122]. While this upregulation suggests a higher antimicrobial capacity in POD animals, somatic cell counts (SCCs) remained similar across breeds.

In POD cows, the (3-hydroxy-3-methylglutaryl-CoA lyase) *HMGCL* gene was downregulated. Notably, this is the first report of HMGCL expression in BMSCs, ranking 5440th among 21,672 bovine genes in normalised counts. This gene is vital for ketogenesis and leucine catabolism, providing energy and influencing ROS production and molecular signalling. Downregulation of HMGCL reduces β-hydroxybutyrate, potentially limiting ROS generation. While this ~2-fold reduction in POD suggests lower oxidative stress, the lack of ROS analysis prevents confirmation. Moreover, beta-hydroxybutyrate might also play a vital role in regulating the barrier immune systems [123]. While SCC values did not significantly differ (the averages were 140,000, 143,000 and 300,000 cells/mL in POD, HO and SM, respectively), the transcriptomic profile of POD animals—including *HMGCL* downregulation—points to distinct innate immune modulation. This suggests

specific regulation of ROS and antimicrobial pathways, aligning with the evolving view of neutrophils as complex regulators of homeostasis and tissue remodelling rather than simple pathogen killers.

Dietary differences, such as those used in the two husbandry systems studied, may influence these patterns, as *HMGCL* expression typically decreases in forage-based diets [124,125].

Considering the network results for POD vs. HO/SM comparison, the SRA stem-loop interacting RNA binding protein (*SLIRP*), ribosomal biosis factor (RBIS), ATP synthase membrane subunit k (*ATP5MK*) genes were found to be downregulated. In humans, SLIRP was identified as a mitochondrial protein with a relevant role in regulating mitochondrial messenger RNA (mRNA) translation and homeostasis [126,127]. The RBIS encoded protein in humans has been demonstrated to be a trans-acting factor in ribosome biogenesis required for efficient 40S and 60S subunit production [128]. The ATP5MK protein is required for dimerisation of the ATP synthase complex and, as such, regulates ATP synthesis in the mitochondria [129]. These results could suggest a slightly reduced mitochondrial protein synthesis in POD relative to the other intensive reared breeds.

5. Conclusions

In this study, our results showed that the SM × HO crossbreds exhibited enhanced transcriptomic plasticity and suggested an improved metabolic efficiency. Moreover, the enrichment in immune and epigenetic signals suggests a robust biological framework that may support health, adaptability and longevity under intensive production conditions. In contrast, the Podolica breed reveals a molecular strategy that prioritises metabolic resilience and resource allocation over maximising yield. This profile is in line with adaptation to an extensive and low-input farming system. Overall, these findings highlight how the integration of functional traits and crossbreeding can balance high productivity with metabolic resilience and animal welfare, supporting more sustainable and resilient dairy production systems.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ruminants6010016/s1. Figure S1: Barplot of the 15 most significant signals (adjust p-value ≤ 0.1) GO term (BP, CC and MF) for upregulated genes in SM × HO of D120 vs. D60. Figure S2: Barplot of the 15 most significant signals (adjust p-value ≤ 0.1) GO term (CC and MF) for downregulated genes in SM × HO of D120 vs. D60. Table S1: Statistics on the 60 RNA sequencing samples analysed. [DIM group: 60 or 120; RIN: RNA integrity number; total reads (R1 + R2): number of total raw reads obtained from the sequencing; QC (%): percentage of reads after quality control procedure; mapped (%): % of reads mapped on the reference genome; PropPaired (%): % of reads properly paired; Ribosomial (% expression data): % of expression associated with rRNA or ribosomal protein (removed in the working dataset)]. Table S2: the correlation and relative p-values among the top 25 expressed genes. [In the sheet "Correlations", the yellow cells represent the genes associated with the fat content in the milk; the text in red represents the significant correlations]. Table S3: Parameters analysed in milk samples considering the cattle breeds or the two sampling times (means ± SE). Table S4: Parameters analysed in milk samples considering both the breeds and the sampling time (means ± SE). Table S5: Statistics on DEGs ($|\log_2 FC| \geq 0.58$ & FDR ≤ 0.05) considering breed, sampling time or their interaction from Deseq2 output. [Ensembl: Ensembl gene ID; Gene symbol: gene name; Description: gene description; baseMean: the average expression level of a gene across all samples after accounting for library size differences; log2FoldChange: logarithm (base 2) of the fold change in gene expression between two groups, indicating the magnitude and the direction (positive: upregulation, negative: downregulation) of the change in expression; lfcSE: log2 fold change standard error; stat: Wald statistic, a measure of the difference in gene expression normalized by its standard error; p-value: unadjusted p-value associated with the Wald statistic;

padj: adjusted *p*-values, which are corrected for multiple testing using methods like the Benjamini–Hochberg procedure; Lpadj: negative logarithm (base 10) of padj.]. Table S6: DEGs ($|\log_2 FC| \geq 0.58$ & FDR ≤ 0.05) comparing POD with (i) HO and SM at D60, (ii) HO, SM and SM $\times$ HO at 120, and (iii) the common in all the comparisons. [Ensembl: Ensembl gene ID; Gene symbol: gene name; Description: gene description; log2FoldChange: logarithm (base 2) of the fold change in gene expression between two groups, indicating the magnitude and the direction (positive: upregulation, negative: downregulation) of the change in expression]. Table S7: Functional enrichment analysis results using DEGs from D120 vs. D60 in SM $\times$ HO comparison. The results were obtained using DAVID and STRING web tools. Table S8: Functional enrichment analysis results using DEGs from D120 vs. D60 in POD comparison. The results were obtained using DAVID and STRING web tools. Table S9: Functional enrichment analysis results using DEGs from SM vs. HO at D60 comparison. The results were obtained using DAVID and STRING web tools. Table S10: Functional enrichment analysis results using DEGs from SM $\times$ HO vs. HO at D60 comparison. The results were obtained using DAVID and STRING web tools. Table S11: Functional enrichment analysis results using DEGs from POD vs. intensive breeds (HO and SM) at D60 comparison. The results were obtained using DAVID and STRING web tools. Table S12: Functional enrichment analysis results using DEGs from POD vs. intensive breeds (HO, SM and SM $\times$ HO) at D120 comparison. The results were obtained using DAVID and STRING web tools.

Author Contributions: Conceptualization, A.C.; methodology A.C. and C.M.; formal analysis, A.C. and M.M.; investigation, A.C. and C.M.; data curation, A.C. and M.M.; writing—original draft preparation, A.C.; writing—review and editing, C.M. and M.M.; visualization, A.C.; supervision, G.C.; funding acquisition, A.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Italian Ministry of Agriculture (MIPAAF) in the national research projects REDDBOV (DM 19735/7303/12) and MIQUALAT (D.M. 16844/7100/2019).

Institutional Review Board Statement: Ethical review and approval were not applied for this study because the milk samples were collected only through the normal routine milking of animals without any interference with animal health or welfare. The dairy herds involved in this study were raised through routine standard farming practices without interfering with their welfare.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original data presented in this study are openly available in SRA with BioProject ID PRJNA1425127.

Acknowledgments: We acknowledge Matilde Giombini for help in the management of the reference part of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Gross, J.J. Dairy Cow Physiology and Production Limits. *Anim. Front.* **2023**, *13*, 44–50. [CrossRef] [PubMed]
2. Solodneva, E.V. Lactation Curves as a Tool for Monitoring the Health and Performance of dairy cows—A Mini-Review. *Sel'skokhozyaistvennaya Biol.* **2022**, *57*, 257–271. [CrossRef]
3. Ferreira, A.M.; Bislev, S.L.; Bendixen, E.; Almeida, A.M. The Mammary Gland in Domestic Ruminants: A Systems Biology Perspective. *J. Proteom.* **2013**, *94*, 110–123. [CrossRef] [PubMed]
4. Dado-Senn, B.; Skibiel, A.L.; Fabris, T.F.; Zhang, Y.; Dahl, G.E.; Peñagaricano, F.; Laporta, J. RNA-Seq Reveals Novel Genes and Pathways Involved in Bovine Mammary Involution during the Dry Period and under Environmental Heat Stress. *Sci. Rep.* **2018**, *8*, 11096. [CrossRef]
5. Fan, Y.; Han, Z.; Lu, X.; Idriss Arbab, A.A.; Nazar, M.; Yang, Y.; Yang, Z. Short Time-Series Expression Transcriptome Data Reveal the Gene Expression Patterns of Dairy Cow Mammary Gland as Milk Yield Decreased Process. *Genes* **2021**, *12*, 942. [CrossRef]
6. Liang, Y.; Gao, Q.; Wang, H.; Guo, M.; Arbab, A.A.I.; Nazar, M.; Li, M.; Yang, Z.; Karrow, N.A.; Mao, Y. Identification and Characterization of Circular RNAs in Mammary Tissue from Holstein Cows at Early Lactation and Non-Lactation. *Biomolecules* **2022**, *12*, 478. [CrossRef]

7. Arora, R.; Sharma, A.; Sharma, U.; Girdhar, Y.; Kaur, M.; Kapoor, P.; Ahlawat, S.; Vijh, R.K. Buffalo Milk Transcriptome: A Comparative Analysis of Early, Mid and Late Lactation. *Sci. Rep.* **2019**, *9*, 5993. [CrossRef]
8. Beckett, L.; Xie, S.; Thimmapuram, J.; Tucker, H.A.; Donkin, X.S.S.; Casey, X.T. Mammary Transcriptome Reveals Cell Maintenance and Protein Turnover Support Milk Synthesis in Early-Lactation Cows. *Physiol Genom.* **2020**, *52*, 435–450. [CrossRef]
9. Yang, J.; Jiang, J.; Liu, X.; Wang, H.; Guo, G.; Zhang, Q.; Jiang, L. Differential Expression of Genes in Milk of Dairy Cattle during Lactation. *Anim. Genet.* **2015**, *47*, 174–180. [CrossRef]
10. Bhat, S.A.; Ahmad, S.M.; Ibeagha-Awemu, E.M.; Bhat, B.A.; Dar, M.A.; Mumtaz, P.T.; Shah, R.A.; Ganai, N.A. Comparative Transcriptome Analysis of Mammary Epithelial Cells at Different Stages of Lactation Reveals Wide Differences in Gene Expression and Pathways Regulating Milk Synthesis between Jersey and Kashmiri Cattle. *PLoS ONE* **2019**, *14*, e0211773. [CrossRef]
11. Mumtaz, P.T.; Bhat, B.; Ibeagha-Awemu, E.M.; Taban, Q.; Wang, M.; Dar, M.A.; Bhat, S.A.; Shabir, N.; Shah, R.A.; Ganie, N.A.; et al. Mammary Epithelial Cell Transcriptome Reveals Potential Roles of LncRNAs in Regulating Milk Synthesis Pathways in Jersey and Kashmiri Cattle. *BMC Genom.* **2022**, *23*, 176. [CrossRef]
12. Wickramasinghe, S.; Rincon, G.; Islas-Trejo, A.; Medrano, J.F. Transcriptional Profiling of Bovine Milk Using RNA Sequencing. *BMC Genom.* **2012**, *13*, 45. [CrossRef] [PubMed]
13. Asselstine, V.; Miglior, F.; Suárez-Vega, A.; Fonseca, P.A.S.; Mallard, B.; Karrow, N.; Islas-Trejo, A.; Medrano, J.F.; Cánovas, A. Genetic Mechanisms Regulating the Host Response during Mastitis. *J. Dairy Sci.* **2019**, *102*, 9043–9059. [CrossRef] [PubMed]
14. Alhussien, M.N.; Dang, A.K. Milk Somatic Cells, Factors Influencing Their Release, Future Prospects, and Practical Utility in Dairy Animals: An Overview. *Vet. World* **2018**, *11*, 562–577. [CrossRef] [PubMed]
15. Li, N.; Richoux, R.; Boutinaud, M.; Martin, P.; Gagnaire, V. Role of Somatic Cells on Dairy Processes and Products: A Review. *Dairy Sci. Technol.* **2014**, *94*, 517–538. [CrossRef]
16. Seo, M.; Lee, H.J.; Kim, K.; Caetano-Anolles, K.; Jeong, J.Y.; Park, S.; Oh, Y.K.; Cho, S.; Kim, H. Characterizing Milk Production Related Genes in Holstein Using RNA-Seq. *Asian-Australas. J. Anim. Sci.* **2016**, *29*, 343–351. [CrossRef]
17. Cánovas, A.; Rincón, G.; Bevilacqua, C.; Islas-Trejo, A.; Brenaut, P.; Hovey, R.C.; Boutinaud, M.; Morgenthaler, C.; Vanklompenberg, M.K.; Martin, P.; et al. Comparison of Five Different RNA Sources to Examine the Lactating Bovine Mammary Gland Transcriptome Using RNA-Sequencing. *Sci. Rep.* **2014**, *4*, 5297. [CrossRef]
18. Toral, P.G.; Hervás, G.; Suárez-Vega, A.; Arranz, J.J.; Frutos, P. Isolation of RNA from Milk Somatic Cells as an Alternative to Biopsies of Mammary Tissue for Nutrigenomic Studies in Dairy Ewes. *J. Dairy Sci.* **2016**, *99*, 8461–8471. [CrossRef]
19. Sharma, N.; Singh, N.K.; Bhadwal, M.S. Relationship of Somatic Cell Count and Mastitis: An Overview. *Asian-Aust. J. Anim. Sci* **2011**, *24*, 429–438. [CrossRef]
20. Zecconi, A.; Meroni, G.; Sora, V.; Mattina, R.; Cipolla, M.; Zanini, L. Total and Differential Cell Counts as a Tool to Identify Intramammary Infections in Cows after Calving. *Animals* **2021**, *11*, 727. [CrossRef]
21. Roche, J.R.; Berry, D.P.; Bryant, A.M.; Burke, C.R.; Butler, S.T.; Dillon, P.G.; Donaghy, D.J.; Horan, B.; Macdonald, K.A.; Macmillan, K.L. A 100-Year Review: A Century of Change in Temperate Grazing Dairy Systems. *J. Dairy Sci.* **2017**, *100*, 10189–10233. [CrossRef] [PubMed]
22. Brito, L.F.; Bedere, N.; Douhard, F.; Oliveira, H.R.; Arnal, M.; Peñagaricano, F.; Schinckel, A.P.; Baes, C.F.; Miglior, F. Review: Genetic Selection of High-Yielding Dairy Cattle toward Sustainable Farming Systems in a Rapidly Changing World. *Animal* **2021**, *15*, 100292. [CrossRef] [PubMed]
23. Menta, G.; Venuti, M. Esempi Di Sostenibilita' Di Alcune Aziende Zootecniche Di Montagna In Cui Si Allevano Bovine Di Razza Pezzata Rossa Italiana. 2014. Available online: https://www.sozooalp.it/fileadmin/superuser/Quaderni/quaderno_8/12_Menta_SZA8.pdf (accessed on 28 December 2025).
24. Piasentier, E.; Menta, G.; Degano, L. Passato, Presente E Futuro Della Pezzata Rossa Italiana Sull'arco Alpino. 2010. Available online: https://www.sozooalp.it/fileadmin/superuser/Quaderni/quaderno_6/15_Piasentier_SZA6.pdf (accessed on 28 December 2025).
25. Knob, D.A.; Alessio, D.R.M.; Thaler Neto, A.; Mozzaquatro, F.D. Reproductive Performance and Survival of Holstein and Holstein × Simmental Crossbred Cows. *Trop. Anim. Health Prod.* **2016**, *48*, 1409–1413. [CrossRef] [PubMed]
26. De Matteis, G.; Scatà, M.C.; Catillo, G.; Grandoni, F.; Rossi, E.; Meo Zilio, D.; Crisà, A.; Lopreiato, V.; Trevisi, E.; Barile, V.L. Comparison of Metabolic, Oxidative and Inflammatory Status of Simmental × Holstein Crossbred with Parental Breeds during the Peripartal and Early Lactation Periods. *J. Dairy Res.* **2021**, *88*, 253–260. [CrossRef]
27. Quinto, M.; Sevi, A.; Di Caterina, R.; Albenzio, M. Quality of Milk and Caciocavallo Cheese from farms Rearing Podolica and Italian Friesian Cows. *Ital. J. Food Sci.* **2003**, *15*, 485–498.
28. Cosentino, C.; D'adamo, C.; Naturali, S.; Pecora, G.; Paolino, R.; Musto, M.; Adduci, F.; Freschi, P. Podolian Cattle: Reproductive Activity, Milk and Future Prospects. *Ital. J. Agron.* **2018**, *13*, 200–207. [CrossRef]
29. ANABIC (Associazione Nazionale Allevatori Bovini Italiani Da Carne). Standard Della Razza Podolica. Available online: http://www.anabic.it/servizio_tecnico/podolica.pdf (accessed on 28 December 2025).

30. Lovallo, C.; Marchitelli, C.; Napolitano, F.; Claps, S.; Crisà, A. Sialyloligosaccharides Content in Mature Milk of Different Cow Breeds. *Sustainability* **2022**, *14*, 2805. [CrossRef]
31. Boutinaud, M.; Rulquin, H.; Keisler, D.H.; Djiane, J.; Jammes, H. Use of Somatic Cells from Goat Milk for Dynamic Studies of Gene Expression in the Mammary Gland. *J. Anim. Sci.* **2002**, *80*, 1258–1269.
32. Babraham Bioinformatics Babraham Bioinformatics. Available online: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed on 28 December 2025).
33. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef]
34. Rosen, B.D.; Bickhart, D.M.; Schnabel, R.D.; Koren, S.; Elsik, C.G.; Tseng, E.; Rowan, T.N.; Low, W.Y.; Zimin, A.; Couldrey, C.; et al. De Novo Assembly of the Cattle Reference Genome with Single-Molecule Sequencing. *Gigascience* **2020**, *9*, giaa021. [CrossRef]
35. Dobin, A.; Davis, C.A.; Schlesinger, F.; Drenkow, J.; Zaleski, C.; Jha, S.; Batut, P.; Chaisson, M.; Gingeras, T.R. STAR: Ultrafast Universal RNA-Seq Aligner. *Bioinformatics* **2013**, *29*, 15–21. [CrossRef] [PubMed]
36. Zerbino, D.R.; Achuthan, P.; Akanni, W.; Amode, M.R.; Barrell, D.; Bhai, J.; Billis, K.; Cummins, C.; Gall, A.; Girón, C.G.; et al. Ensembl 2018. *Nucleic Acids Res.* **2018**, *46*, D754–D761. [CrossRef] [PubMed]
37. Pertea, M.; Pertea, G.M.; Antonescu, C.M.; Chang, T.-C.; Mendell, J.T.; Salzberg, S.L. StringTie Enables Improved Reconstruction of a Transcriptome from RNA-Seq Reads. *Nat. Biotechnol.* **2015**, *33*, 290–295. [CrossRef] [PubMed]
38. Love, M.I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef]
39. Bionaz, M.; Loor, J.J. Gene Networks Driving Bovine Milk Fat Synthesis during the Lactation Cycle. *BMC Genom.* **2008**, *9*, 366. [CrossRef]
40. Bardou, P.; Mariette, J.; Escudié, F.; Djemiel, C.; Klopp, C. SOFTWARE Open Access Jvenn: An Interactive Venn Diagram Viewer. *BMC Bioinformatics* **2014**, *15*, 293. [CrossRef]
41. Huang, D.W.; Sherman, B.T.; Lempicki, R.A. Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources. *Nat. Protoc.* **2009**, *4*, 44–57. [CrossRef]
42. Sherman, B.T.; Hao, M.; Qiu, J.; Jiao, X.; Baseler, M.W.; Lane, H.C.; Imamichi, T.; Chang, W. DAVID: A Web Server for Functional Enrichment Analysis and Functional Annotation of Gene Lists (2021 Update). *Nucleic Acids Res.* **2022**, *50*, W216–W221. [CrossRef]
43. Szklarczyk, D.; Gable, A.L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N.T.; Morris, J.H.; Bork, P.; et al. STRING V11: Protein–Protein Association Networks with Increased Coverage, Supporting Functional Discovery in Genome-Wide Experimental Datasets. *Nucleic Acids Res.* **2019**, *47*, D607–D613. [CrossRef]
44. String. Available online: https://string-db.org/ (accessed on 1 December 2025).
45. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* **2003**, *13*, 2498–2504. [CrossRef]
46. Doncheva, N.T.; Morris, J.H.; Gorodkin, J.; Jensen, L.J. Cytoscape StringApp: Network Analysis and Visualization of Proteomics Data. *J. Proteome Res.* **2019**, *18*, 623–632. [CrossRef]
47. Chin, C.-H.; Chen, S.-H.; Wu, H.-H.; Ho, C.-W.; Ko, M.-T.; Lin, C.-Y. CytoHubba: Identifying Hub Objects and Sub-Networks from Complex Interactome. *BMC Syst. Biol.* **2014**, *8*, S11. [CrossRef] [PubMed]
48. Yu, G.; Wang, L.-G.; Han, Y.; He, Q.-Y. ClusterProfiler: An R Package for Comparing Biological Themes Among Gene Clusters. *OMICS* **2012**, *16*, 284–287. [CrossRef] [PubMed]
49. Sabia, E.; Braghieri, A.; Pacelli, C.; Di Trana, A.; Coppola, A. Perception of Ecosystem Services from Podolian Farming System in Marginal Areas of Southern Italy. *Agriculture* **2023**, *14*, 28. [CrossRef]
50. Sycheva, O.V.; Anisimova, E.I.; Omarov, R.S.; Shlykov, S.N. Simmental Cattle Breed Lactation Features of Various Productive Types. *IOP Conf. Ser. Earth Environ. Sci.* **2021**, *848*, 012070. [CrossRef]
51. Ahmed, R.H.; Schmidtmann, C.; Mugambe, J.; Thaller, G. Effects of the Breeding Strategy Beef-on-Dairy at Animal, Farm and Sector Levels. *Animals* **2023**, *13*, 2182. [CrossRef]
52. Rinaldi, S.; Contò, M.; Claps, S.; Marchitelli, C.; Renzi, G.; Crisà, A.; Failla, S. Milk Fat Depression and Trans-11 to Trans-10 C18:1 Shift in Milk of Two Cattle Farming Systems. *Sustainability* **2022**, *14*, 977. [CrossRef]
53. Crisà, A.; Marchitelli, C.; Failla, S.; Contò, M. Determination of *N* -Acetylneuraminic and *N* -Glycolylneuraminic Acids in Unprocessed Milk of Four Cattle Breeds. *J. Dairy Res.* **2022**, *89*, 299–301. [CrossRef]
54. De Matteis, G.; Scatà, M.C.; Grandoni, F.; Crisà, A.; O'Brien, M.B.; Meade, K.G.; Catillo, G. Effect of IL8 Haplotype on Immunological Traits in Periparturient Dairy Cows. *Vet. Immunol. Immunopathol.* **2021**, *238*, 110288. [CrossRef]
55. Zilio, D.M.; Steri, R.; Catillo, G.; Buttazzoni, L. Establishment of a Crossbreed Simmental × Holstein Experimental Herd and First Assessment of Heterosis Effects on Technical and Biological Parameters. *Ital. J. Anim. Sci.* **2017**, *16*, 1–280. [CrossRef]
56. Scatà, M.C.; Grandoni, F.; Barile, V.L.; Catillo, G.; De Matteis, G. Simmental × Holstein Crossbred: Comparison of Immunological Traits with Parental Breeds during Peripartum and Early Lactation Period. *J. Dairy Res.* **2020**, *87*, 424–428. [CrossRef] [PubMed]

57. Yue, S.; Wang, Z.; Wang, L.; Peng, Q.; Xue, B. Transcriptome Functional Analysis of Mammary Gland of Cows in Heat Stress and Thermoneutral Condition. *Animals* **2020**, *10*, 1015. [CrossRef] [PubMed]
58. Grajales, S.M.B.; Zuluaga, J.J.E.; Herrera, A.L.; Osorio, N.R.; Vergara, D.M.B. RNA-Seq Differential Gene Expression Analysis in Mammary Tissue from Lactating Dairy Cows Supplemented with Sunflower Oil. *Anim. Prod. Sci.* **2020**, *60*, 758–771. [CrossRef]
59. Fantini, A. L'ancestrale Mungitura Della Podolica. Ruminantia 2019. Available online: https://archivio2023-2024.ruminantia.it/lancestrale-mungitura-della-podolica/ (accessed on 28 December 2025).
60. Natrella, G.; De Palo, P.; Maggiolino, A.; Faccia, M. A Study on Milk and Caciocavallo Cheese from Podolica Breed in Basilicata, Italy. *Dairy* **2023**, *4*, 482–496. [CrossRef]
61. Zorc, M.; Dolinar, M.; Dovč, P. A Single-Cell Transcriptome of Bovine Milk Somatic Cells. *Genes* **2024**, *15*, 349. [CrossRef]
62. Ren, J.; Zhang, Z.; Tang, M.; Wen, Z.; Luo, C.; Qiang, Z.; Cai, X.; Wang, H.; Wang, Q.; Ji, Y.; et al. A Single-Cell Transcriptomic Study of Milk Cells from Dairy Cows with Divergent Lactation Performance. *Sci. Rep.* **2025**, *15*, 34803. [CrossRef]
63. Farrell, H.M.; Jimenez-Flores, R.; Bleck, G.T.; Brown, E.M.; Butler, J.E.; Creamer, L.K.; Hicks, C.L.; Hollar, C.M.; Ng-Kwai-Hang, K.F.; Swaisgood, H.E. Nomenclature of the Proteins of Cows' Milk-Sixth Revision. *J. Dairy Sci.* **2004**, *87*, 1641–1674. [CrossRef]
64. Sigl, T.; Meyer, H.H.D.; Wiedemann, S. Gene Expression of Six Major Milk Proteins in Primary Bovine Mammary Epithelial Cells Isolated from Milk during the First Twenty Weeks of Lactation. *Czech J. Anim. Sci.* **2012**, *57*, 469–480. [CrossRef]
65. Bionaz, M.; Loor, J.J. Gene Networks Driving Bovine Mammary Protein Synthesis during the Lactation Cycle. *Bioinform. Biol. Insights* **2011**, *5*, 83–98. [CrossRef]
66. Çardak, A.D. Effects of genetic variants in milk protein on yield and composition of milk from Holstein-Friesian and Simmentaler cows. *S. Afr. J. Anim. Sci.* **2005**, *35*, 41–47. [CrossRef]
67. Bonfatti, V.; Di Martino, G.; Cecchinato, A.; Vicario, D.; Carnier, P. Effects of β-κ-Casein (CSN2-CSN3) Haplotypes and β-Lactoglobulin (BLG) Genotypes on Milk Production Traits and Detailed Protein Composition of Individual Milk of Simmental Cows. *J. Dairy Sci.* **2010**, *93*, 3797–3808. [CrossRef] [PubMed]
68. Pistoia, A.; Casarosa, L.; Poli, P.; Mani, D.; Ferruzzi, G. Caratteristiche Qualitative Del Latte e Del Formaggio Caciocavallo Nella Razza Bovina Podolica. *Large Anim. Rev.* **2015**, *21*, 63–66.
69. Perna, A.; Intaglietta, I.; Simonetti, A.; Gambacorta, E. Physico-Chemical Characteristics and Clotting Properties of Milk from Podolica Cows. *Anim. Sci. J.* **2016**, *87*, 1017–1024.
70. Kaya, U.; Özkan, H.; Yazlık, M.O.; Çamdeviren, B.; Güngör, G.; Karaaslan, İ.; Dalkıran, S.; Keçeli, H.H.; Akçay, A.; Yakan, A. Comparative evaluation of major milk quality parameters of Holstein and Simmental cows at different lactation stages under similar environmental conditions. *Med. Weter.* **2023**, *79*, 1–11. [CrossRef]
71. Hinz, K.; O'Connor, P.M.; Huppertz, T.; Ross, R.P.; Kelly, A.L. Comparison of the Principal Proteins in Bovine, Caprine, Buffalo, Equine and Camel Milk. *J. Dairy Res.* **2012**, *79*, 185–191. [CrossRef]
72. Shi, H.; Zhu, J.; Luo, J.; Cao, W.; Shi, H.; Yao, D.; Li, J.; Sun, Y.; Xu, H.; Yu, K.; et al. Genes Regulating Lipid and Protein Metabolism Are Highly Expressed in Mammary Gland of Lactating Dairy Goats. *Funct. Integr. Genom.* **2015**, *15*, 309–321. [CrossRef]
73. Lemay, D.G.; Pollard, K.S.; Martin, W.F.; Freeman Zadrowski, C.; Hernandez, J.; Korf, I.; German, J.B.; Rijnkels, M. From Genes to Milk: Genomic Organization and Epigenetic Regulation of the Mammary Transcriptome. *PLoS ONE* **2013**, *8*, e75030. [CrossRef]
74. Verardo, V.; Crisá, A.; Ochando-Pulido, J.M.; Martínez-Férez, A. Oligosaccharides from Colostrum and Dairy By-Products: Determination, Enrichment, and Healthy Effects. In *Studies in Natural Products Chemistry*; Elsevier: Amsterdam, The Netherlands, 2018; Volume 57, pp. 157–178.
75. Valk-Weeber, R.L.; Deelman-Driessen, C.; Dijkhuizen, L.; Eshuis-De Ruiter, T.; Van Leeuwen, S.S. In Depth Analysis of the Contribution of Specific Glycoproteins to the Overall Bovine Whey N-Linked Glycoprofile. *J. Agric. Food Chem.* **2020**, *68*, 6544–6553. [CrossRef]
76. Le Provost, F.; Cassy, S.; Hayes, H.; Martin, P. Structure and Expression of Goat GLYCAM1 Gene: Lactogenic-Dependent Expression in Ruminant Mammary Gland and Interspecies Conservation of the Proximal Promoter. *Gene* **2003**, *313*, 83–89. [CrossRef]
77. Crisà, A.; Ferrè, F.; Chillemi, G.; Moioli, B. RNA-Sequencing for Profiling Goat Milk Transcriptome in Colostrum and Mature Milk. *BMC Vet. Res.* **2016**, *12*, 264. [CrossRef]
78. Hanus, O.; Samkova, E.; Křížova, L.; Hasoňova, L.; Kala, R. Role of Fatty Acids in Milk Fat and the Influence of Selected Factors on Their Variability—A Review. *Molecules* **2018**, *23*, 1636. [CrossRef] [PubMed]
79. Crisà, A.; Marchitelli, C.; Pariset, L.; Contarini, G.; Signorelli, F.; Napolitano, F.; Catillo, G.; Valentini, A.; Moioli, B. Exploring Polymorphisms and Effects of Candidate Genes on Milk Fat Quality in Dairy Sheep. *J. Dairy Sci.* **2010**, *93*, 3834–3845. [CrossRef] [PubMed]
80. Suburu, J.; Shi, L.; Wu, J.; Wang, S.; Samuel, M.; Thomas, M.J.; Kock, N.D.; Yang, G.; Kridel, S.; Chen, Y.Q. Fatty Acid Synthase Is Required for Mammary Gland Development and Milk Production during Lactation. *Am. J. Physiol. Endocrinol. Metab.* **2014**, *306*, E1132–E1143. [CrossRef] [PubMed]

81. Marchitelli, C.; Contarini, G.; De Matteis, G.; Crisà, A.; Pariset, L.; Scatà, M.C.; Catillo, G.; Napolitano, F.; Moioli, B. Milk Fatty Acid Variability: Effect of Some Candidate Genes Involved in Lipid Synthesis. *J. Dairy Res.* **2013**, *80*, 165–173. [CrossRef]
82. Bernard, L.; Leroux, C.; Chilliard, Y. Expression and Nutritional Regulation of Stearoyl-CoA Desaturase Genes in the Ruminant Mammary Gland: Relationship with Milk Fatty Acid Composition. In *Stearoyl-CoA Desaturase Genes in Lipid Metabolism*; Springer: New York, NY, USA, 2013; pp. 161–193. ISBN 9781461479697.
83. Lu, J.; Argov-Argaman, N.; Anggrek, J.; Boeren, S.; van Hooijdonk, T.; Vervoort, J.; Hettinga, K.A. The Protein and Lipid Composition of the Membrane of Milk Fat Globules Depends on Their Size. *J. Dairy Sci.* **2016**, *99*, 4726–4738. [CrossRef]
84. Chong, B.M.; Reigan, P.; Mayle-Combs, K.D.; Orlicky, D.J.; McManaman, J.L. Determinants of Adipophilin Function in Milk Lipid Formation and Secretion. *Trends Endocrinol. Metab.* **2011**, *22*, 211–217. [CrossRef]
85. Li, Y.H.; Zhou, H.; Cheng, L.; Zhao, J.; Hickford, J.G.H. Variation in PLIN2 and Its Association with Milk Traits and Milk Fat Composition in Dairy Cows. *J. Agric. Sci.* **2020**, *158*, 774–780. [CrossRef]
86. Dudemaine, P.L.; Thibault, C.; Alain, K.; Bissonnette, N. Genetic Variations in the SPP1 Promoter Affect Gene Expression and the Level of Osteopontin Secretion into Bovine Milk. *Anim. Genet.* **2014**, *45*, 629–640. [CrossRef]
87. Prigent, M.; Barlat, I.; Langen, H.; Dargemont, C. IκBα and IκBα/NF-KB Complexes Are Retained in the Cytoplasm through Interaction with a Novel Partner, RasGAP SH3-Binding Protein 2. *J. Biol. Chem.* **2000**, *275*, 36441–36449. [CrossRef]
88. Ahlawat, S.; Arora, R.; Sharma, U.; Sharma, A.; Girdhar, Y.; Sharma, R.; Kumar, A.; Vijh, R.K. Comparative Gene Expression Profiling of Milk Somatic Cells of Sahiwal Cattle and Murrah Buffaloes. *Gene* **2021**, *764*, 145101. [CrossRef]
89. Pradeep, J.; Monika, S.; Ankita, S.; Umesh, K.S.; Amit, K.; Ashok, M.; Mishra, B.P.; Sandeep, M.; Kataria, R.S.; Kaushik, J.; et al. Expression Analysis of Solute Carrier (SLC2A) Genes in Milk Derived Mammary Epithelial Cells during Different Stages of Lactation in Sahiwal (Bos Indicus) Cows. *Adv. Dairy Res.* **2014**, *2*, 117. [CrossRef]
90. Considine, T.; Healy, Á.; Kelly, A.L.; McSweeney, P.L.H. Hydrolysis of Bovine Caseins by Cathepsin B, a Cysteine Proteinase Indigenous to Milk. *Int. Dairy J.* **2004**, *14*, 117–124. [CrossRef]
91. Bevilacqua, C.; Helbling, J.C.; Miranda, G.; Martin, P. Translational Efficiency of Casein Transcripts in the Mammary Tissue of Lactating Ruminants. *Reprod. Nutr. Dev.* **2006**, *46*, 567–578. [CrossRef] [PubMed]
92. Berberat, P.O.; Katori, M.; Kaczmarek, E.; Anselmo, D.; Lassman, C.; Ke, B.; Shen, X.; Busuttil, R.W.; Yamashita, K.; Csizmadia, E.; et al. Heavy Chain Ferritin Acts as an Anti-apoptotic Gene That Protects Livers from Ischemia-reperfusion Injury. *FASEB J.* **2003**, *17*, 1724–1726. [CrossRef] [PubMed]
93. Ali, A.; Rehman, M.U.; Mushtaq, S.; Ahmad, S.B.; Khan, A.; Karan, A.; Bashir Wani, A.; Ganie, S.A.; Mir, M.U.R. Biochemical and Computational Assessment of Acute Phase Proteins in Dairy Cows Affected with Subclinical Mastitis. *Curr. Issues Mol. Biol.* **2023**, *45*, 5317–5346. [CrossRef] [PubMed]
94. Anderson, C.L.; Chaudhury, C.; Kim, J.; Bronson, C.L.; Wani, M.A.; Mohanty, S. Perspective-FcRn Transports Albumin: Relevance to Immunology and Medicine. *Trends Immunol.* **2006**, *27*, 343–348. [CrossRef]
95. Behl, J.D.; Verma, N.K.; Tyagi, N.; Mishra, P.; Behl, R.; Joshi, B.K. The Major Histocompatibility Complex in Bovines: A Review. *ISRN Vet. Sci.* **2012**, *2012*, 872710. [CrossRef]
96. Kiang, J. Heat Shock Protein 70 KDa Molecular Biology, Biochemistry, and Physiology. *Pharmacol. Ther.* **1998**, *80*, 183–201. [CrossRef]
97. Liu, Q.; Liang, C.; Zhou, L. Structural and Functional Analysis of the Hsp70/Hsp40 Chaperone System. *Protein Sci.* **2020**, *29*, 378–390. [CrossRef]
98. Wu, S.; Fan, J.; Tang, F.; Chen, L.; Zhang, X.; Xiao, D.; Li, X. The Role of RIM in Neurotransmitter Release: Promotion of Synaptic Vesicle Docking, Priming, and Fusion. *Front. Neurosci.* **2023**, *17*, 1123561. [CrossRef]
99. Bello, O.D.; Zanetti, M.N.; Mayorga, L.S.; Michaut, M.A. RIM, Munc13, and Rab3A Interplay in Acrosomal Exocytosis. *Exp. Cell Res.* **2012**, *318*, 478–488. [CrossRef]
100. Capuco, A.V.; Choudhary, R.K. Symposium Review: Determinants of Milk Production: Understanding Population Dynamics in the Bovine Mammary Epithelium. *J. Dairy Sci.* **2020**, *103*, 2928–2940. [CrossRef] [PubMed]
101. Zoldan, K.; Schneider, J.; Moellmer, T.; Fueldner, C.; Knauer, J.; Fuerll, M.; Starke, A.; Specht, M.; Reiche, K.; Hackermueller, J.; et al. Discovery and Validation of Immunological Biomarkers in Milk for Health Monitoring of Dairy Cows-Results from a Multiomics Approach. *Adv. Dairy Res.* **2017**, *5*, 182. [CrossRef]
102. Diks, S.H.; Sartori da Silva, M.A.; Hillebrands, J.-L.; Bink, R.J.; Versteeg, H.H.; van Rooijen, C.; Brouwers, A.; Chitnis, A.B.; Peppelenbosch, M.P.; Zivkovic, D. D-Asb11 Is an Essential Mediator of Canonical Delta–Notch Signalling. *Nat. Cell Biol.* **2008**, *10*, 1190–1198. [CrossRef] [PubMed]
103. Brisken, C.; O'Malley, B. Hormone Action in the Mammary Gland. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a003178.
104. Rupp, R.; Boichard, D. Genetics of Resistance to Mastitis in Dairy Cattle. *Vet. Res.* **2003**, *34*, 671–688. [CrossRef]
105. Dai, W.; Zou, Y.; White, R.R.; Liu, J.; Liu, H. Transcriptomic Profiles of the Bovine Mammary Gland during Lactation and the Dry Period. *Funct. Integr. Genom.* **2018**, *18*, 125–140. [CrossRef]

106. Favorit, V.; Hood, W.R.; Kavazis, A.N.; Skibiel, A.L. Graduate Student Literature Review: Mitochondrial Adaptations across Lactation and Their Molecular Regulation in Dairy Cattle. *J. Dairy Sci.* **2021**, *104*, 10415–10425. [CrossRef]
107. Long, X.; Chen, L.; Xiao, X.; Min, X.; Wu, Y.; Yang, Z.; Wen, X. Structure, Function, and Research Progress of Primary Cilia in Reproductive Physiology and Reproductive Diseases. *Front. Cell Dev. Biol.* **2024**, *12*, 1418928. [CrossRef]
108. McDermott, K.M.; Liu, B.Y.; Tlsty, T.D.; Pazour, G.J. Primary Cilia Regulate Branching Morphogenesis during Mammary Gland Development. *Curr. Biol.* **2010**, *20*, 731–737. [CrossRef]
109. Bisutti, V.; Vanzin, A.; Pegolo, S.; Toscano, A.; Gianesella, M.; Sturaro, E.; Schiavon, S.; Gallo, L.; Tagliapietra, F.; Giannuzzi, D.; et al. Effect of Intramammary Infection and Inflammation on Milk Protein Profile Assessed at the Quarter Level in Holstein Cows. *J. Dairy Sci.* **2024**, *107*, 1413–1426. [CrossRef] [PubMed]
110. Hoeksema, M.; Van Eijk, M.; Haagsman, H.P.; Hartshorn, K.L. Histones as Mediators of Host Defense, Inflammation and Thrombosis. *Future Microbiol.* **2016**, *11*, 441–453. [CrossRef] [PubMed]
111. Kaplan, M.J.; Radic, M. Neutrophil Extracellular Traps: Double-Edged Swords of Innate Immunity. *J. Immunol.* **2012**, *189*, 2689–2695. [CrossRef] [PubMed]
112. Jiang, L.Y.; Sun, H.Z.; Guan, R.W.; Shi, F.; Zhao, F.Q.; Liu, J.X. Formation of Blood Neutrophil Extracellular Traps Increases the Mastitis Risk of Dairy Cows During the Transition Period. *Front. Immunol.* **2022**, *13*, 880578. [CrossRef]
113. Pisanu, S.; Cubeddu, T.; Pagnozzi, D.; Rocca, S.; Cacciotto, C.; Alberti, A.; Marogna, G.; Uzzau, S.; Addis, M.F. Neutrophil Extracellular Traps in Sheep Mastitis. *Vet. Res.* **2015**, *46*, 59. [CrossRef]
114. Cheng, Z.; Buggiotti, L.; Salavati, M.; Marchitelli, C.; Palma-Vera, S.; Wylie, A.; Takeda, H.; Tang, L.; Crowe, M.A.; Wathes, D.C.; et al. Global Transcriptomic Profiles of Circulating Leucocytes in Early Lactation Cows with Clinical or Subclinical Mastitis. *Mol. Biol. Rep.* **2021**, *48*, 4611–4623. [CrossRef]
115. Vanselow, J.; Yang, W.; Herrmann, J.; Zerbe, H.; Schuberth, H.J.; Petzl, W.; Tomek, W.; Seyfert, H.M. DNA-Remethylation around a STAT5-Binding Enhancer in the AS1-Casein Promoter Is Associated with Abrupt Shutdown of a AS1-Casein Synthesis during Acute Mastitis. *J. Mol. Endocrinol.* **2006**, *37*, 463–477. [CrossRef]
116. Singh, K.; Erdman, R.A.; Swanson, K.M.; Molenaar, A.J.; Maqbool, N.J.; Wheeler, T.T.; Arias, J.A.; Quinn-Walsh, E.C.; Stelwagen, K. Epigenetic Regulation of Milk Production in Dairy Cows. *J. Mammary Gland Biol. Neoplasia* **2010**, *15*, 101–112. [CrossRef]
117. Johnstone, M.E.; Leck, A.L.; Lange, T.E.; Wilcher, K.E.; Shephard, M.S.; Paranjpe, A.; Schutte, S.; Wells, S.I.; Kappes, F.; Salomonis, N.; et al. DEK Promotes Mammary Hyperplasia and Is Associated with H3K27me3 Epigenetic Modifications. *Life Sci. Alliance* **2025**, *8*, e202503230. [CrossRef]
118. Beyenbach, K.W.; Wieczorek, H. The V-Type H+ ATPase: Molecular Structure and Function, Physiological Roles and Regulation. *J. Exp. Biol.* **2006**, *209*, 577–589. [CrossRef]
119. Futai, M.; Sun-Wada, G.H.; Wada, Y.; Matsumoto, N.; Nakanishi-Matsui, M. Vacuolar-Type ATPase: A Proton Pump to Lysosomal Trafficking. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **2019**, *95*, 261–277. [CrossRef]
120. Lu, N.; Zhou, Z. Membrane Trafficking and Phagosome Maturation during the Clearance of Apoptotic Cells. *Int. Rev. Cell Mol. Biol.* **2012**, *293*, 269–309. [PubMed]
121. Castellano, F.; Molinier-Frenkel, V. An Overview of L-Amino Acid Oxidase Functions from Bacteria to Mammals: Focus on the Immunoregulatory Phenylalanine Oxidase IL4I1. *Molecules* **2017**, *22*, 2151. [CrossRef] [PubMed]
122. Nagaoka, K.; Aoki, F.; Hayashi, M.; Muroi, Y.; Sakurai, T.; Itoh, K.; Ikawa, M.; Okabe, M.; Imakawa, K.; Sakai, S. L-Amino Acid Oxidase Plays a Crucial Role in Host Defense in the Mammary Glands. *FASEB J.* **2009**, *23*, 2514–2520. [CrossRef] [PubMed]
123. Qi, J.; Gan, L.; Fang, J.; Zhang, J.; Yu, X.; Guo, H.; Cai, D.; Cui, H.; Gou, L.; Deng, J.; et al. Beta-Hydroxybutyrate: A Dual Function Molecular and Immunological Barrier Function Regulator. *Front. Immunol.* **2022**, *13*, 805881. [CrossRef]
124. Zhao, K.; Chen, Y.H.; Penner, G.B.; Oba, M.; Guan, L.L. Transcriptome Analysis of Ruminal Epithelia Revealed Potential Regulatory Mechanisms Involved in Host Adaptation to Gradual High Fermentable Dietary Transition in Beef Cattle. *BMC Genom.* **2017**, *18*, 976. [CrossRef]
125. Novak, T.E.; Rodriguez-Zas, S.L.; Southey, B.R.; Starkey, J.D.; Stockler, R.M.; Alfaro, G.F.; Moisá, S.J. Jersey Steer Ruminal Papillae Histology and Nutrigenomics with Diet Changes. *J. Anim. Physiol. Anim. Nutr.* **2019**, *103*, 1694–1707. [CrossRef]
126. Trisciuoglio, D.; Desideri, M.; Farini, V.; De Luca, T.; Di Martile, M.; Tupone, M.G.; Urbani, A.; D'Aguanno, S.; Del Bufalo, D. Affinity Purification-Mass Spectrometry Analysis of Bcl-2 Interactome Identified SLIRP as a Novel Interacting Protein. *Cell Death Dis.* **2016**, *7*, e2090. [CrossRef]
127. Singh, V.; Moran, J.C.; Itoh, Y.; Soto, I.C.; Fontanesi, F.; Couvillion, M.; Huynen, M.A.; Churchman, L.S.; Barrientos, A.; Amunts, A. Structural Basis of LRPPRC–SLIRP-Dependent Translation by the Mitoribosome. *Nat. Struct. Mol. Biol.* **2024**, *31*, 1838–1847. [CrossRef]

128. Badertscher, L.; Wild, T.; Montellese, C.; Alexander, L.T.; Bammert, L.; Sarazova, M.; Stebler, M.; Csucs, G.; Mayer, T.U.; Zamboni, N.; et al. Genome-Wide RNAi Screening Identifies Protein Modules Required for 40S Subunit Synthesis in Human Cells. *Cell Rep.* **2015**, *13*, 2879–2891. [CrossRef]
129. Ohsakaya, S.; Fujikawa, M.; Hisabori, T.; Yoshida, M. Knockdown of DAPIT (Diabetes-Associated Protein in Insulin-Sensitive Tissue) Results in Loss of ATP Synthase in Mitochondria. *J. Biol. Chem.* **2011**, *286*, 20292–20296. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

https://doi.org/10.3390/ruminants6010016