Transcriptome profiles of whole blood in Italian Holstein and Italian Simmental lactating cows diverging for genetic merit for milk protein

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ABSTRACT

The aim of the research was to investigate the variation of gene expression in the blood of lactating cows in relation to the genetic merit for productive traits. For the study, 24 Italian Holstein (IH) cows from a single farm and 24 Italian Simmental (IS) cows from a second farm were selected. Cows were in mid-lactation, and farms had similar management and feeding conditions. For each breed, cows were separated into 3 classes of estimated breeding value (EBV) for milk protein yield (EBVp), namely, 8 cows with low EBVp (low group, LG), 8 cows with high EBV (high group, HG), and the 8 cows closest to the median (medium group, MG). Gene expression was measured on blood with a whole-transcriptome bovine microarray, and data of LG and HG were expressed relative to MG. The number of differentially expressed genes between the low and high EBVp groups were 443 for IS and 281 for IH cows. The IS cows had a greater number of genes (398 vs. 241 in IH cows) with opposite expression in the high and low EBV groups, compared with the MG. In IS cows, the largest number of pathways affected were within the KEGG subcategories of “energy metabolism,” “lipid metabolism,” and “metabolism of cofactors and vitamins.” Furthermore, the “glutathione metabolism” pathway was markedly affected, with *GSTM1* (from glutathione-S-transferase family), *GSS* (glutathione synthetase), and *G6PD* (glucose-6-phosphate dehydrogenase) upregulated in the IS HG but downregulated in the IS LG group. The “intestinal immune network for IgA production” pathway had 2 bovine leukocytes antigen (*BoLA-DQ*) genes, and the B-cell activating factor of the TNF superfamily (*TNFSF13B*) upregulated in the IS LG group and downregulated in the IS HG group. The IH cows had the highest number of affected pathways in the “metabolism” and “organismal systems” categories, the latter with 5 out of 10 subcategories relative to the immune system. Within the “T

cell receptor signaling pathway,” the IH HG cows had upregulation of the *CD1d*, *CD4*, and *CD3* antigens and downregulation of the *NFκB* subunit *Rela* gene. This study revealed that specific metabolic and immunological pathways are directly related to genetic merit, and could be considered a signature of quantitative selection. Starting from these preliminary observations, the comparison of differentially expressed genes among animals with different EBVp seems a promising approach to unravel molecular response at a blood level associated with productive traits.

Key words: genetic merit, lactating cow, blood gene expression, estimated breeding value

INTRODUCTION

Over the past decades, genetic selection through EBV has improved milk, fat, and protein yields. A large portion of these improvements likely is a result of changes in digestive and metabolic functions, along with better nutritional management, but many of the genetic and genomic variations underpinning these improvements are not completely elucidated (Pareek et al., 2011; McNamara, 2012). In the modern high-yielding dairy cow, the observed reduction in reproductive performance and higher disease susceptibility represent a negative consequence of the selection programs based primarily on productive traits (Snijders et al., 2001; Berry et al., 2011), and underscore the need to better understand the biological mechanisms that link productive performance and health of dairy cows.

Several studies on genetics and immunology are currently ongoing to gather information to refocus breeding programs to improve the health and reproductive performance of cows (Park et al., 2004; Banos et al., 2013). As a general worldwide trend, breeding programs that in the past selected aggressively for increased milk, fat, and protein productions now include traits such as fertility, udder health, and functional survival (Miglior et al., 2005; Oltenacu and Broom, 2010). In different lineages of Holstein cattle, evidence indicates that milk yield is not the only factor strictly responsible for reproductive failure (Snijders et al., 2001). Furthermore,

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Table 1. Characteristics of cows involved in the study and divided according to the estimated breeding values for milk protein yield ($n = 8$ cows per group)

Item	Italian Simmental ¹			Italian Holstein ¹		
	LG	MG	HG	LG	MG	HG
Parity	2.7 ± 0.6	3.2 ± 0.6	3.5 ± 0.8	2.3 ± 0.8	2.6 ± 0.9	2.6 ± 0.7
DIM at sampling	132 ± 28	142 ± 40	152 ± 27	159 ± 32	152 ± 29	142 ± 39
BW at sampling	687 ± 30	688 ± 33	665 ± 53	637 ± 39	632 ± 35	620 ± 60
EBVp ¹	-2.0 ± 2.7 ^C	7.4 ± 1.6 ^B	21.2 ± 6.2 ^A	-2.3 ± 7.4 ^C	24.5 ± 7.1 ^B	45.9 ± 3.4 ^C

^{A-C}Means within a row with different superscripts significantly differ ($P < 0.01$) within the breed; if not indicated, means in the same row within breed do not significantly differ.

¹Estimated breeding value for milk protein yield (EBVp) group: LG = 8 cows with the lowest EBVp of the Italian Simmental (IS) and Italian Holstein (IH) herds; MG = 8 cows closest to the median EBVp of the IS and IH herds; HG = 8 cows with the highest EBVp of the IS and IH herds.

recent data suggest that selection for healthier animals based on naturally superior immunity, without lowering productivity, is feasible (Mallard et al., 2011).

Recent studies have placed more emphasis on the effects of DNA polymorphisms associated with milk traits and immune response on reproductive performance and milk production. For instance, Demeter et al. (2009) reported an association between the *DGAT1* polymorphism and the rate of nonreturn after insemination in Dutch Holstein-Friesian cows. Cochran et al. (2013) studied the association between SNP of Holstein bulls and the pregnancy rate of the daughters, and their effects on productive traits, uncovering a negative correlation for 11 SNP. Polymorphisms that associate with milk yield and composition also were identified in immune-related genes (Beecher et al., 2010).

If the link between DNA polymorphisms and phenotypic traits is biologically relevant, transcriptional profiles in cells or tissues from cows with divergent genetic merit for productive traits would be expected to differ. Phenotype to genotype relationships rely on the statistical association among DNA variants with traits recorded in a large number of animals, but actually do not provide evidence of the physiologic changes for which the genotypes are believed to be responsible.

As a first approach to better understand the link between genetic merit for production traits and the cellular transcriptome, the present study used a whole-transcriptome microarray and bioinformatics to compare gene expression profiles in peripheral blood leukocytes of healthy Italian Simmental (**IS**) and Italian Holstein (**IH**) lactating cows diverging for EBV for milk protein (**EBVp**).

MATERIALS AND METHODS

Animals and Allocation to the Experimental Groups

Twenty-four healthy IS and 24 IH pluriparous cows between 80 and 180 DIM were selected based on their

EBV for milk protein yield from a commercial farm of 125 lactating IS and a commercial farm of 145 lactating IH. Farms were located in the northeast of Italy (Friuli Venezia Giulia region). The cow EBV values for milk protein yield (kg), provided from the Italian breeder associations of IS (ANAPRI, <http://www.anapri.eu/>) and IH (ANAFI, <http://www.anafi.it/>), were used to select cows and to define 3 EBV groups per breed. For each farm 24 cows were selected, 8 with the lowest EBVp (low group, **LG**), 8 with the highest EBVp (high group, **HG**) and the 8 with an EBVp value closest to the median value of EBVp (medium group, **MG**) of the herd. A more detailed description about the cow characteristics and EBV is presented in Table 1.

The farms had free-stall barns and the animals had ad libitum access to a TMR twice daily at around 0700 and 1700 h. All cows had free access to fresh water. Diets were formulated to meet the estimated nutrient requirements for lactation (INRA, 2010).

Sampling Procedures

One sample of about 50 mL of milk was collected from all cows during the normal routine of morning milking (beginning of milking from 0500 to 0600 h) and was immediately refrigerated and then frozen at -20°C until analysis. Blood was collected on the same day after the morning milking in a time span of 60 min, when cows had ad libitum access to fresh water and spontaneously moved to the feed headlock fence for the morning meal. The cows of the 2 herds were sampled within 10 d.

Blood from all cows was sampled from the coccygeal vein in three 10-mL vacuum tubes; one contained K3-EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium) for plasma collection, and one had no anti-coagulant for serum collection and a PAXgene Blood RNA System tube (Preanalytix, Hombrechtikon, Switzerland) for RNA extraction. The blood from the first 2 tubes was centrifuged within 1 h at 755 × *g* for 10 min

at 20°C and the plasma and serum samples were stored at -20°C until further analysis. The PAXgene tubes were handled and stored following the manufacturer's instructions.

Analysis of Plasma and Milk

The analyses of plasma total protein, albumin, glucose, urea, creatinine and AST were performed using a Roche Cobas 6000 analyzer with proprietary kits (TP2, ALB2, GLUC2, UREAL, creatinine plus, and ASTL; F. Hoffmann-La Roche AG, Basel, Switzerland). Globulin content was calculated as the difference between total protein and albumin, and then the albumin to globulin ratios were also calculated. Hemoglobin was analyzed with a Beckmann Coulter Gen-S analyzer (Beckmann Coulter Inc., Fullerton, CA). Plasma NEFA, BHBA, glutathione peroxidase (**GPx**), and total antioxidant status (**TAS**) were measured with Randox kits (Randox Laboratories Limited, Crumlin, UK). The GPx was expressed as units of hemoglobin (U/g of Hb). Plasma Zn was analyzed with a colorimetric assay (product code 17640H, Sentinel CH. SpA, Milan, Italy). Milk protein, fat, and lactose content and SCC were measured with mid-infrared spectroscopy (Fourier Transform Instrument, FT6000, Foss Electric, Hillerød, Denmark).

Statistical Analysis of Biochemical and Milk Data

All the data were stored in a spreadsheet using Microsoft Office Excel (2010, Microsoft Corp., Redmond, WA), and the descriptive statistics and analyses were performed with SPSS (SPSS, 1997). Normality of data was tested by the Kolmogorov-Smirnov nonparametric test. Only SCC was not normally distributed, and for this variable a $\log_2$ transformation was used before statistical analysis. A general linear analysis was used within each breed to determine differences in milk yield and its composition and plasma metabolites, using the following model:

$$y_i = \mu + \text{group}_i + b \times \text{DIM} + \varepsilon,$$

where y = dependent variable; μ = general mean; group = fixed effect of the EBVP group, with i from 1 to 3 (LG, MG, and HG for the IS and IH); b = the linear component of DIM; and ε = the residual error.

Microarray Protocol, Statistical Analysis, and Data Mining

After RNA extraction, all samples were deemed of good/excellent integrity, showing an RNA Integrity

Number (RIN) >6.5. The RNA samples were labeled using a 2-dye Low Input Quick Amp Labeling Kit (Agilent, Santa Clara, CA). The cDNA was obtained for each sample by a reverse-transcriptase reaction adding 200 ng of RNA, 0.8 μ L of T7 Promotor and DNase-RNase-free water to a volume of 5.3 μ L. Six annotated Bovine (V2) Gene Expression Microarrays, 4×44K (Agilent), 3 for each farm, were used for the gene expression analysis. Hybridizations were performed balancing across arrays and slides the samples from each EBVP quartile. Within each breed, every slide provided at least one cow of every EBVP group, alternating the Cy3 and Cy5 dyes for each group across the slides. Every slide had 4 microarrays, with one Cy3 and Cy5 labeled samples each (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2014-9049>). After hybridization, arrays were scanned with a ScanArray 4000 (GSI-Lumonics, Billerica, MA) dual-laser confocal scanner and images were processed and edited to extract the intensity data using Agilent Features Extraction Software.

For the statistical analysis, data from 24 microarrays from the 2 breeds were imported to GeneSpring GX software (Agilent), and a quantile normalization was performed to signal intensity values to avoid potential biases not due to biological effects (Bolstad et al., 2003). Afterward, the signal intensity data were organized by breed and EBVP group. The gene annotations from the gal file were then uploaded according to the probe identifiers. The intermediate EBVP groups of IS (IS MG) and IH (IH MG) were used as a reference within each breed to evaluate differential gene expression of the extreme groups (LG, lowest EBVP and HG, highest EBVP). A permutation matrix was created by calculating for each gene of each cow of the LG and HG the ratios with the corresponding gene of the MG group. The resulting linear expression ratios were then $\log_2$ transformed ($\log_2$ ratios).

A tab-delimited file was uploaded to the MeV software (version 4.9.0; <http://mev-tm4.sourceforge.net/>; Saeed et al., 2003), and used to perform for each breed a t -test of the expression $\log_2$ ratios between the LG/MG and HG/MG groups. The P -values were based on the t -distribution with a critical value of 0.05, and an adjusted Bonferroni multiple testing correction was then applied. Data mining on the list of significant genes was performed using the Kyoto Encyclopedia of Genes and Genomes pathways (KEGG; <http://www.genome.jp/kegg/>) with the Database for Annotation, Visualization and Integrated Discovery tool (DAVID; <http://david.abcc.ncifcrf.gov/>; Hosack et al., 2003).

RESULTS

Milk Yield and Composition

As expected, both IH and IS high EBVp cows (HG) had higher ($P < 0.05$ for IH and <0.01 for IS) 305-d milk yield compared with the intermediate and the lower EBVp groups (MG and LG). The 305-d milk protein and fat yields were significantly higher ($P < 0.01$) for the HG of IS and for HG of IH cows (Table 2). Furthermore, except fat concentration and SCC, all milk components in IH were significantly different across the EBVp groups (milk and fat yield $P < 0.05$; protein concentration and yield $P < 0.01$). Neither the IS nor the IH EBVp groups had a different 305-d SCC log.

Blood Biochemistry

Biochemical analyses of blood were within the reference values for physiological conditions, confirming the positive health status of the cows (Table 3; Kaneko et al., 1997; Gonzalez et al., 2011). In IS, a dual-purpose cow selected for both milk and meat traits, blood NEFA significantly differed across the EBVp groups ($P < 0.01$), with the highest value (0.12 vs. 0.09 and 0.1 mEq/L) detected for the intermediate group (MG; $P < 0.01$). In the IH cows, BHBA concentration in the LG, MG, and HG EBVp groups was higher (0.75 mmol/L), intermediate (0.64), and lower (0.59), respectively ($P < 0.05$).

The covariate for DIM in the GPx for IS cows between the EBVp groups was significant ($P < 0.05$), but not in the single group comparison, being higher, intermediate, and lower in IS LG, IS MG, and IS HG (246.14, 240.50, and 232.86 U/g of Hb, respectively). Similarly, the covariate was significant for blood urea for IH cows ($P < 0.01$), with the lowest value in the cows with intermediate EBVp (IH MG group, $P < 0.05$).

Differentially Expressed Genes and Effect on KEGG Pathways

After the Bonferroni correction with a P -value <0.05 , IS cows had 443 differentially expressed uniquely annotated genes (DEG) between the low (IS LG) and high (IS HG) EBVp groups, whereas 281 were detected for the comparison in the IH groups. Details of the number of up- and downregulated genes compared with the MG group are reported in Table 4. Overall, IS cows had more DEG between the experimental groups and the reference IS MG groups in comparison with IH (443 vs. 281). For both breeds, the cows with high genetic merit

Table 2. Effects of genetic merit (LG, MG, HG) and covariate for DIM on milk yield and composition in lactating Italian Simmental (IS) and Italian Holstein (IH) cows

Item	Unit	IS ¹				P-value				IH ¹				P-value			
		LG	MG	HG		RMSE ²	Group	DIM		LG	MG	HG		RMSE	Group	DIM	
Milk yield	kg/d	29.80	31.41	34.03		3.97	0.077	0.055		29.53 ^a	30.74 ^a	35.34 ^b		3.72	0.031	0.353	
Fat	%	4.36	4.01	3.75		0.61	0.164	0.467		3.55	3.37	4.03		0.73	0.205	0.757	
Fat	kg/d	1.30	1.26	1.28		0.26	0.959	0.492		1.07 ^a	1.03 ^a	1.41 ^b		0.28	0.041	0.756	
Protein	%	3.50	3.47	3.49		0.20	0.888	0.185		2.74 ^a	3.06 ^b	3.07 ^b		0.17	0.003	0.100	
Protein	kg/d	1.04	1.09	1.18		0.10	0.072	0.117		0.81 ^a	0.94 ^b	1.08 ^c		0.14	0.004	0.992	
SCC	log	5.82	4.37	5.38		1.79	0.283	0.652		3.29	3.78	3.50		1.50	0.773	0.221	
Milk yield	kg/305 d	8,105 ^c	8,733 ^b	9,962 ^a		1,316.1	0.006	0.539		9,141 ^c	9,671 ^b	10,660 ^{ab}		1,321.7	0.010	0.411	
Fat	kg/305 d	318 ^c	327 ^b	381 ^a		14.2	0.007	0.517		333 ^c	367 ^b	453 ^a		41.1	0.000	0.489	
Protein	kg/305 d	289 ^c	309 ^b	355 ^a		13.2	0.003	0.243		279 ^c	295 ^b	344 ^a		29.3	0.001	0.531	

^{a-c}Means with different superscripts on the same row differ significantly for $P < 0.05$ within IS or IH breed.
¹Estimated breeding value for milk protein yield (EBVp) group: LG = 8 cows with the lowest EBVp of the IS and IH herds; MG = 8 cows closest to the median EBVp of the IS and IH herds; HG = 8 cows with the highest EBVp of the IS and IH herds.
²RMSE = root mean square error.

Table 3. Effects of genetic merit (LG, MG, HG) and DIM on plasma and blood parameters in Italian Holstein (IH) and Italian Simmental (IS) lactating cows

Item	Unit	IS ¹			P-value			IH ¹			P-value		
		LG	MG	HG	RMSE ²	Group	DIM	LG	MG	HG	RMSE	Group	DIM
Total protein	g/L	76.57	76.38	76.57	4.70	0.976	0.409	81.00	82.63	79.75	3.96	0.411	0.461
Albumin (Alb)	g/L	36.86	38.00	37.14	2.38	0.595	0.416	37.83	38.88	39.50	1.59	0.340	0.062
Globulin (Glo)	g/L	39.71	38.38	39.43	5.74	0.895	0.732	43.17	43.75	40.25	4.35	0.371	0.174
Alb/Glo	ratio	0.95	1.01	0.96	0.17	0.795	0.954	0.90	0.90	0.98	0.10	0.464	0.086
Urea	mmol/L	4.57	4.01	4.17	0.67	0.374	0.424	4.00 ^a	3.46 ^b	3.65 ^{ab}	0.51	0.096	0.007
Creatinine	μmol/L	93.71	99.38	89.57	11.54	0.313	0.406	67.67	67.00	68.75	7.15	0.878	0.865
AST ³	IU/L	61.86	70.13	80.71	14.93	0.072	0.482	87.50	94.25	85.38	12.09	0.270	0.107
Zinc	μmol/L	11.29	10.36	10.66	1.46	0.519	0.686	14.15	14.70	13.26	1.69	0.345	0.147
TAS ⁴	mmol/L	1.14	1.16	1.14	0.00	0.654	0.392	1.17	1.18	1.18	0.00	0.981	0.309
Hemoglobin	g/dL	9.77	10.81	10.03	1.05	0.158	0.824	11.37	10.88	10.63	0.79	0.365	0.227
GPx ⁵	U/g of Hb	246.14 ^a	240.50 ^a	232.86 ^b	49.51	0.998	0.038	253.50	269.25	306.75	47.44	0.106	0.599
Cortisol	pmol/mL	8.59	9.29	8.62	6.45	0.967	0.699	4.39	5.91	4.04	3.49	0.490	0.271
Glucose	mmol/L	3.46	3.30	2.99	0.32	0.098	0.118	3.25	3.29	3.30	0.20	0.713	0.092
NEFA	mEq/L	0.09 ^a	0.12 ^b	0.10 ^{ab}	0.00	0.001	0.733	0.11	0.11	0.12	0.00	0.932	0.339
BHBA	mmol/L	0.57	0.49	0.51	0.10	0.547	0.222	0.75 ^a	0.64 ^a	0.59 ^b	0.10	0.032	0.304

^{a,b}Means with different superscripts in the same row differ significantly at $P < 0.05$ within IS or IH breed.¹Estimated breeding value for milk protein yield (EBVp) group: LG = 8 cows with the lowest EBVp of the IS and IH herds; MG = 8 cows closest to the median EBVp of the IS and IH herds; HG = 8 cows with the highest EBVp of the IS and IH herds.²RMSE = root mean square error.³AST = aspartate amino transferase.⁴TAS = total antioxidant status.⁵GPx = glutathione peroxidase.

(HG groups) had a greater number of up- and down-regulated genes, and IS LG cows had the opposite trend with more down- than upregulated genes. This trend resulted in a greater number of genes with opposite expression (LG/MG vs. HG/MG) in the dual-purpose IS cows. Data mining with the DAVID tool was performed to identify KEGG pathways affected in cows with a negative (IS and IH LG groups) and positive (IS and IH HG groups) EBV for milk protein.

KEGG Pathways and Relevant Genes in IS Cows

Relative to IS MG, the most affected pathways and the significant genes detected in the IS LG and IS HG groups of IS cows are reported in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2014-9049>). The largest number of pathways affected belong to the “metabolism” category. In particular, the “energy metabolism,” “lipid metabolism,” and “metabolism of cofactors and vitamins” subcategories had the highest number of genes.

Both the “cellular processes” and “organismal systems” categories had 6 pathways most affected, the former with “regulation of actin cytoskeleton” and “endocytosis” pathways containing the highest number of genes (10 and 10, respectively), whereas the latter had 6 and 5 genes in “vascular smooth muscle contraction” and “progesterone-mediated oocyte maturation” pathways, respectively.

Three genes in the “glutathione metabolism” pathway, *GSTM1* (an enzyme of the glutathione-S-transferase family), *GSS* (glutathione synthetase), and *G6PD* (glucose-6-phosphate dehydrogenase), were upregulated in the IS HG group.

Within the subcategory “immune system,” also the “intestinal immune network for IgA production” pathway had differentially expressed genes in IS cows. In particular, 2 *BoLA-DQ* antigens (*BoLA-DQB* and *BoLA-DQA5*) and the B-cell activating factor of the TNF superfamily (*TNFSF13B*) were up- and downregulated in the LG and HG group, respectively. On the contrary, the transforming growing factor B1 (*TGFB1*) had an opposite regulation in both the groups, being down- and upregulated in LG and HG.

Among other differentially expressed genes *DGAT2*, a diacylglycerol acyltransferase different from *DGAT1* but also represented in the “glycerolipid metabolism” pathway, was up- and downregulated in the LG and HG, respectively. Within the same pathway, *LPAATα* (lysophosphatidic acid acyltransferase) had opposite expression compared with *DGAT2* (i.e., down- and upregulated in the LG and HG, respectively, compared with MG).

Table 4. Summary of the differentially expressed genes (DEG; $P < 0.05$) in the Italian Simmental (IS) and Italian Holstein (IH) groups¹

Item	IS		IH	
	LG/MG	HG/MG	LG/MG	HG/MG
Upregulated	200	252	154	168
Downregulated	243	191	127	113
Genes with opposite expression ²	398		241	
Total DEG	443		281	

¹For each breed, the differentially regulated genes are calculated as ratios of LG to MG and HG to MG groups. Estimated breeding value for milk protein yield (EBVp) group: LG = 8 cows with the lowest EBVp of the IS and IH herds; MG = 8 cows closest to the median EBVp of the IS and IH herds; HG = 8 cows with the highest EBVp of the IS and IH herds.

²Genes that in the 2 extreme groups (Q1 and Q3) have opposite regulation (i.e., upregulated and downregulated) when compared with the Q2 groups.

KEGG Pathways and Relevant Genes in IH Cows

The most affected pathways based on the DEG in the LG and HG groups of IH cows, compared with the IH MG, are reported in Supplemental Table S2 (<http://dx.doi.org/10.3168/jds.2014-9049>). The highest number of affected pathways was observed for the “organismal systems” and “metabolism” categories. The subcategories “purine metabolism” and “xenobiotics biodegradation and metabolism” had the highest number of represented genes within the “metabolism” category. Regarding the “organismal system” category, 5 out of 10 subcategories belong to the “immune system,” and among those also the pathways “T cell receptor signaling,” “hematopoietic cell lineage,” and “B cell receptor signaling.” The “cellular processes” category had 3 pathways and the “regulation of actin cytoskeleton” and “focal adhesion” had the highest number of DEG, 8 and 6, respectively.

Within the “T cell receptor signaling pathway,” the IH HG cows had upregulation of the *CD1d*, *CD4*, and *CD3*, and *MALT1* and *RelA* genes, represented in both T and B cell receptor signaling pathways, had different expression regulation in IH LG and HG groups. In particular, *MALT1*, coding for a paracaspase, was downregulated in both groups compared with the IH MG whereas *RelA*, coding for the p65 member of the *NFκB* transcription factor, was upregulated in IH LG and downregulated in HG.

The expression of the *ACSL1* gene in IH was greater HG and lower in LG compared with the MG group. The pro-opiomelanocortin gene (*POMC*) had the same expression pattern as *ACSL1*, being upregulated in IH HG and downregulated in LG. Also the *CYP4A11* oxidoreductase regulation gene had the same variation between groups as *ACSL1*, whereas *ACOX3* had an opposite regulation, with downregulation in IH LG and upregulation in HG.

DISCUSSION

The study aimed to investigate potential molecular biomarkers in blood related to different genetic merit and productive potential in lactating dairy cows. For this reason, we enrolled cows at mid-lactation, when animals are no longer in the transition period.

Whole blood is a complex mixture of cells and does not necessarily reflect molecular events at an organ level, but is easier to sample in comparison with tissue and involves limited handling of animals.

Blood metabolite (Table 3) values were within the reference reported for healthy cows after peak lactation (Kaneko et al., 1997; Gonzalez et al., 2011) and allowed a first consideration with respect to the different productive potential of IS and IH used in this study. In the IH breed, which has been bred for high milk, fat, and protein yields, the BHBA was lower ($P < 0.05$) in the highest producing IH HG group (0.75, 0.64, and 0.59 mmol/L in LG, MG, and HG groups, respectively, Table 3) suggesting a greater efficiency of energy utilization. Our previous study with a larger population (155 cows) revealed that breed and genetic merit for a productive trait have an effect on blood metabolic profiles at mid-lactation (Sgorlon et al., 2015; accepted). In this study, blood TAS was significantly affected by breed, being higher in IH than IS cows (1.19 vs. 1.14 mmol/L, $P < 0.001$). In both breeds, plasma BHBA was negatively associated with the EBVp as covariate ($P < 0.05$) and plasma glucose in the IS breed also was negatively associated with the EBVp ($P < 0.05$).

Considering that IS and IH cows originated from 2 different farms, a statistical comparison of the whole blood cell transcriptome between breeds is not straightforward, even though the results obtained in the 2 breeds can help to underline the different genetic background between IH cows, highly specialized for milk production, and IS cows, a dual-purpose breed selected

for both meat and milk production. Because our study involved end-point sampling, it is also not possible to determine with certainty if the responses observed are a cause of metabolites and hormonal effects or vice versa. However, in healthy cows in a balanced metabolic condition, even small differences in gene transcription levels could be the result of the different genetic background.

At the molecular level, the inherently different metabolism of the 2 breeds agrees with the higher number of DEG detected and with the enrichment of the “metabolism” category for IS (dual purpose) cows with lower and higher EBVp for milk protein compared with IH cows (443 vs. 281, Table 4). Compared with the intermediate IS EBVp group (IS MG), the DEG of LG and HG cows were represented, mostly with opposite expression, across 8 pathways belonging to the “metabolism” category (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2014-9049>). Four of them related to energy metabolism or metabolism of major nutrients (lipids, carbohydrates, amino acids). The IH cows had 7 KEGG pathways in the “metabolism” category, but none of them involved in energy or carbohydrate metabolism (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-9049>). The lower number of genes represented in these pathways for IH cows supports the idea of a general difference in overall metabolism between EBVp and breeds. In contrast, the specialized high-milk-producing IH cows had more KEGG pathways involved with the “immune system” subcategory compared with the IS cows and most of the DEG had opposite expression in the extreme EBVp groups (IH LG and HG, relative to MG).

Pathway Interpretation in IS Cows

The downregulation of the 3 genes (*GSS*, *GSTM1*, and *G6PD*) in the IS LG group within the “glutathione metabolism” pathway (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-9049>) suggests a reduction of de novo GSH synthesis and a less efficient reduction of the oxidized glutathione disulfide (*GSSG*). Oxidative stress conditions were not apparent when evaluating blood parameters (Table 3), but besides the antioxidant defense from the reactive oxygen species related to the GPx activity, the *GSH* can be related to its important role in modulation of the immune response as reported in humans (Townsend et al., 2003). This point agrees with the expression of *TGFβ1*, *TNFSF13B*, and *BoLA* (antigen-presenting peptides) genes, which belong to the “intestinal immune network for IgA production” pathway and had opposite expression in cows with different genetic merit. The *BoLA-DQ* complex plays a relevant role for enhanced immune response in dairy cows. The *BoLA-DQ5* and *BoLA-DQA5* map in

the chromosome 23 (BTA23:25855146–25863045 and BTA23:25404020–25411642, respectively), within the 250,000-bp flank region (Hapmap44002-BTA-110636, 25178791 bp) of a SNP of the *ELOVL5* gene, which was found highly associated with antibody-mediated immune response in Holstein cows (Thompson-Crispi et al., 2014).

Aukrust et al. (1995) working in humans reported the activation of the tumor necrosis factor system subsequent to the reduction of *GSH* in CD4+ lymphocytes. In the IS LG group, the overexpression of the 13B-TNF super family proteins could have led to the activation of the class II of histocompatibility antigens of the major histocompatibility complex (BoLA antigens presenting peptides) and to a response of the B cell. Such response would have been favored by the inhibition of the anti-proliferating *TGF-β1*, which was upregulated in the IS HG. It appears that in IS cows differences in EBV for milk protein were associated with the control of anti-oxidant activity, likely due to the different metabolic requirements for higher milk production and immune function.

In IS cows also *LPAATα* (*AGPAT1*) and *DGAT2* genes, composing “glycerolipid metabolism,” had an opposite expression level in LG and HG groups. The *LPAATα* and *DGAT2* genes are involved in triacylglycerol (TAG) synthesis via the glycerol phosphate pathway, and the latter is responsible for the last step in TAG synthesis. In mice, *DGAT2* inactivation resulted in lethal neonatal lipopenia and skin barrier abnormalities (Stone et al., 2004). In contrast, transgenic overexpression in liver caused hepatic steatosis, with increased amount of TAG, DAG, ceramides, and unsaturated long-chain acyl-CoA FA (Monetti et al., 2007). These findings suggest that the *DGAT1* and *DGAT2* play fundamentally different roles in mammalian TAG metabolism (Stone et al., 2004). The *DGAT2*-specific functions are still poorly understood in livestock, but some recent evidence associated it with carcass fat in commercial feedlot steers (Li et al., 2009) and more recently a genome-wide association study on IH have indicated a marker of milk protein percentage close to the *DGAT2* gene (Capomaccio et al., 2015).

The *LPAATα* gene, an acylglycerolphosphate acyltransferase, catalyzes the acylation of lysophosphatidic (LPA) to phosphatidic acid (LA), a TAG precursor but also a crucial compound for phospholipid synthesis. In dairy cows, as well as in humans, LPA signaling has been recognized also to have multiple effects on reproductive functions (Kowalczyk-Zieba et al., 2012; Wocławek-Potocka et al., 2014) and several of the known effects of LPA, such as proliferation and IL-2 production (Xu et al., 1995), are consistent with a potential proinflammatory or proimmune function. In

this context, we could interpret the downregulation of LPA in SQ3 as control mechanism of the inflammatory response, consistent with the upregulation of the glutathione genes previously discussed. Furthermore, differences in expression of these genes could be related to the significantly higher level of blood NEFA in IS HG compared with the MG. Overall, the anabolic and energy storing propensity was confirmed in IS cows, which had the lowest genetic merit for a milk trait.

Pathway Interpretation in IH Cows

The *CD4* gene, coding for a cluster of differentiation molecules of T leukocytes, was upregulated in the IH HG group. The CD4+ lymphocytes play a protective role at the onset of *Staphylococcus aureus* induced mastitis (Rivas et al., 2000), and this pathogen is often associated with high SCC and subclinical mastitis, although the genetic correlation is low (Sørensen et al., 2009). Recently, an epigenetic downregulation of CD4 cluster expression has been reported in Holstein cows with clinical mastitis (Wang et al., 2013), underscoring its role in the control of mammary health. Mastitis resistance is to some extent under genetic control (Detilleux, 2002; Olmos et al., 2009), and SCC in the IH breeding program accounts for 10%. The CD4 gene maps in chromosome 5 (BTA5:103995316–104017746 bp) in a region close to a SNP (rs109019599, BTA5:106713273 bp) associated with functional traits in dairy cows (Chessa et al., 2015). The downregulation of *RelA* in the IH HG cows, encoding a subunit of the *NFκB* nuclear factor of inflammatory activation, would suggest a lower activation of inflammatory cascade in cows with high genetic merit (Olmos et al., 2009).

Compared with the cows with lower EBVp, the IH HG cows had significantly higher milk and milk component yields but not SCC. Hence, the upregulation of *CD4* and *CD3G* in HG cows could be the effect of either the activation of the immune response, not evidenced by the blood parameters, or the different clusters of surface receptor molecules expressed within the T leukocytes population.

The *ACSL1* gene, a member of the acyl-CoA synthetase long-chain FA family highly expressed in bovine tissues (Bionaz and Loores, 2008), belongs to “adipocytokine,” “PPAR,” and “fatty acids metabolism” pathways and was upregulated in IH HG cows. Association analysis (Widmann et al., 2011) indicated that *ACSL1* is a candidate gene for FA composition and lipid metabolism in skeletal muscle of cattle. For *POMC*, a gene highly involved in feed intake and also upregulated in IS HG and downregulated in LG, association analysis indicated a QTL (*BMS607*) in the flanking region within 196,000-bp span (Bagnato et al., 2008). These

associations would indicate that selection for productive traits in IH cows enhances lipid metabolism with a negative effect on intake regulation.

CONCLUSIONS

The present study is a first attempt to compare blood chemistry and gene expression profiles in whole blood of Italian Simmental and Italian Holstein healthy cows with diverging EBV for milk protein yield. This study revealed that specific metabolic and immunological pathways are directly related to genetic merit, and could be considered a signature of quantitative selection. Further research at the molecular level is thus required to identify and understand the biological effects of genetic selection for productive traits on molecular response of immune and metabolic status at a systemic level.

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