UNIVERSITÀ DEGLI STUDI DI UDINE

Agricultural Science and Biotechnology PhD school
Cycle XXVI
Coordinator: prof. Mauro Spanghero

DOCTOR OF PHYLOSOPHY DISSERTATION

RELATION OF RUMEN MICROBIOME AND BLOOD TRANSCRIPTOME WITH THE GENETIC MERIT IN ITALIAN SIMMENTAL AND ITALIAN HOLSTEIN COWS

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ACADEMIC YEAR 2013/2014

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Sandri M, Manfrin C, Pallavicini A, Stefanon B, 2014. **Microbial biodiversity of the liquid fraction of rumen content from lactating cows**. Animal 13:1-8.

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doi:10.1017/S1751731114000056

animal

Microbial biodiversity of the liquid fraction of rumen content from lactating cows

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(Received 4 September 2012; Accepted 17 December 2013)

Host and dietary interactions with the rumen microbiome can affect the efficacy of supplements, and their effect on the composition of the bacterial population is still unknown. A 16S rRNA metagenomic approach and Next-Generation Sequencing (NGS) technology were used to investigate the bacterial microbiome composition in the liquid fraction of the rumen content collected via stomach tubing. To investigate biodiversity, samples were taken from three groups of four lactating dairy cows given a supplement of either 50 g of potato protein (Ctrl group), or 50 g of lyophilized Saccharomyces cerevisiae *(LY group) or 50 g of dried* S. cerevisiae *(DY group) in a potato protein support. Rumen samples were collected after 15 days of dietary treatments and milk production was similar between the three groups. Taxonomic distribution analysis revealed a prevalence of the* Firmicutes *phylum in all cows (79.76%) and a significantly (*$P < 0.05$*) higher presence of the genus* Bacillus *in the DY group. Volatile fatty-acid concentration was not significantly different between groups, possibly because of relatively high inter-animal variability or limited effect of the treatments or both, and the correlation analysis with bacterial taxa showed significant associations, in particular between many* Firmicutes *genera and butyrate. Limited differences were observed between dietary treatments, but the lack of microbiome data before yeast administration does not allow to draw firm conclusions on the effect of dietary treatments.*

Keywords: rumen bacteria community, *Saccharomyces cerevisiae*, Next-Generation Sequencing, 16S rRNA, dairy cows

Implications

Next-Generation Sequencing technology offers the opportunity to gather information regarding the rumen microbiome by comparing repository databases of rumen bacteria 16S rRNA gene sequences generated through different experiments. The high extent of sequencing attained through this research provides valuable information on the microbial biodiversity of the liquid phase of the rumen and represents a contribution to research in rumen microbiology and dairy feed supplements. However, the effectiveness of yeast supplements to modify the rumen bacterial microbiome requires further investigation.

Introduction

Bacteria account for more than 95% of the total number of rumen microorganisms (Brulc *et al.,* 2009) and their metabolism has a significant effect on animal maintenance and performance. Although rumen bacterial populations have flexible metabolic capabilities, dietary composition and physical features of feedstuffs have a major influence on microbial diversity. Particularly, although the total microbial number often remains similar, changes might occur in terms of the microbiome composition or species makeup (Fernando *et al.,* 2010). The ecology of rumen microbes is based on a dynamic interaction between the host and the diet and is strictly related to the vital functions of the animal, such as its immune functions, regulation of extracellular signalling and competition with opportunistic pathogenic bacteria (Khafipour *et al.,* 2009). These aspects increase the complexity in understanding the rumen ecology and its adaptation to different environments, which is also probably subject to the interplay between the rumen microbes and the host genome, as has been reported for other mammals (Turnbaugh *et al.*, 2006).

Dietary supplements are widely used in dairy cows to increase their productivity through improving rumen metabolism. Yeasts, in particular different strains or commercial products of *Saccharomyces cerevisiae*, have been tested on dairy cows with a number of positive effects such as increased dry matter intake (DMI) and milk production (Wohlt *et al.,*1998; Dann *et al.,* 2000); improved diet digestibility (Erasmus *et al.*, 1992; Marden *et al.*, 2008); stabilized rumen pH (Bach *et al.,* 2007); and stimulated rumen bacteria growth (Newbold *et al.,* 1995). However, there are also many studies with no or

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negative responses to yeast supplementation (Mwenya *et al*., 2005; Longuski *et al.,* 2009; Chung *et al.,* 2011), probably related to the strong influence of the basal diet, the variation in yeast strains and the different commercial formulations.

Meta-analysis (Desnoyers *et al.,* 2009; Robinson and Erasmus, 2009) of published lactation experiments with *S. cerevisiae* supplementation has shown an increase in milk production of 0.9 kg milk/day or 1.2 g milk/kg BW, but many factors were claimed to interfere with the results (e.g. strain and level of supplementation, DMI, dietary NDF, % of dietary concentrates). Moreover, theories about the physiological effects of live *S. cerevisiae* supplementation in dairy cows differ widely, and detailed investigations of the effects of yeasts on rumen microbiome composition are lacking.

High-throughput sequencing (HTS), such as pyrosequencing, is effective in obtaining sequences of a large number of microorganisms simultaneously, allowing metagenomic investigations of different microbiomes. Massive and parallel pyrosequencing of microbial communities allows the investigation of the variability of potential 'rumen phenotypes' and the bases of the diet–rumen microbiome interactions. A continually increasing amount of data on the rumen metagenome is being generated from HTS techniques, but little information is available regarding the rumen content fractions, which are likely to represent microorganisms related to the digestion of different substrates, that is, the soluble components in the liquid fraction and dietary fiber in the solid fraction (Pitta *et al.,* 2010).

In this study, a gene-centric metagenomic approach is used to investigate the microbiome composition of the liquid fraction of the rumen content obtained from 12 lactating dairy cows supplemented with potato protein or with a similar amount of lyophilized or dried *S. cerevisiae*. The aim is to provide insight into the bacterial composition and its biodiversity, as a basis for further investigations.

Material and methods

Animals, milk production, diets and sample collection

Twelve lactating Friesian cows housed in the same barn and fed the same diet were used for the experiment. Animals were of the first to third parity from 60 to 240 days in milk. Animals had *ad libitum* access to water and were offered a total mixed ration (17.3 kg DM/head per day), formulated to cover the nutrient requirements for lactation (INRA, 1988). The diet consisted of 532 g/kg DM corn silage, 255 g/kg DM lucerne forage, 154 g/kg DM cereals (60 : 40 corn and barley meal proportion), 44 g/kg DM protein supplement (soy bean meal and hydrolyzed potato protein), and 15 g/gk DM vitamin and mineral supplement. Animals were fed twice a day, at 0700 and 1700 h and the supplements were given individually once daily with the morning feeding. Milk production was recorded individually for 30 days, starting from 15 days before the beginning of the experimental period.

At the beginning of the experiment, cows were split into three groups of four animals each, paired for parity, milk yield and stage of lactation. The average milk yield was 29.5 ± 4 kg/day. The three groups were randomly assigned to experimental treatments: Group LY (yeast form 1), Group DY (yeast form 2) and a Control Group (Ctrl; potato protein). Cows in the LY group received 50 g/head per day of a commercial live lyophilized brewer's yeast (DM 95.5%, CP 46.0%, L 4.7%, CF 0.5%, ash 6.0%; vital count 1×10^{10} cfu/g). Those in the DY group were given a supplement of 50 g/head per day of dried brewer's yeast (DM 95.0%, CP 32.0%, L 1.8%, CF 0.2%, ash 28%). The control group received 50 g/head per day of hydrolyzed potato protein (DM 90.0%, CP 86.0%, L 3.6% CF 0.6% and Ash 3.1%). After 14 days of supplementation, rumen contents were collected 1 h after the morning feeding with an oesophageal probe. A sample of about 40 g of rumen contents was collected, which were separated immediately into two aliquots of about 20 g each, one without fixative for DNA extraction and the second (20 ml) with 1 ml of 85% ortophosphoric acid (H_2PO_4) for volatile fatty-acid (VFA) determination. The aliquots were frozen at −80°C until analysis or DNA extraction.

Sample fractioning and VFA analysis

For DNA extraction, the aliquots of whole rumen content were centrifuged for 5 mins at $750 \times \mathbf{g}$ to separate the solid phase from the liquid phase. The supernatant was transferred to a tube and centrifuged for 20 mins at $12\,000 \times \mathbf{g}$. The pellet formed was washed with buffer solution (NaCl 0.9%), centrifuged again and the supernatant discarded. For VFA analyses, the second aliquot of the whole rumen contents was centrifuged at $20\,000 \times \mathbf{g}$ for 30 min at 10°C. The supernatant was filtered using a 25 mm syringe filter with a 0.45 μm polypropylene membrane (VWR International Srl, PA, USA) and the filtrate analyzed by HPLC at 220 nm (LC 200 Perkin-Elmer pump connected to a model ISS-100 autosampler (20 μl loop) with a UV-Vis model LC 95 Perkin-Elmer detector, Perkin-Elmer, Norwalk, CN, USA). Separations were achieved using an Aminex HPX-87 H column (300 mm × 7.8 mm) and one pre-column (Bio-Rad, Hercules, CA, USA) with the thermostat at 40°C.

For data collection and integration, Turbochrom software with two NCI 900 PE Nelson Perkin Elmer interfaces was used. For a complete analysis of VFA by HPLC, a 40 min isocratic programme was run with H_2SO_4 0.008 N as a mobile phase at a flow rate of 0.6 ml/min. Peaks of compounds were identified by comparing retention times of standard mixtures with those of the samples and quantification was based on peak area measurements on the basis of an external standard.

Metagenomic DNA extraction

The DNA from the pellets of the liquid fraction was extracted with a Fecal DNA MiniPrep kit (Zymo Research; Irvine, CA, USA) following the manufacturer's instructions, including a bead beating step. Pre-amplification of DNA concentration in the samples was measured with a Quant-iT dsDNA BR Assay (Invitrogen, Carlsbad, CA, USA). The primers used for the

Eubacteria 16S rRNA amplification were F8 AGAGTTTG ATCCGGCTCAC (Baker *et al*., 2003) and R534 ATTACCG CGGCTGCTGGC (Liu *et al*., 2007), which produced amplicons of about 500 base pairs (bps). The primers were chosen to amplify the eubacteria 16S rRNA regions V1–V3, as reported by Liu *et al*. (2007 and 2008), as being the most reliable to produce community clusters and to accurately assign taxonomy in a large data set (Kumar *et al*., 2011; Vilo and Dong, 2012). The primers above were added with multiplex identifier tags and 454 adapters A and B to allow pooling of the amplicons generated from different individuals before sequencing. PCR amplification was initially performed in a CFX96 Real-Time PCR detection system (Bio Rad, Hercules, CA, USA) using the EvaGreen™ dye (Biotium, Hayward, CA, USA) and two units of the Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes, Varta, Finland) according to the manufacturer's instructions. Optimal PCR conditions were found with the following thermal profile: an initial 1-min denaturation step at 95°C, followed by 25 cycles at 95° for 20 s, 65°C for 15 s and 72°C for 30 s. Amplification products were analysed with a 65°/95°C melting curve. The same thermal profile was thus used for metagenomic DNA (3 to 11 ng) amplification with Mastercycler® epgradient S (Eppendorf, Hamburg, Germany), adding a final extension step at 72°C for 1 min. The samples were checked on TBE 2% gel and extracted and purified with HiYeld™ Gel/PCR DNA Fragments Extraction kit (RBC Bioscience, Taipei, Taiwan), according to the manufacturer's instructions. After purification, sample concentrations were measured by a Quant-iT dsDNA BR Assay (Invitrogen, Carlsbad, CA, USA) and the pooled samples were sent to an external sequencing service for pyrosequencing on half a plate of a Roche 454 GS FLX Titanium series. Collection and sequence information were deposited at GenBank SRA (short read archive) under accession number SRA049734.1.

Metadata analysis

Trimmed fastA files from pyrosequencing Roche 454 GS FLX Titanium series were analysed with the online RDP Classifier 2.3 (http://rdp.cme.msu.edu; Ribosomal Dataset Project; Cole *et al*., 2008) for Operational Taxonomic Units (OTUs) identification, rarefaction curves and α-biodiversity calculations, by targeting our samples on bacterial small-subunit 16S rRNA. Hierarchical clustering analysis was applied for OTUs definition, whereas for the analysis of α-biodiversity the nonparametric Chao1 estimator (Chao, 1984) and the Shannon Index, H′ (Shannon and Weaver, 1949) were used.

For taxonomic analysis, the community visualization tool for the Eubacteria domain at the genus taxonomic level, including not assigned taxa from the online VAMPS service (http://vamps.mbl.edu) was used (data not shown). Sequences were then annotated and assigned with the GAST assignment method, which blasts each high-quality tag against a reference database of hypervariable sequences of the regions V1-V3 (RefV1-V3) from the 16S rRNA database (Ref16SSU). First, the tag was aligned against the top 100 BLAST hits and the Ref V1-V3 matches having the minimum pairwise distance to the tag were selected. For each best Ref V1-V3 match, all sources from Ref 16SSU sequences were selected and a consensus agreement of $\geqslant$66% of selected Ref 16SSU sources was calculated. The consensus taxonomy was then applied to the tag.

Statistical analysis

The differences of total VFA contents (mMol/l) and their molar percentages in the rumen fluid samples between the groups were evaluated using the ANOVA model with 'group' as a fixed factor. Pearson's coefficients indicated the correlation between the microbiome composition and the VFA proportions in the rumen contents. All the statistical analyses were performed with SPSS (SPSS, 1997).

Results

During the recording period, the mean ± s.d. milk yields did not significantly differ between treatments and were 28.9 ± 4.4, 29.1 ± 4.5 and 30.1 ± 4.3, respectively, for the LY, DY and Ctrl groups.

Sequencing data and biodiversity analysis

The number of reads sequenced per sample showed a good reliability for sequencing depth, further confirmed by the small difference in number, length and average quality score of sequences of different samples. The mean number of 16S sequence reads per sample was 30 245 with a mean length of 458 bps (minimum 455; maximum 461). To compare alternative definitions, the classification of sequences into OTUs and biodiversity indexes of Chao1 and H′ were calculated with a stair-step cut-off of 0.02 from 0.00 to 1.6 (data not shown). The analysis of the results indicate that the 0.10 cut-off (10% similarity among sequences) leads to comparable mean values between Chao1 and OTU values (i.e. estimated and detected (Table 1). The rarefaction curves (Figure 1) for a cut-off of 0.02 indicate the very high-sequencing depth and the reliability of the results, independently from the number of tag sampled. No significant differences between the groups are shown for OTU and Chao1 at the selected cut-offs. In addition, the Shannon biodiversity index (H′) for species richness in the fluid fraction does not significantly vary between treatments, indicating a large variability between cows or a limited effect of the treatment.

16S rRNA annotations, phylogenetic analysis and effect of yeast supplementation

Taxonomic annotations (352 031 sequences for the 12 samples) were performed for the bacteria domain at the different levels and the assigned sequences belonging to abundant phylotype members, having a frequency higher than 1% (Fuhrman, 2009), are reported in Table 2. Within the annotated sequences, *Firmicutes* is the most represented phylum (79.76%), followed by *Fusobacteria* (8.60%), *Bacteroidetes* (3.84%), *TM7, Tenericutes* and *Actinobacteria*. Members at the phylum level having a frequency lower than 1%

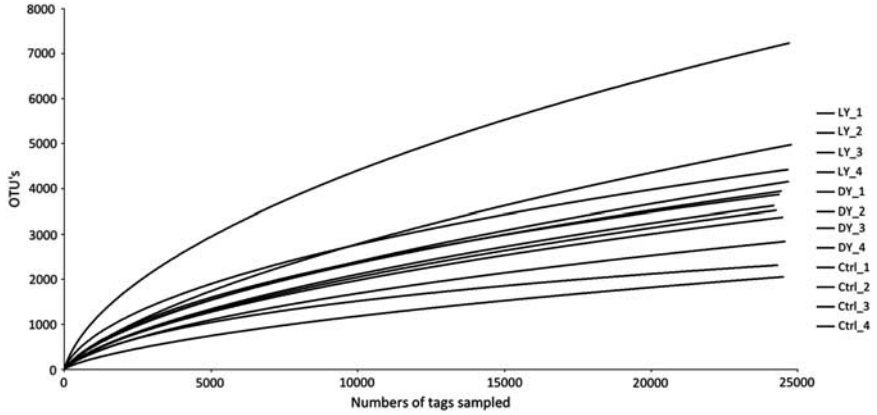

Figure 1 Rarefaction curves of all 12 samples at 0.02 distance cut-off. Distance cut-off indicates the similarity threshold among sequences needed to be clustered in an Operational Taxonomic Unit. (A 0.1 distance cut-off indicates sequences similarity of 90%).

Table 1 *Number of OTU, Shannon (H′) and Chao_1 biodiversity indexes and rarefaction data of bacterial microbiome measured in rumen liquid fluid of cows daily supplemented with 50 g LY, 50 g DY or with 50 g potato protein (Ctrl) using increasing distance cut-off values*

		Experimental group				
		Mean				
Item	Cut-off	LY	DY	Ctrl	s.e.	CV (%)
OTUs						
	0.02	2974	4512	4101	390	10.10
	0.06	1101	1634	1478	143	10.18
	0.10	505	752	677	64	9.93
H′						
	0.02	5.5	6.3	6.2	0.19	3.17
	0.06	3.7	4.7	4.7	0.26	5.95
	0.10	3.1	4.0	3.9	0.25	6.82
Chao_1						
	0.02	6178	9225	8271	813	10.30
	0.06	1611	2274	2027	188	9.54
	0.10	592	850	773	70	9.48
Rarefaction						
	0.02	4511	2973	4099	390	10.10
	0.06	1634	1101	1477	143	10.19
	0.10	752	504	677	64	9.93

LY = lyophilized yeast; DY = dried yeast; OTU = Operational Taxonomic Units; Ctrl = Control.
Differences between means were never significant ($P > 0.05$)
Distance cut-off indicates the level of similarity (units) among sequences needed to be clustered in an OTU. (A 0.1 distance cut-off indicates sequences similarity of 90%).

(rare members, included in the 'others' in Table 2), are *Chloroflexi* (0.54%), *Proteobacteria* (0.44%), *Cyanobacteria* (0.22%), *Spirochaetes* (0.20%) and *Verrucomicrobia* (0.17%). A small amount (0.26%) of the coding sequences could not be assigned to a phylum. Many sequences are identified only down to the order, class or family level (e.g. *Ruminococcaceae*, *Lachnospiraceae*). Among the identified genera, the most represented are *Bacillus* (26.03%), *Sneathia* (8.60%), *Streptococcus* (8.28%) and *Succiniclasticum* (3.20%). No genera were assigned to any of the two highly represented families, *Lachnospiraceae* and *Ruminococcaceae*. Annotated genera having a low frequency are *Lactobacillus*, *Enterococcus* and *Mogibacterium* (1.70%, 1.35% and 1.02%, respectively) and *Prevotella* (2.32%). For more than 30 genera, the percentage in the liquid phase was lower than 1%, and these genera are included in the 'others' in Table 2 (Fuhrman, 2009). Within these genera, the most represented (range 0.4% to 1.0%) are *Butyrivibrio* (0.86%), *Lysinibacillus* (0.70%), *Ruminococcus* (0.66%), *Marvinbryantia* (0.47%) and *Clostridium* (0.45%), all belonging to the phylum *Firmicutes*, and *Atopobium* (0.56%) belonging to the phylum *Actinobacteria*.

The comparisons of individual bacterial microbiomes indicated that the classes *Bacilli* and *Clostridia* of *Firmicutes* phylum were common in the 12 samples. The genus *Bacillus* was detected in 10 of the 12 cows, with the highest prevalence for all the cows of DY treatment. Members of the genus *Streptococcus* were identified in seven cows, and was the most abundant taxa in some cows (up to 35.66%). Families *Lactobacillaceae* and *Enterococcaceae* and their respective genera *Lactobacillus* and *Enterococcus* were detected in samples of a limited number of cows ($n = 5$).

The family *Ruminococcaceae* is present with a non-assigned genus in all samples ranging from 2.32% to 20.63%. Within this family, *Ruminococcus* and *Faecalibacterium* are the most identified genera (Table 2). Within the same class, the *Lachnospiraceae* family is also common to all samples, but the most abundant genus are not identified and the other genera are *Marvinbryantia*, *Blautia*, *Butyrivibrio*, *Syntrophococcus* and *Acetitomaculum* (Table 2).

Bacteria belonging to the class *Clostridia* family *Veillonellaceae* genus *Succiniclasticum* were represented in all except for one cow, which also showed the lowest level of biodiversity (data not shown). Other identified genera such as *Selenomonas, Schwarzia* and *Mogibacterium* are detected only in few cows (Table 2).

Within the phylum *Bacteroidetes*, only the *Bacteroidia* class is detected, with two families identified, *Prevotellaceae*, accounting for 2.86% of the total sequences, and *Rikenellaceae,* accounting for <1%.

Bacteria belonging to *Prevotellaceae* and *Rikenellaceae* families are represented in eight and two cows, respectively, although the most prevalent genus identified (*Prevotella*)

Table 2 *Average taxonomic frequencies distributions and number of assigned sequences (Seq) at a phylum, family and genus levels of microbiome in rumen liquid fractions and effect of yeasts supplementation at the genus level*

Taxonomy level												
Phylum		Family		Genus								
						LY		DY		Ctrl		MSE
	%		%		%	%	n	%	n	%	n	
Firmicutes	79.8	Bacillaceae	26.1	Bacillus	26.0	31.7[ab]	(4)	61.8[a]	(4)	4.7[b]	(2)	3.1
		Lachnospiraceae	16.9	Not assigned	13.9	14.7	(4)	8.6	(2)	16.3	(4)	70.6
		Ruminococcaceae	12.8	Not assigned	11.1	10.2	(4)	7.3	(4)	12.8	(4)	26.6
		Streptococcaceae	8.3	Streptococcus	8.3	7.0	(3)	1.1	(1)	15.6	(3)	105.3
		Veillonellaceae	4.4	Succiniclasticum	3.2	2.5	(4)	2.5	(3)	5.5	(4)	6.5
		Erysipelotrichaceae	2.4	Not assigned	2.0	1.6[ab]	(4)	1.4[a]	(2)	2.5[b]	(4)	0.4
		Lactobacillaceae	1.7	Lactobacillus	1.7	1.7	(2)	1.1	(1)	2.5	(1)	6.3
		Not assigned	1.6	Not assigned	1.6	1.5	(0)	1.1	(3)	2.3	(4)	0.9
		Enterococcaceae	1.4	Enterococcus	1.4	1.3	(2)	2.5	(2)	0.8	(1)	5.5
		Not assigned	1.3	–	–	0.6	–	0.4	–	0.8	–	0.1
		Incertae sedis		Mogibacterium	1.0	1.0	(3)	0.7	(1)	1.4	(2)	0.3
Fusobacteria	8.6	Leptotrichiaceae	8.6	Sneathia	8.6	0.0	(0)	0.0	(0)	12.8	(1)	217.3
Bacteroidetes	3.8	Prevotellaceae	2.9	Prevotella	2.3	3.7	(3)	1.8	(2)	1.7	(3)	3.1
TM7	2.3	Not assigned	2.3	Not assigned	2.3	2.1[ab]	(3)	1.4[a]	(3)	3.5[b]	(4)	1.4
Tenericutes	2.2	Not assigned	2.2	Not assigned	2.2	1.5[ab]	(2)	1.2[a]	(3)	3.3[b]	(4)	1.7
Actinobacteria	1.3	–	–	–	–	–	–	–	–	–	–	–
Others < 1%*	1.7	Others < 1%	5.6	Others < 1%	10.3	13.6	–	5.1	–	9.7	–	–
Not assigned < 1%†	0.3	Not assigned < 1%	1.7	Not assigned < 1%	4.1	5.3	–	2.0	–	3.8	–	–
Total	100.0		100.0		100.0	100.0		100.0		100.0		
Seq No.	351 107		324 415		221 145	74 582		75 262		81 301		

LY = lyophilized yeast; DY = dried yeast; Ctrl = Control; MSE = mean square error.
Results of statistical analysis (ANOVA) and frequency values between the LY group (lyophilized yeast), DY group (dried yeast) and Control group (potato protein) is reported only at a genus level, as data for phylum and family are not significant between groups (frequency are the average for the 12 rumen samples).
In brackets, the number of cows in each experimental group that presented the annotated sequences.
*Others < 1%: sequences assigned to other Taxa having a frequency lower than 1%.
†Non-assigned < 1%: sequences not assigned belonging to Taxa having a frequency lower than 1%.
[a,b]Significant difference ($P < 0.05$).

never exceeded 4% of the bacteria in the liquid fraction rumen content.

The genus *Sneathia* is found only in one cow of the Ctrl group, which accounted for more than 50% of the bacterial metagenome.

Limited differences between the DY group with the LY and Ctrl groups are observed for the bacterial microbiome at the genus level (Table 2).

In particular, differences ($P < 0.05$) are observed between the DY group and the Ctrl group for the genus *Bacillus,* for a non-assigned genus of *Erysipelotrichaceae* family and for two non-assigned genera belonging to the *TM7* and *Tenericutes* phyla, respectively.

Among the rare members, *Firmicutes Ruminococcus* was also under-represented in the DY group and showed frequencies of 0.27%, 0.91% and 0.63% in the DY, LY and Ctrl groups, respectively ($P < 0.05$).

Rumen VFA's analysis and bacterial microbiome associations
The average content of VFA in the rumen liquid (Table 3) is 95.2 in LY, 95.1 in DY and 89.6 mMol/l in the Ctrl group ($P > 0.05$).

The highest and most significant Pearson correlations are found between the molar proportions of butyrate in the rumen liquid and the bacterial microbiome composition both at the phylum, family and genus level (Table 4). Negative correlations with butyrate are calculated for *Firmicutes Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae, Erysipelotrichaceae* families, for *Mogibacterium* genus and for TM7 phylum. Only for the *Bacillaceae* family in the *Firmicutes* phylum, a positive correlation with butyrate is shown. This phylum also shows uniquely significant correlations with acetate and propionate, with opposite signs. Significant correlations are also shown for valerate with the *Bacteroidetes* family *Rikenellaceae*, for isovalerate with the genus *Butyrivibrio* and for isobutyrate with an unknown genus of *Clostridiaceae* family.

Discussion

Sequencing and biodiversity
The composition of the microbial population in the rumen affects the degradation of feeds, DMI, host nutrient supply and animal health (Fernando *et al.,* 2010; Kong *et al.,* 2010;

Mosoni *et al.,* 2011). The advent of Next-Generation Sequencing (NGS) offers a breakthrough opportunity to go deeper into this knowledge (Woelders *et al.,* 2011) and new rumen research in this field has recently been published (Brulc *et al*., 2009; Kong *et al*., 2010; Jami and Mizrahi, 2012; Ross *et al*., 2012). As a first attempt to depict the variation of the microbiome composition subsequent to yeast supplementation, NGS was applied to the rumen fluid fraction. The rumen fluid fraction contains small non-degraded feed particles, metabolites, free microbes and microbial remnants, and the richness of bacterial populations is considered to be much lower than that of bacteria adherent to large particles of feeds (Kong *et al.,* 2010). As oesophageal tubing was the mode of collection in the current experiment, analyses have been limited to the rumen liquid fraction as this collection technique does not generate representative samples of the solid phase of the rumen content, as this fraction is mainly composed of an intermediate layer of heterogeneous digesta (Hobson, 1988) at different stages of fermentation.

From the rarefaction curves (Figure 1), it appears that the sequence depth reached in the current experiment allowed an in-depth investigation of the biodiversity of the rumen samples. As a consequence of the sequencing depth, the OTUs classified from the rumen microbiomes in the present experiment (Table 1) are higher than in other studies. In particular, in the studies by Kong *et al.* (2010) and Fernando *et al.* (2010), which used technologies producing lower sequencing depths (clone libraries and T-RFLP), the OTUs at 0.03 cut-off were 613 in the first study and 315 or 398, depending on the experimental group, in the second study. The H′ data reported by Kong *et al.* (2010), for a cut-off of 0.03, ranged from 4.50 to 4.62 for the liquid fraction and from 4.74 to 4.78 for the particulate fraction. The reported values are very close to the H′ of Table 1 for a 0.6 cut-off.

Table 3 *Mean concentrations (± s.d.) at the Time 15 of total volatile fatty acids (mMol/l), molar proportion of individual fatty acid (%) and ratio of fatty acids in rumen fluid of the LY group (lyophilized yeast), DY group (dried yeast) and Ctrl (potato protein) group*

	LY		DY		Ctrl	
	Mean	s.d.	Mean	s.d.	Mean	s.d.
Total VFAs (mMol/l)	95.2	16.26	95.1	12.90	89.6	4.46
Acetate (%)	68.7	4.17	67.6	3.41	67.6	1.36
Proprionate (%)	19.8	3.50	21.5	3.57	22.3	1.90
Butyrate (%)	9.6	0.87	9.2	0.84	8.2	0.33
Isovalerate (%)	0.8	0.15	0.8	0.15	0.8	0.10
Valerate (%)	0.6	0.13	0.6	0.11	0.6	0.06
Isobutyrate (%)	0.5	0.09	0.4	0.05	0.4	0.03
Isoacids (%)	1.9	0.22	1.8	0.12	1.9	0.09
C3/C2	0.3	0.08	0.3	0.07	0.3	0.03
C4/C2	0.1	0.02	0.1	0.01	0.1	0.01
(C3 + C4)/C2	0.4	0.09	0.5	0.07	0.5	0.03

Ctrl = Control.
Comparison of the means between groups were not significant ($P > 0.05$).
C3/C2: propionate to acetate ratio.
C4/C2: butyrate to acetate ratio.
(C3 + C4)/C2 ratio: propionate plus butyrate to acetate ratio.

However, a full analysis of the microbiome composition is not only dependent on the use of NGS, but also on the number of reads per sample. The number of OTUs estimated from rarefaction analysis by Pitta *et al.* (2010) were 647 and 808. The authors used a cut-off of 0.03 and an average depth of pyrosequences of 2141 and 3046 for liquid and solid fractions, respectively. This value is considerably lower than the mean value of 3862 OTUs reported in Table 1 for the 0.02 cut-off (average of experimental groups). However, data obtained in different experiments are not straightforward to compare, as they are affected by environmental conditions (i.e. feeding, management, healthy status, etc.), sampling technique, depth of sequencing and, probably, animal genetic.

Identification and distribution of 16S rRNA assigned sequences in the experimental groups

According to the assignment of 16S rRNA sequences to the NCBI database (Table 2) genera of the phylum, *Firmicutes* are the most represented and the genus *Bacillus* is the most abundant in both yeast-supplemented groups (LY and DY groups $P < 0.05$ in comparison with the Ctrl group). Conversely, genera of the phylum *Bacteroidetes* (*Prevotella* and *Rikenella*) are detected at lower frequencies.

Instead, Pitta *et al.* (2010), comparing Bermuda grass hay with grazed winter wheat diets fed to cows, reported that genera of the phylum *Bacteroidetes* were the most abundant in the rumen bacterial community followed by genera of *Firmicutes*, both in the solid and the liquid fractions. The same authors reported that the prevalence of the genera *Prevotella* and *Rikenella* were not different between both rumen fractions and diets. In a study by Kong *et al.* (2010), the composition of the bacterial communities in the rumen of cows fed lucerne or triticale differed between the tightly attached to particulate populations compared with the planktonic and those loosely attached to particulates. Differences in sampling procedures and dietary composition can partly explain the variation of the rumen microbiome composition in comparison with the present study. A further difference can be related to the higher sequencing depth and to the 16S region sequenced (V1-V3), which probably lead to a more complete and different description of the microbial phylogenic distribution. This is confirmed by Brulc *et al.* (2009), who obtained a more accurate bacterial partitioning on the same samples by increasing the sequencing depth with the use of NGS. Brulc *et al.* (2009) obtained, from samples collected in the same period (1 h after morning feeding) and 454 sequencing, a percentage of *Firmicutes* in the liquid fraction lower than the value reported in this study (62% *v.* 79%).

Data on the effects of *S. cerevisiae* on microbiome composition are not available in the literature, and some evidence, summarized by Desnoyers *et al.* (2009) and Robinson and Erasmus (2009), does not allow to postulate how yeast can affect the rumen bacterial microbiome. The results presented in Table 2 for the three experimental diets indicate a limited influence of dried yeast (DY group) on

Table 4 *Coefficients of correlation between the abundance of bacterial taxa and molar percentage of volatile fatty acids in the rumen content (n = 12)*

Phylum	Family	Genus	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
Bacteroidetes			–	–	–	–	–	–
	Rikenellaceae		–	–	–	–	–	0.624
		Genus_NA	–	–	–	–	–	0.613
Firmicutes			0.654	−0.634	–	–	–	–
	Bacillaceae		–	–	–	0.589	–	–
	Lachnospiraceae		–	–	–	−0.628	–	–
		Butyrivibrio	–	–	–	–	−0.585	–
		Genus_NA	–	–	–	−0.638	–	–
	Ruminococcaceae		–	–	–	−0.599	–	–
		Genus_NA	–	–	–	−0.661	–	–
	Veillonellaceae		–	–	–	−0.592	–	–
	Erysipelotrichaceae		–	–	–	−0.613	–	–
	Clostridiaceae		–	–	–	–	–	–
		Genus_NA	–	–	−0.672	–	–	–
	Family_NA	Mogibacterium	–	–	–	−0.627	–	–
TM7			–	–	–	−0.638	–	–
	Family_NA		–	–	–	−0.638	–	–
		Genus_NA	–	–	–	−0.638	–	–

Pearson correlation is reported for phylum, families and genus.
Only significant coefficients ($P < 0.05$) are reported.

rumen bacteria, which is significant ($P < 0.05$) for the percentages of *Bacillus* ($P < 0.05$), for a non-assigned genus of *Erysipelotrichaceae,* and for a non-assigned genus of the phyla *TM7* and *Tenericutes*, even if the metabolic meaning of these variations is currently unknown. The average VFA content and their molar proportions and ratios (Table 3) are not significantly affected by the lyophilized or the dried form of *S. cerevisiae* inclusions. These parameters were analyzed at time 0 and did not differ between the groups. For instance, the mean total VFA content (mmol/l) was equal to 82.4 ± 7.19, 85.6 ± 7.93 and 79.3 ± 9.58, and the molar proportions of acetate were equal to 68.5 ± 4.26, 70.4 ± 1.56 and 68.2 ± 3.48 for the Ctrl group, DY group and LY group, respectively. After 15 days (Time 15), the standard deviations of the total VFA content and of the molar proportions of acetate, propionate and butyrate were lower in the Ctrl group in comparison with the DY group and the LY group. Considered together, these data suggest that the effect of yeast supplementation was limited and that the inter-animal variability was not negligible, excluding a firm conclusion on the yeast treatment.

The correlation of *Firmicutes* with acetate and propionate indicates a fibrolytic metabolic pattern of this *phylum* (Sawanon and Kobayashi, 2006; Sawanon *et al.,* 2011), but the understanding of the other correlations reported in Table 4 requires knowledge of the metabolic pathways of each bacterial strain within the rumen.

The negative correlation of families and genera of the *Firmicutes* and *TM7* with butyrate concentrations suggests that either the bacteria of these phyla can use the butyrate for their metabolism or that they are competing for the same substrate with butyrate-producing species.

Conclusions

This work represents a preliminary study to investigate the rumen microbiome of liquid fraction in lactating cows fed with or without yeast supplementation. The high depth of pyrosequencing allows to depict the microbial biodiversity of the liquid rumen contents, which represents a further contribution to the knowledge of the rumen ecosystem. The inclusion of 50 g of dry or lyophilized yeast in the diet had minor effects on the rumen microbiome, but has to be considered as preliminary conclusion, given the limited number of cows allotted to dietary treatments and the lack of rumen samples collected before the yeast supplementation. However, the present study can provide further information on the composition of the rumen microbiome in the liquid fraction and should be viewed as a starting point for future studies.

Acknowledgement

Research funded by FESR and Italy within the program for the 'Cooperazione Transfrontaliera Italia Slovenia 2007–2013'.

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SECOND PART

Abstract

Phenotypic selection of livestock over last 50 years, in particular of dairy cows, has been effective in increase productions and improve animal nutrients utilization.

The effects of the phenotypic improvement, in particular on the rumen microbial profile and on the gene expression remains largely unknown.

In this study a picture of the effects of the estimated breeding value for a productive trait on cows rumen metagenome and blood transcriptome of healthy Italian Holstein and Italian Simmental lactating cows is presented.

Preliminarily, two Italian Holstein and two Italian Simmental commercial farms were selected in the Friuli Venezia Giulia region for having similar management and feeding conditions.

Within each farm healthy cows, ranging from two to five calvings and from 70 to 180 days in milk were selected. Within each farm cows were then grouped in three classes of Estimated Breeding Value for milk protein (EBVp). The EBV classes were defined within each breed and farm by sorting the EBV values into quartiles, where the first (Q1) and the third (Q3) quartiles correspond to the ±25% extremes EBV values versus the mean of the distribution. Three quartiles (Q1, Q2 and Q3) resulted in each farm.

Milk yields and compositions form 135 cows were obtained from the official recording service and the same day blood, urine and rumen content were sampled during the morning meal and subjected to biochemical analysis.

These data were used to asses the relationship between EBVp and productive traits and metabolic condition. Based on these results, two subsets of cows were chosen to investigate rumen microbiome and blood gene expression.

For rumen microbiome composition two EBVp minus and two EBVp plus variant cows for each farm and each breed (overall 16 cows) were selected. The massive parallel High Throughput Sequencing of the whole rumen contents and the annotation procedure of FASTQ base called files allowed to describe the classify the taxonomy of the bacterial and archaeal communities. Statistical analysis applied to the abundance profiles at the different taxonomic levels indicated a significant effect of breed and EBVp. Within *Bacteria* domain Italian Simmental cows had significant ($P<0.05$) higher presence of bacteria belonging to *Actinobacteria* and lower bacteria of the *Clostridia* class in comparison to Italian Holstein cows. Rumen contents of Italian Simmental cows were also significantly richer ($P<0.001$) of archaeal *Methanobacteria* than Italian Holstein cows. Overall, the EBVp resulted positively related with *Bacteroidetes* ($P<0.01$) and negatively with methanogen *Euryarchaeota* phyla.

The investigation of gene expression profile at the blood level was performed in eight cows for each EBVp quartile (eight Q1, eight Q2 and eight Q3) for one Italian Simmental and one Italian Holstein farm (overall 24 Italian Simmental and 24 Italian Holstein cows).

For the dual purpose Italian Simmental cows the most relevant affected pathways were related to glutathione metabolism and intestinal IgA production. In the Q3 group GSS and TGF1B genes were significantly ($P<0.05$) over expressed in comparison to the Q1 group.

For the Italian Holstein cows the most relevant affected pathways were related to T cell receptor and adipocytokine signalling pathways. In the Q3 group CD3G, CD4 and ACSL1 genes were significantly ($P<0.05$) over expressed in comparison to the Q1 group.

At the best of our knowledge, this approaches has never been used and a direct comparison with published studies is not straight full, but some preliminary considerations from the data gathered from our study can be drawn.

Despite of the limited number of cows included in the study, the rumen metagenomic data indicate that microbial population is significantly affected by genetic merit other than breed.

The analysis of blood gene expression showed that also specific metabolic and immunitary pathways are directly related to the genetic merit, and can be considered a signature of the quantitative selection.

Starting from this preliminary observations, the approaches are promising since they can identified phenotypic biomarkers that can be used for genomic selection, based on rumen microbial profile and differentially expressed genes more than on DNA variants.

Project overview

1.The estimated breeding value and the improvement of dairy cows performance

Estimated breeding value (EBV) is the most important statistical method utilized from the mid-20th century in livestock genetic selection. Its development is due to the advent of quantitative genetics based on principle of heredity and modern statistical theory [1].

Estimated breeding value is an estimate of the genetic merit for an animal for a given trait or series of traits based on the evaluation of all available data on the performance of an animal and close relatives, for a trait. Moreover, using traditional methods of genetic evaluation, the true breeding value (or true genetic merit) is not known.

In practice, the phenotype of an individual and a substantial number of its relatives is recorded to compute the likelihood that the individual is transmitting a favourable set of alleles for the trait of interest.

Essentially, to report an EBV for a particular trait, the statistical genetics method calculates an average of all genetic loci contributing to a trait as by the individual [2].

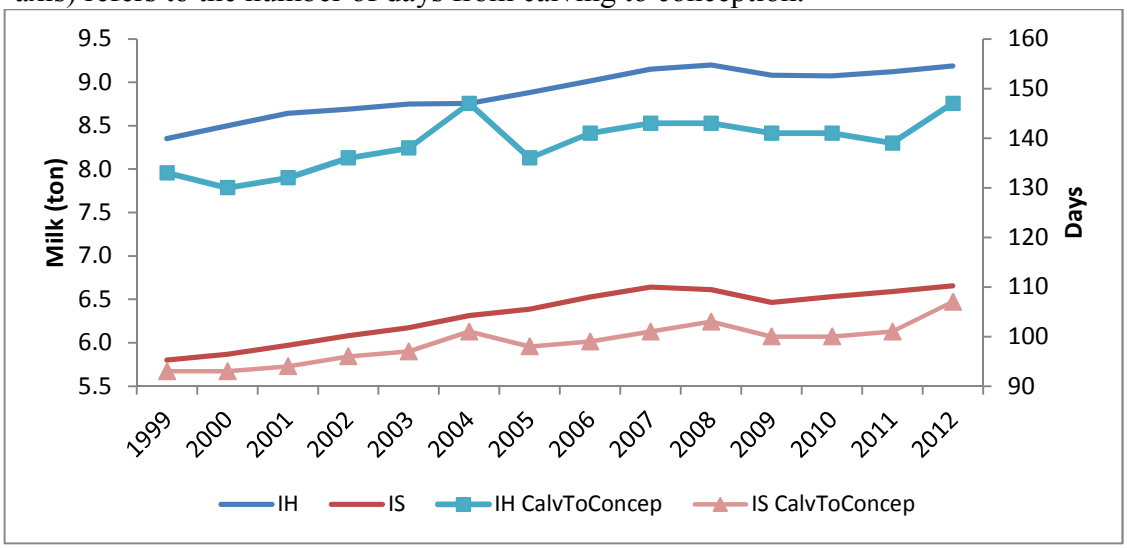
The EBVs for the traits aimed to be selected are then combined to calculate a selection index in which each of them has a specific weight depending on their assigned importance and selection aims.

The dramatic increase in milk yield per cow in the last 20 years is the result of the rapid progress in genetic and management (Figure 1).

Although current international breeding goals in dairy cows include functional traits such as fertility, udder health, type traits and functional survival (Figure 2), breeding programs, in

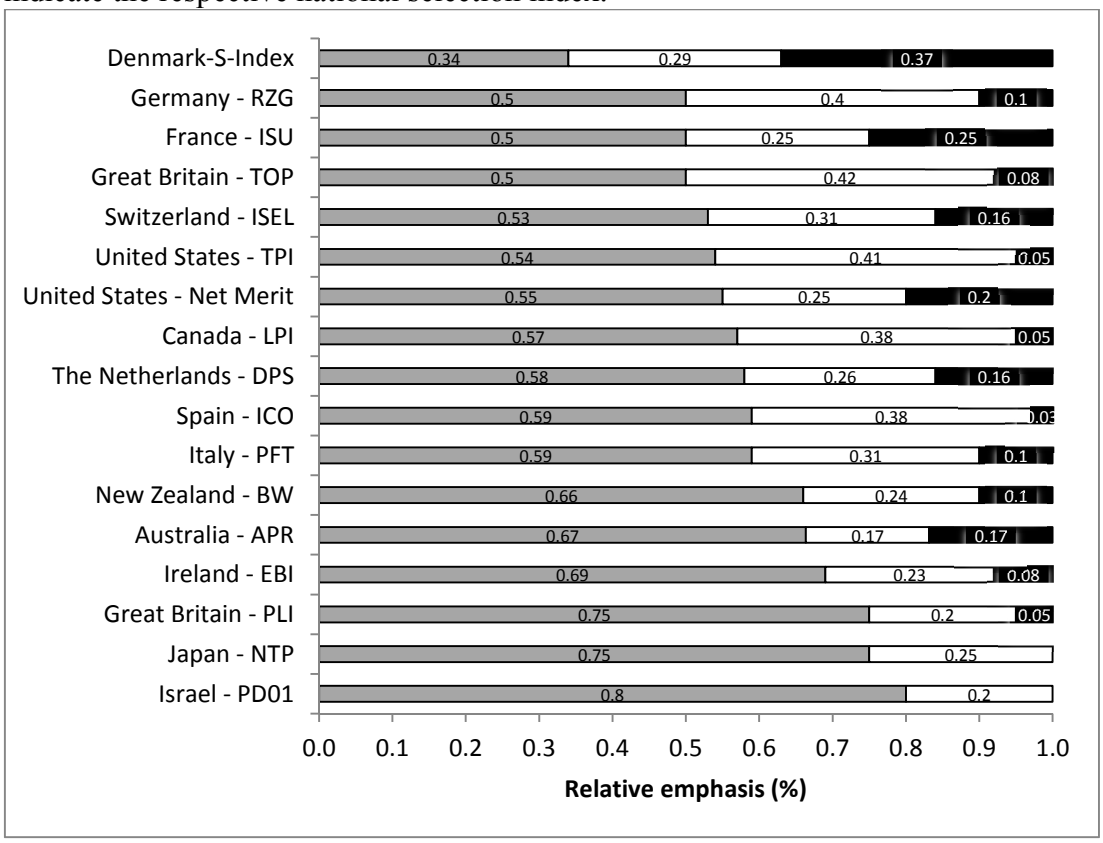
particular for Holstein, in the past selected aggressively for increased milk production. The main reasons for this shift were the European quota-based milk marketing system and price constraints, together with increasing producer and consumer concerns associated with the observed deterioration of the health and reproduction of dairy cows [3].

Figure 1. Trend of the productive and reproductive performances of the Italian Holstein (IH) and the Italian Simmental (IS) dairy cattle across last 13 years. Milk yield (left axis) refers to the standardized 305d lactation and CalvToConcep (right axis) refers to the number of days from calving to conception.



Source: AIA

Figure 2: Relative emphasis on longevity (grey), durability (white), and health and reproduction (black) components in Holstein selection indexes of countries in August 2003 (from Miglior 2005)[3]. More recent data are lacking due to the Interbull company property and secrecy policy. Acronims close to the countries indicate the respective national selection index.



2. *The selection index of the Italian Holstein breed (Frisona Italiana)*

The current selection index for the Italian Holstein breed is the PFT (Productivity, Functionality and Type) (http://www.anafi.it/). It combines milk quality and functionality to determine a 49:51 ratio between the weights of the productive and functional traits (Table 1).

Table 1. The Italian Holstein selection index, as published by the Italian Association of Frisona Italiana breeders (ANAFI).

PRODUCTIVITY		FUNCTIONALITY	
Trait	Weight	Trait	Weight
Milk yield	0	TYPE	4
Fat yield	8	ICM	13
Protein yield	36	IAP	6
Fat %	2	Somatic cells	10
Protein %	3	Functional longevity	8
		Fertility	10

TYPE: index derived from 15 defined linear traits; ICM: Udder composite index; IAP: Feet & Legs composite index

3. The selection index of the Italian Simmental breed (Pezzata Rossa Italiana)

The current selection index for the Italian Simmental breed is the IDA (Dual purpose index) (http://www.anapri.eu/).

This breed is selected to produce both milk and beef, although the dairy traits has become prevalent. Thereof in the selecting index, together with traits related to milk production and functionality, aspects related to the production of meat are considered (Table 2).

Table 2. The Italian Simmental selection index, as published by the Italian Association of Pezzata Rossa Italiana breeders (ANAPRI)

MILK PRODUCTION		BEEF PRODUCTION		FITNESS		FUNCTIONALITY	
Trait	Weight	Trait	Weight	Trait	Weight	Trait	Weight
Protein kg	5	PT beef index	18	Udder	14.5	Milkability	7.5
Fat %	2	Cow muscularity	6	Feet & Legs	5	Somatic cells	-5
Protein %	5						

PT: performance test of the young bulls

4. Sorting criteria of farms and animal cohorts

Two Italian Simmental (farms A and B) and two Italian Holstein (farms C and D) commercial dairy farms located in the North East part of the Po Valley, Italy, were selected. Farms were sorted for having comparable management and diet compositions before and during the experimental period. Briefly, the size of the herds ranged from 270 to 433 animals, and from 126 to 214 lactating cows. A more detailed description of herds characteristic and farms management is presented in Table 3 and Table 4.

The local Farmer and Breeder Association (Associazione Allevatori del Friuli Venezia Giulia, Codroipo, Italy) provided assistance for the selection of farms and information for the individual milk data through the lactation, the reproductive parameters and the managerial aspects. The 4 farms had free stall barns as housing system and the animals were fed *ad libitum* a total mixed ration (TMR) regularly twice a day, at around 0700 h and 1700 h with free access to fresh water. Diets were formulated to cover the nutrient requirements for lactation (INRA, 1988) and the ingredients, chemical compositions and nutrient content are reported in Table 5.

Table 3. Composition of the herds and characteristics of the farms involved in the study. Significantly differences of productive traits refer to EBVp quartiles within each farm.

Breed		It. Simmental		It. Holstein	
Farm		A	B	C	D
Herd size	No	343	270	368	433
Dairy animals	No	183	169	194	215
Heifers	No	65	61	76	97
Lactating cows	No	152	148	155	182
Cows < 70 DIM	No	31	16	14	22
Cows sampled	No	27	33	36	39
	%	22.3	25.6	25.5	24.4
DIM	Mean	126.7	141.4	151	145.8
	sd	33	33.8	33.1	28.5
EBVp	Mean	17.9	8.6	22.6	16.5
	sd	8.6	10.1	18.4	15.7
Housing	type	Free stall	Free stall	Free stall	Free stall
Bedding	type	Concrete	Straw	Concrete	Concrete
Milking	type	Parlour	Parlour	Parlour	Parlour
Productive traits	**EBV**				
Milk yield (t)	Q1	6.82±0.35[a]	8.26±3.39[a]	9.14±3.38[a]	9.44±0.97[a]
	Q2	8.99±4.08[a]	8.64±0.89[a]	9.49±1.30[a]	11.23±3.56[a]
	Q3	9.43±0.99[b]	10.44±1.39[b]	10.50±0.68[b]	12.07±1.83[b]
Milk protein (kg)	Q1	254±2.83[a]	286±119.03[a]	278±101.54[a]	301±37.33[a]
	Q2	297±132.3[b]	307±25.13[b]	296±38.17[b]	364±116.21[b]
	Q3	325±32.86[c]	363±20.83[c]	441±41.29[c]	400±48.13[c]
Milk fat (kg)	Q1	314±46.67[a]	314±130.15[a]	339±234.26[a]	354±32.84[a]
	Q2	340±158.14[a]	318±33.82[a]	386±65.90[a]	408±138.36[a]
	Q3	350±31.26[c]	395±42.07[c]	340±18.10[c]	433±42.43[c]

Milk yield and milk fat a, c: indicate a significant difference for $P<0.01$
Milk protein a, b: indicate a significant difference for $P<0.05$
Milk protein a, c: indicate a significant difference for $P<0.01$
DIM: days in milk; EBVp: estimated breeding value for milk protein

Table 4. Number, DIM (mean±dev.st.) and parity (mean ±dev.st.) of the 135 cows enrolled for the study sorted according to breed, farm and Estimated Breeding Value for milk protein

EBV quartile	**Q1**			**Q2**			**Q3**		
Farm	**n of cows**	**DIM**	**Parity**	**n of cows**	**DIM**	**Parity**	**n of cows**	**DIM**	**Parity**
					It. Simmental				
A	2	124±9	2.5±1	13	118±31	4±1	12	137±35	3±1
B	8	133±28	3±1	19	140±38	3±1	6	150±20	4±1
Total	**10**	**131±27**	**3±1**	**32**	**131±36**	**4±1**	**18**	**141±31**	**3±1**
					It. Holstein				
C	8	155±34	2±1	15	142±29	2±0	13	158±37	3±1
D	12	146±23	3±1	21	152±29	3±1	6	122±23	2±1
Total	**20**	**151±29**	**3±1**	**36**	**148±29**	**3±1**	**19**	**147±37**	**3±1**

DIM: days in milk

Table 5. Composition of the rations offered to the dairy cows and chemical and nutritive contents

Breed		It. Simmental		It. Holstein	
Farm		A	B	C	D
Ingredients					
Lucerne, hay	kg DM/d	3.06	4.45	2.50	4.03
Grass, hay	"				0.90
Corn, silage	"	6.82	6.06	7.82	6.15
Corn cob, silage	"	3.13	3.24		
Lucerne, silage	"	1.50		3.16	
Grass, silage	"		0.71		
Corn, ground	"	0.87	1.04	3.15	4.56
Soybean meal s.e.	"	1.05	0.70		1.75
Rapeseed meal s.e.	"				0.90
Whole Soybean	"	1.25			
Barley, ground	"		0.44		
Bran wheat	"				
Protein Supplement	"	2.10	2.64	0.79	2.25
Protein and Fat Supplement	"			2.38	
Mineral Supplement	"			0.41	
Hydrogenated fat	"	0.14			0.20
Glycol propylenic	"	0.14			
Minerals	"	0.20	0.05	0.14	0.05
DMI	"	20.3	19.3	20.3	20.8
Composition					
CP	%/DM	15.59	15.09	14.67	15.46
EE	%/DM	4.01	2.43	3.33	4.23
Ash	%/DM	7.58	6.28	6.78	5.44
NDF	%/DM	31.87	33.88	34.33	32.47
Starch	%/DM	26.63	27.93	25.84	27.88
PDIA	g/d	945	1012	710	886
PDIN	g/d	2077	1963	1604	1954
PDIE	g/d	2024	1804	1526	1784
NEl	Mcal/d	30.05	27.40	27.27	28.74

CP: crude protein; EE: ether extract; NDF: neutral detergent fiber
PDIE: protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen-fermented OM (INRA, 1988).
PDIN: protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen degraded N (INRA, 1988).
NEl: net energy for lactation

The first criteria to select the cows were healthy status and lactation stage. Within each breed and farm, clinically healthy cows with DIM ranging from 70 to 180 days and with calf order from 2 to 5 were identified.

Accordingly, 60 Italian Simmental (IS) (27 from farm A and 33 from farm B) and 75 Italian Holstein (IH) (36 from farm C and 39 from farm D) lactating cows were selected.
Blood biochemistry and Body Condition Scoring [4] were screened to confirm the healthy status of the animals intended to be enrolled for the study and to uncover preliminary effects of the breed and the EBV.

5. *Cows allocation to the experimental groups*

Cows included in the cohorts were then matched with the respective Estimated Breeding Values for milk protein kg (EBVp) provided by Italian Holstein (ANAFI) and Italian Simmental (ANAPRI) breed associations and hence, according with their EBVp value, were assigned to 3 classes.

We focused on the estimated breeding value for milk protein yield among the different genetic indices because of the economic relevance of this aspect in the dairy herd and because it could be seen as a "synthetic index", the product of milk yield and protein percentage[5]and reflecting, as a consequence, the potential for protein synthesis and milk production in dairy cows.

It is well known that a high positive genetic correlation between milk and protein yield exists[5], while for milk fat and protein percentage the correlation with milk production is negative [6]. At the same time the lack of QTL affecting only protein yield was confirmed by both these studies and Lipkin (2008) in Israeli Holstein cows reported that 68.9 and 76.5% of QTL markers affecting protein yield also were associated with protein percentage and milk yield, respectively.

The EBV classes were defined within each breed and farm by sorting the EBV values into quartiles, where the first and the third quartiles correspond to the ±25% extremes EBV values versus the mean of the distribution. Three quartiles resulted in each farm:

Q1 = cows belonging to the 25% of the lowest EBV values for milk protein kg (EBVp) in the population (breed) included in the study

Q2 = cows between the 25% of the lowest and the 25% of the highest EBV values for milk protein kg (EBVp) in the population (breed) included in the study

Q3 = cows belonging to the 25% of the highest EBV values for milk protein kg (EBVp) in the population (breed) included in the study

A general description of the main traits of the cows according to the EBVp groups is given in Table 4, while relative productive traits are shown in Table 3.

6. Samples collection and statistical analysis

One sample of about 50 ml of milk was collected from all cows in all farms during the normal routine of morning milking (beginning of milking from 5:00 AM to 6:00 AM) contextually to the other samples collection. Milk was immediately refrigerated, with no addition of preservatives, and then frozen at -20°C until analysis.

Blood was collected after the milking, when cows had ad libitum access to fresh water and spontaneously moved to cattle feed headlocks fence for the morning meal after the milking.

Blood from all cows was sampled from the coccygeal vein in three 10 ml vacuum, one with K3-EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium) for plasma collection, one with no preservative for serum collection and a PAXgene Blood RNA System tube (Preanalytix, Hombrechtikon, Switzerland) for RNA extraction. The blood from the first two tubes was centrifuged within 1 h at 3000 RPM for 10 min at 20°C and the plasma and serum samples were stored at –20°C until further analysis. PAXgene tubes were handled and stored following the manufacturer's instructions.

Urines were sampled waiting for spontaneous urination. Ten ml of sample were immediately added with 10% sulphuric acid until a final pH of 3.0 was reached and the amount of acid added recorded. After, the urine samples were filtered using Millipore (0.22 μm) membrane filter, and 3 aliquots of each sample were stored at -20°C until analysis.

A sample of about 40 g of rumen content for each animal enrolled in the relative study was collected with an esophageal probe one hour after the morning feeding and frozen at -80°C without fixative until DNA extraction.

All samples were collected the same day.

General linear model was used for the outcomes of serum, plasma and urine metabolites, with fixed effect for breed, farm nested within breed. The model included a linear regressions for DIM and for EBVp within breed. All tests were 2-sides and significance was based on a $P < 0.05$.

General linear model was used for the outcomes of milk yield and composition with fixed effect for EBVp quartiles, breed and farm nested within breed. All tests were 2-sides and significance was based on a $P < 0.05$.

7. Aim of the study

The aim of the study was to investigate the effect of the phenotypic selection in Italian Holstein and Italian Simmental lactating cows in terms of microbial profile of rumen content and gene expression of peripheral leukocytes. One hundred thirty five clinically healthy lactating cows were selected from four dairy commercial intensive farms having similar management, feeding and milking systems. In order to limit the effect of physiological conditions of the cows, within each farm cows were sorted for being between 70 and 180 days in milk and primiparous animals were excluded. Cows were then ranked for the Estimated Breeding Values for milk protein (EBVp) and the comparisons were performed accordingly.

Specifically, for the gene expression study 24 Italian Holstein and 24 Italian Simmental cows from two farms (overall 48 animals) were chosen and assigned either to a minus (Q1), or to an intermediate (Q2) or to a plus variant (Q3) group, based on their EBVp. The differential expression gene study was then performed between the extreme groups (Q1 and Q3) in comparison to the intermediate (Q2).

For the investigation on rumen metagenomic, 4 cows from two farms were selected for each breed (overall 8 Italian Simmental and 8 Italian Holstein). In particular, the two animals with the extreme positive and negative EBVp values within each farm were chosen.

Chapter 1

Relation of the genetic merit for milk protein with the rumen microbial profile of dairy cows

1.1. Introduction

This section of the thesis concerns the differences in rumen microbial populations (mainly *Bacteria* and *Archaea*) of cows of different breeds and with different genetic merit for milk protein (EBVp).

Phenotypic selection of livestock over last 50 years, in particular of dairy cows, has been effective in increase productions and improve animal nutrients utilization, but the effect of the phenotypic improvement on rumen microbial profile remains largely unknown. High throughput massive parallel sequencing and a system approach give the possibility to obtain sequences of a large number of microorganisms simultaneously and to apply a comparative approach to the ecology of the microbial communities that inhabit the rumen.

Rumen microbial ecology affects several aspects of livestock breeding and management. Feedstuffs utilization, microbial protein and volatile compounds production and methane emissions are of great interest because of their impact on animal metabolism, nutrient energy utilization and thus digestive efficiency. Individual variability in microbial populations of the rumen are likely to rely on an exclusive microbiome-host relation [7] which is, such as differences among breeds, still unknown.

1.2. Aim of the study

The aim of this study is to take a picture of the effects of the estimated breeding value for a productive trait on cows rumen populations of healthy Italian Holstein and Italian Simmental lactating cows.

A massive parallel high throughput sequencing was applied to whole rumen contents of Italian Holstein and Italian Simmental lactating cows with two purposes: 1) To compare the rumen microbial profile of two cattle breeds, different for genetic and partly for productive orientation; 2) to uncover the effect of the phenotypic selection and the genetic merit estimation on rumen microbiome.

Abbreviations

IH	Italian Holstein
IS	Italian Simmental
EBVp	Estimated Breeding Value for milk protein (kg)
ISmin	Italian Simmental cows minus variant for EBVp within the selected farms
ISplus	Italian Simmental cows plus variant for EBVp within the selected farms
IHmin	Italian Holstein cows minus variant for EBVp within the selected farms
IHplus	Italian Simmental cows plus variant for EBVp within the selected farms
EBV+	Cows (It. Simmental and It. Holstein) plus variant for EBVp within the selected farms
EBV-	Cows (It. Simmental and It. Holstein) minus variant for EBVp within the selected farms
H'	Shannon biodiversity index, calculated as $-\Sigma [Pi*\ln(Pi)]$, where Pi = proportion of total sample represented by specie i
HTS	High throughput sequencing
NGS	Next generation sequencing
CV%	Coefficient of variation
VFAs	Volatile fatty acids
AA	Amino acids

1.3. Materials and Methods

Animal selection and rumen content collection

For this study 16 cows, four from each farm (two IS and two IH farms), were selected. In particular, we included the two animals with the extreme positive and negative EBVp values within each farm.

A more detailed description of the cows enrolled in the study is presented in Table 6.

The samples of rumen content for metagenomic analysis were collected as described in the project overview.

Table 6. Individual characteristics and Estimated Breeding values for the cows involved in the study

	Breed															
	Simmental								**Holstein**							
Farm	**A**				**B**				**C**				**D**			
EBV group	**ISmin**		**ISplus**		**ISmin**		**ISplus**		**IHmin**		**IHplus**		**IHmin**		**IHplus**	
Cows characteristics																
ID	16	25	21	26	4	24	9	11	16	33	5	6	11	18	21	22
Parity	3	2	3	2	5	3	4	4	2	2	3	4	3	3	2	2
DIM at sampling	130	117	143	136	140	115	152	154	180	157	179	98	166	157	163	171
BCS at sampling	3.75	3.25	2.75	3.50	2.75	3.00	2.75	2.75	3.25	3.00	2.75	2.75	3.00	2.50	2.75	2.50
EBV for milk protein (kg)	-0.4	-2.6	+43.5	+32.9	-12.3	-1.5	+27.3	+34.3	-2.0	-16.0	+45.0	+47.0	-5.0	-9.0	+27.0	+28.0
Productive traits (305d)																
Milk yield (t)	5.95	7.07	11.06	9.84	7.95	8.68	11.16	12.52	8.52	9.52	11.39	10.03	9.01	9.75	10.16	10.47
Milk protein (kg)	231	256	389	331	267	327	365	400	257	263	350	363	306	320	332	353
Milk fat (kg)	241	347	401	347	295	354	382	442	318	313	502	463	345	359	354	435

DIM: days in milk; BCS: Body Condition Score; EBV: Estimated breeding value; ISmin, ISplus: Italian Simmental cows with negative and positive value for EBV for milk protein, respectively; IHmin, IHplus: Italian Holstein cows with negative and positive value for EBV for milk protein, respectively.

Metagenomic DNA extraction, processing and sequencing

For DNA extraction whole rumen contents were cleaned basing on the protocol applied by Fortin [8] to marine sediments, with the following steps: 3mins centrifugation at 3000 xg, a first washing with a 50mM Tris-HCl pH8.3, 200mM NaCl, 5mM Na_2 EDTA, 0.05% Triton X-100 buffer solution, a second washing with 50mM Tris-HCl pH8.3, 200mM NaCl, 5mM Na_2 EDTA buffer solution and a third washing with a 10mM Tris-HCl pH8.3, 0.1mM Na_EDTA classc TE buffer solution. The DNA from the washed whole rumen content was extracted with a Fecal DNA MiniPrep kit (Zymo Research; Irvine, CA, USA) following the manufacturer's instructions, including the bead beating step. Pre-amplification DNA concentration in the samples was measured with a Nanodrop-1000 assay (Thermo Scientific, Wilmington, DE, USA).

DNA samples were further cleaned up through repeated Ethanol precipitation using 3 M sodium acetate pH 5.2. For every 1 µg DNA, we dilute the genomic DNA to 20 ng/ul on a final volume of 55 ul and using a Covaris S2 station we sheered the genomic DNA in AFA microtubes 200 Cycles per bursty, Intensity 5 cycles of 40 seconds in frequency sweeping mode at 4°C. The obtained samples were processed according to TruSeq DNA Sample Prep V2 Low Throughput Protocol. Briefly, each defragmented DNA were blunt ended and phosphorylated, and a single 'A' nucleotide was added to the 3' ends of the fragments in preparation for ligation to an adapter that has a single-base 'T' overhang. Indexing Adapter ligation at both ends of the genomic DNA fragment confers different sequences at the 5' and 3' ends of each strand in the genomic fragment. Size-selected DNA was PCR amplified to enrich for fragments that have adapters on both ends. The resulting sample library was again purified and size-selected by AMPure XP beads. The final purified products were quantitated prior to

seeding clusters on a flow cell using both qPCR (Kapa Library Quantification Kit) and BioAnalyzer High Sensitivity DNA Chip (Agilent). DNA library samples were bound to complementary adapter oligos grafted on the surface of the flow cell. The templates were copied from the hybridized primer by 3' extension using a high fidelity DNA polymerase. The paired-end flow cell contains proprietary oligos that enable selective cleavage of the forward DNA strand after resynthesis of the reverse strand. The reverse strand was regenerated by bridge amplification with the Paired-End Module. After resynthesis of the reverse strand, the original forward strand was cleaved and the reverse strand sequenced for the second read. All cluster generation steps were performed on automated cBot Cluster Generation System using the Paired-End Cluster Generation Kit v4. Sequencing was then performer using a 200-Cycle Paired-End Run TruSeq SBS Kit on a HiScanSQ System. Thanks to HiSeq Control Software (HCS), the analysis was performed during the chemistry and imaging cycles of a sequencing run. The output from a sequencing run (FASTQ) was a set of quality-scored bases called files which are generated from the raw image files and contain the base calls per cycle.

Poor quality sequences was removed from the data set based on the Phred quality score of the nucleotide. The Phred quality score is defined as Q, where Q = -10log10(probability of a base calling error). The reads <50 bp long or with an average Q <20 were discarded.

Metadata analysis

Sequencing data in the format of FASTQ bases called files were analyzed with the online MG-RAST (http://metagenomics.anl.gov/) pipeline [9] for ribosomal RNA (rRNA) genes annotation. After uploading, data were preprocessed by using SolexaQA software package to trim low-quality regions from FASTQ data [10]. The software checked whether these quality scores match actual error rates by mapping reads back to a haploid reference sequence that

was de novo assembled from the same read dataset. The default quality threshold (i.e., Phred quality score) for the reads of the SolexaQA was P = 0.05 (or equivalently, Q ≈ 13, or 1 base call error every 20 nucleotides).

A dereplication step was performed using a simple k-mer approach to rapidly identify all 20 character prefix identical sequences, in order to remove artificial duplicate reads.

The MG-RAST pipeline provided also a screening stage to remove the reads that were near-exact matches to the genomes of a handful of model organisms, including fly, mouse, cow, and human. The screening stage used Bowtie [11] (a fast, memory-efficient, short read aligner), and only reads that do not match the model organisms passed into the next stage of the annotation pipeline.

rRNA reads were detected through an initial BLAT search against a reduced RNA database that efficiently identifies RNA. The reduced database was a 90% identity clustered version of the SILVA database and is used merely to rapidly identify sequences with similarities to ribosomal RNA. The rRNA-similar reads were then clustered at 97% identity, and the longest sequence picked as the cluster representative. rRNA annotation with the MG-RAST pipeline was performed through a BLAT of the longest cluster representative obtained from the 97% identity clustering step, against the M5rna database, integrating SILVA [12], Greengenes [13] and RDP [14] databases.

Finally, microorganisms abundance profiles were generated from the data, applying a 60% minimum identity and 15 alignment length cut-offs. Rarefaction curves and alpha diversity Shannon diversity Index (H') estimation [15], calculated based on protein taxonomic annotation and reported as the number of associated species, were also obtained for all the samples.

For abundance profiling and statistical analysis the taxa belonging to protozoa and viruses were excluded from the dataset, in order to focus on the most abundant and species enriched populations present in the rumen microbiome. The abundance profiles were calculated using the frequency data within each taxonomic level. Similarity heatmaps were calculated in MG-RAST pipeline separately for Bacteria and Archaea at the phylum and class taxonomic levels using 60% minimum identity cutoff and normalized data with a ward type clustering at Bray-Curtis distance.

Statistical analysis

For each sample and each taxonomic level, absolute abundance data from MG-RAST were normalized to relative abundance, and reported as percentage of the total abundance.

Due to the high number of taxa represented at very low frequencies, for taxonomic statistical analysis a 1% cut-off to the abundance of the samples at each taxonomic level was applied, in order to reduce the background noise.

The data were then computed for descriptive and statistical analysis with the SPSS package [16]. A general linear model was used for the outcomes of each taxa from phylum to genus taxonomic level, with fixed effect for breed and farm nested within breed, including a linear regressions for EBVp.

1.4. Results and discussion

Sequencing data and biodiversity analysis: an overview

The purified DNA extracted from the rumen contents had good quality across the samples, even with variable concentration. In all the samples 260/280 and 260/230 ratios were, except for the 260/230 ratio in one sample, >1, while the average DNA concentration was 162±92 ng/µl (details are shown in Table 7).

Table 7. Quality parameters of the purified metagenomic DNA from the rumen contents of each Italian Simmental (IS) and Italian Holstein (IH) cow enrolled in the study.

Farm	EBV group	Cow ID	ng/µl	$A_{260/280}$	$A_{260/230}$
A	ISmin	16	60	1.30	1.13
		25	86	1.56	1.30
	ISplus	21	220	1.88	1.11
		26	109	1.61	1.25
B	ISmin	4	63	1.21	1.38
		24	204	1.74	1.21
	ISplus	9	174	1.80	1.51
		11	405	1.73	1.94
C	IHmin	16	122	1.92	1.27
		33	281	1.70	1.92
	IHplus	5	176	1.44	1.39
		6	107	1.40	0.96
D	IHmin	11	203	1.71	1.71
		18	220	1.62	1.79
	IHplus	21	25	1.92	1.36
		22	151	1.73	1.46

ISmin, ISplus: Italian Simmental cows with negative and positive value for EBV for milk protein, respectively; IHmin, IHplus: Italian Holstein cows with negative and positive value for EBV for milk protein, respectively. $A_{260/280}$: ratio of sample absorbance at 260 and 280 nm; $A_{260/230}$: ratio of sample absorbance at 260 and 230 nm

From the sequencing process, the number of reads obtained per sample showed a good reliability for sequencing depth, further confirmed by the different number of sequences between samples.

The mean number of reads sequenced per sample was 64,135,820 ± 14,745,272 with a mean length of 100 bps. Detailed information for each sample about the original number of reads, those passed the quality check, the identified features and the general Shannon biodiversity Index (H') calculated at 0.1 cutoff, are shown in Table 8.

Table 8. Number of sequences, quality check (QC) result, identified features and Shannon biodiversity Index (H') for each rumen sample.

					Identified features			
Farm	EBV group	Cow ID	n of sequences	passed the QC	rRNA identified features	Proteins	Functional categories	H'
A	ISmin	16	92,723,508	76,699,288	69,844	27,455,563	14,879,493	5.26
		25	72,000,000	61,207,303	56,207	22,115,836	11,918,947	5.23
	ISplus	21	72,000,000	61,990,955	59,468	22,965,664	12,381,253	5.31
		26	72,000,000	61,017,760	56,283	22,063,975	11,921,331	5.28
B	ISmin	4	72,000,000	65,717,253	65,098	25,521,180	14,140,123	4.87
		24	32,666,672	30,650,987	36,539	12,572,232	6,791,720	5.43
	ISplus	9	69,533,726	62,661,833	82,274	26,001,530	13,257,114	5.40
		11	36,753,986	34,134,951	43,074	13,893,457	7,285,960	5.54
C	IHmin	16	53,444,544	49,299,116	63,832	19,820,514	10,490,922	5.47
		33	70,196,724	65,728,754	85,000	25,146,629	13,426,979	5.53
	IHplus	5	71,374,794	65,911,348	74,469	26,943,313	14,538,787	5.58
		6	62,414,278	57,441,800	72,312	22,486,990	11,565,066	5.03
D	IHmin	11	57,364,738	54,180,593	68,348	19,480,179	10,046,720	5.55
		18	53,658,686	51,129,479	59,585	20,759,272	11,076,585	5.49
	IHplus	21	72,000,000	66,869,336	72,546	26,383,346	13,817,795	5.45
		22	66,041,466	60,489,221	69,145	24,068,869	12,886,405	5.55

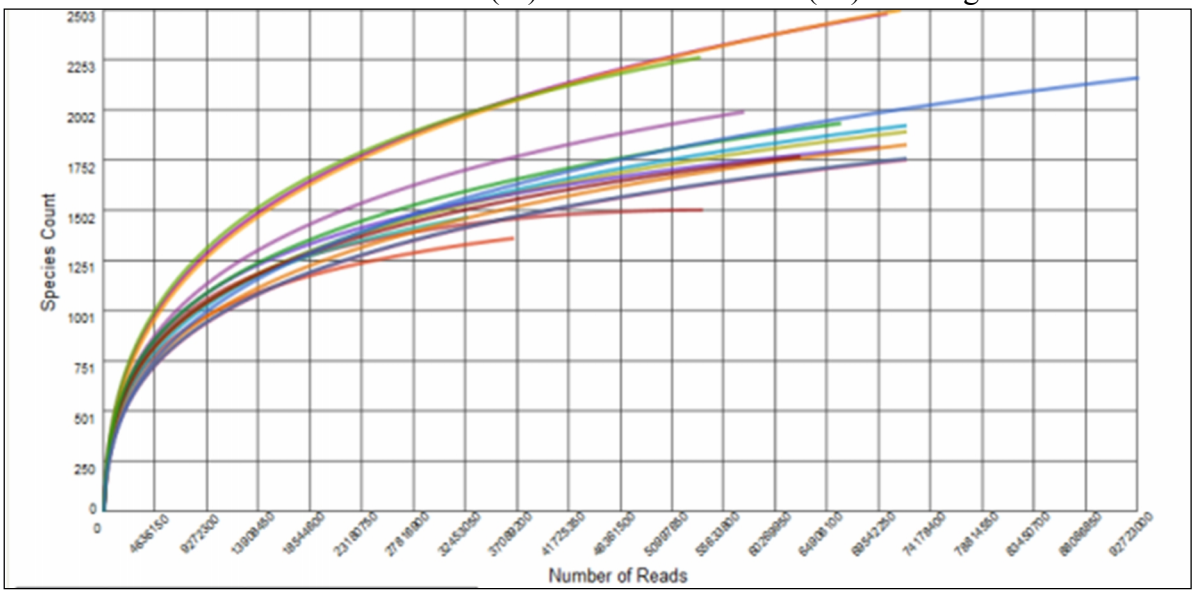
ISmin, ISplus: Italian Simmental cows with negative and positive value for EBV for milk protein, respectively; IHmin, IHplus: Italian Holstein cows with negative and positive value for EBV for milk protein, respectively.

The rarefaction curves (Figure 3), showing the species belonging to all the microbial domains (bacteria, archaea, protozoa, virus), indicate a good sequencing depth, even though a slight slope was still present at the end of most the curves, indicating that a fraction of the species diversity remained to be discovered. Rarefaction plot serves also as a means of comparing species richness between samples in a way independent of the sampling effort. The H' indexes mean value for a 0.10 cutoff was 5.38±0.2 (min 4.87 max 5.58), denoting a considerable biodiversity, that was similar for the rumen content of all the 16 cows sampled. Furthermore, H' did not appear to be particularly affected by the number of reads per sample. In fact, the samples with lowest number of reads showed H' values similar to the samples with the highest reads number. It has to be considered that rarefaction curves and H' indexes were inferred using the sequences after the technical quality check of the sequencing but before the SolexaQA check, and thereof technical artifacts not yet removed may have partly biased the results, depending on the starting quality of the reads. There are not many publications reporting the H' indexes for rumen microbial populations [17] and several factors, such as rumen material (e.g. liquid or solid fraction), as well as molecular techniques (e.g. cloning, qPCR, target genes or HTS sequencing) and taxonomic annotation can, independently from diet consistently affect the results, which are actually difficult to compare. In our recent study on bacterial metagenomic of the liquid fraction of rumen content from IH lactating cows [18], a pyrosequencing NGS approach was used. Resulted indicated a good sequencing depth that allowed to get reliable information on biodiversity measured by H' index. At 0.1 cutoff, the same used in the present study, H' values were 3.1, 4.0 or 3.9 for the two groups supplemented with two types of yeasts and for the control group, respectively. Hence, current

results, reporting higher H' indices in whole rumen content and with a massive sequencing approach, appear to be consistent and indicate a good sequencing effort.

Moreover, the H' data reported by Kong et al., [17], referred only to the bacterial population of different rumen content fractions, ranged from 4.50 to 5.58 for 0.03 cutoff. In our study, a mean value of 5.38 was obtained with a much more stringent 0.10 cutoff, definitely indicating a higher biodiversity. This result is reasonable considering that a NGS technology was applied, uncovering also microorganisms different from bacteria.

Figure 3. Rarefaction curves of annotated species richness, calculated at 0.10 cut-off, in the rumen content of Italian Simmental (IS) and Italian Holstein (IH) lactating cows.



Ribosomal RNA (rRNA) taxonomic annotation and phylogenetic analysis

The taxonomic distribution in IH and IS of the sequences annotated in phylotypes members having a frequency higher than 1% [19] in at least one sample at the phylum, class and order taxonomic levels is shown in Tables 9 and 10 for the bacterial and archaeal domains, respectively.

Within the Bacteria domain, the phyla most represented either in IH and IS were *Firmicutes* (41.39±10.05 and 34.62±10.68%, respectively), and *Bacteroidetes* (37.52±12.14 and 28.80±21.43%). These results agree with other previously reported [17,20-23]. *Bacteroidetes* were significantly affected by farm ($P<0.001$), which likely explains the high standard deviation, and were positively related to the EBVp covariate ($P<0.01$). Another bacterial phylum present in IH rumen contents was *Actinobacteria* (7.15±5.50%), which was significantly ($P<0.05$) lower than in IS cows (13.63±10.02%).

A negative effect of EBVp covariate was observed for *Proteobacteria* phylum, which resulted also significantly ($P<0.05$) higher in IH than in IS, despite of the low frequencies (0.30±0.50 and 0.03±0.01, respectively).

Within the archaeal domain the *Euryarchaeota* was the unique represented phylum (12.81±4.10 and 22.30±9.25% in IH and IS cows, respectively). This phylum had also more pronounced effects compared to bacterial phyla, showing either a significant higher value in IS, the effect of the farm ($P<0.001$) and a negative effect of the EBVp covariate ($P<0.05$). The annotation of *Euryarchaeota* phylum at lower taxonomic levels showed that *Methanobacteria* class and its *Mathanobacteriales* order constituted the vaste majority of the archaeal population in rumen samples, furthermore maintaining the same significant effects.

At the class taxonomic level, *Firmicutes Clostridia* showed the significant ($P<0.05$) effect of the breed, being higher in IH than in IS (39.64±9.79 vs 30.22±8.64), and of the farm ($P<0.001$). *Clostridiales* was its unique represented order, and the increased significance ($P<0.01$) of the different abundance between the breeds can be noted. *Negativicutes* order *Selenomonadales*, *Erysipelotrichi* order *Erysipelotrichales* and *Bacilli* order *Lactobacillales* were the others classes and relative orders belonging to *Firmicutes* phylum that were reported in microbiomes. However, none of them showed significant effects.

Bacteroidia order *Bacteroidales* represented most of the *Bacteroidetes* phylum at the order level and maintained the significant effect of the farm ($P<0.001$) and of the EBVp covariate ($P<0.01$). Class *Actinobacteria* was significantly higher ($P<0.05$) in IS than in IH cows (13.6±10.69 vs 7.12±5.48%), but at the order level showed two populations. *Actinobacteria* order *Bifidobacteriales* remained significantly different between breeds (11.36±10.49 and 4.67±5.57% in IS and IH, respectively), while *Coriobacteriales* were more similar between breeds showing the significant effect of the farm ($P<0.01$).

Table 9. Bacterial composition and effect of breed, farm and genetic merit at Phylum, Class and Order taxonomic levels in the rumen contents of Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 4 commercial farms in North-East of Italy.

Items			Breed				Effects			SEM
			IH		IS		Breed	Farm	COV(EBVp)	
Phylum	Class	Order	mean	st. dev	mean	st. dev				
Firmicutes			41.39	10.05	34.62	10.68	NS	NS	-0.161	2.740
	Clostridia		39.64	9.79	30.22	8.64	*	***	-0.009	2.540
		Clostridiales	39.64	9.79	30.22	8.64	**	***	-0.009	2.540
	Negativicutes		1.53	0.59	1.06	1.31	NS	NS	0.009	0.253
		Selenomonadales	1.53	0.59	1.06	1.31	NS	NS	0.009	0.253
	Erysipelotrichi		0.24	0.45	0.18	0.51	NS	NS	0.006	0.117
		Erysipelotrichales	0.24	0.45	0.18	0.51	NS	NS	0.006	0.117
	Bacilli		0.14	0.16	3.29	9.17	NS	NS	-0.082	1.618
		Lactobacillales	0.12	0.16	3.27	9.16	NS	NS	-0.082	1.616
Bacteroidetes			37.52	12.14	28.80	21.43	NS	***	0.038 **	4.569
	Bacteroidia		37.42	12.13	28.74	22.89	NS	***	0.038 **	4.564
		Bacteroidales	37.42	12.13	28.74	22.89	NS	***	0.038 **	4.564
Actinobacteria			7.15	5.50	13.63	10.02	*	NS	-0.096	2.221
	Actinobacteria (class)		7.12	5.48	13.60	10.69	*	NS	-0.095	2.215
		Bifidobacteriales	4.67	5.57	11.36	10.49	*	NS	-0.076	2.205
		Coriobacteriales	2.35	1.59	2.19	0.58	NS	**	-0.019	0.290
Proteobacteria			0.30	0.50	0.03	0.01	*	NS	-0.008 *	0.075

* indicates a significant effect for P<0.05; ** indicates a significant effect for P<0.01; *** indicates a significant effect for P<0.001.
Signs indicate the relationship, positive or negative, between the parameter and the effect.
COV(EBVp): statstical effect of the covariate for the Estimated Breeding Value for milk protein.

Table 10. Archaeal composition and effect of breed, farm and genetic merit at Phylum, Class and Order taxonomic levels in the rumen content of Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 4 commercial farms in North-East of Italy.

Items			Breed				Effects			SEM
			IH		IS		Breed	Farm	COV(EBVp)	
Phylum	Class	Order	mean	st. dev	mean	st. dev				
Euryarchaeota			12.81	4.10	22.30	9.25	***	***	-0.116 *	2.200
	Methanobacteria		12.77	4.08	22.24	9.85	***	***	-0.015 *	2.194
		Methanobacteriales	12.77	4.08	22.24	9.85	***	***	-0.115 *	2.194

* indicates a significant effect for P<0.05; ** indicates a significant effect for P<0.01; *** indicates a significant effect for P<0.001.
Signs indicate the relationship, positive or negative, between the parameter and the effect.
COV(EBVp): statstical effect of the covariate for the Estimated Breeding Value for milk protein.

At the family taxonomic level, *Prevotellaceae* (phylum *Bacteroidetes*) resulted to be the most abundant in both the breeds (33.77±10.56 and 26.74±20.45% in IH and IS cows, respectively). The high standard deviations are likely to explain the significant effect of the farm ($P<0.001$), even though a consistent individual variability was present (CV% 31.08 in IH, 62.29 in IS), with a maximum and minimum abundances of 7.49 and 62.29% respectively, both observed in IS cows (data not shown). EBVp covariate had also a positive effect on *Bacteroidales* family *Prevotellaceae*, as reported for its taxonomic superior levels ($P<0.01$). Within class *Bacteroidales* also the *Bacteroidaceae* family was present, with lower frequencies (3.48±1.63 and 1.93±2.42% in IH and IS, respectively) significant differences between breeds ($P<0.01$), farms ($P<0.001$) and, as the companion family, positively related to EBVp ($P<0.01$). *Methanobacteriaceae* (*Euryarchaeota* phylum) showed a significant higher value in IS than in IH (22.18±9.82 vs 12.70±4.05%) ($P<0.001$), and also the negative effect of the EBVp covariate was confirmed ($P<0.05$).

Within the order of *Clostridiales,* the families of *Eubacteriaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiaceae* were present. All of them resulted significantly affected by the farm ($P<0.01$), leading to speculate a greater effect going down to lower taxonomic levels of environment and diet on the phylotypes. *Ruminococcaceae* resulted also higher in IH than in IS (10.70±4.96 vs 6.50±3.46%).

Finally, at the genus level the most abundant of the *Bacteria* domain was *Prevotella* spp. (family *Prevotellaceae*) (IH 33.93±10.55%; IS 27.24±20.22%), in agreement with previous studies [21,22]. This is explained by the fact that although *Bacteroidetes* was the second most abundant phylum, the diversity within this phylum was low. Others genera represented were *Eubacterium* (family *Eubacteriaceae*) (IH 14.30±3.09%; IS 12.99±2.82%), *Butyrivibrio*

(family *Lachnospiraceae*) (IH 10.96±4.15%; IS 8.96±2.31%), *Ruminococcus* (family *Ruminococcaceae*) (IH 10.76±5.03%; IS 6.72±3.48%) and *Bifidobacterium* (family *Bifidobacteriaceae*) (IH 4.66±5.54%; IS 11.30±10.37%).

Except for *Bifidobacterium*, all the other genera were significantly different between farms ($P<0.01$). Moreover, *Prevotella* and *Butyrivibrio* resulted positively related to EBVp ($P<0.05$ and $P<0.001$), while *Ruminococcus* and *Bifidobacterium* had significantly different abundance between breeds ($P<0.01$ the first and $P<0.05$ the second).

The strong effect of the farm is reasonable considered the differences, even though not very pronounced, between the diets fed to the animals.

Butyrivibrio and *Prevotella* and spp. belong to the principal rumen polisaccharides-utilizing bacteria, producing butyrate, acetate and propionate in different proportion depending from the substrate [24]. *Butyrivibrio spp.* and in particular *B. fibrisolvens,* the unique *Butyrivibrio* identified in our samples (data not shown), is also among the predominant hemicellulolytic [25] and proteolytic [26] rumen bacteria. They are not cellulosolytic, even though some of them have shown to adhere to the substrate, and they not grow on starch medium [25]. *P. ruminicola*, the most represented *Prevotella* specie in our study (data not shown), ferment starch [27-29] besides fermenting dextrin, pectin, and xylan [30,31], but not cellulose, and hydrolase a variety of proteins and peptides [32].

The non-cellulosolytic activity of these bacteria has a fundamental role together with cellulolytic bacteria, also in the coordinate action to breakdown cell wall components of fibrous feeds [28,33]. In fact, fibrolytic bacteria provide noncellulosolytic organisms with small-length cellodextrins and cellobiose [34], and this cross-feeding may enhance fiber digestion by

removing the products of cellulose hydrolysis [35]. This cooperation seems to give an explanation to the significant higher presence of both *Prevotella* and *Butyrivibrio* spp. in the rumen of the cows with positive EBVp, that would utilize more efficiently carbohydrates released after cellulose degradation.

Within the *Archaea* domain only the genus *Methanobrevibacter* was observed. It resulted, as its ascendant phylotypes, significantly higher in IS than IH cows (21.47±10.36 vs 11.85±3.73%, respectively), negatively related to the EBVp covariate ($P<0.05$), but not affected by the farm. The similarity of the relative abundance of the *Euryarchaeota* phylotypes at each taxonomic level up to the genus confirms, as previously reported, the low diversity of archaeal compared to bacterial rumen community and the dominance of the *Methanobrevibacter* genus [21,23]. Recent evidences would indicate that ruminal methanogenesis is not strongly linked to *Archaea* number [21,36], and that significant differences in methane emissions from cows are not necessarily associated to a different or lower presence of *Archaea* [23].

Rumen ecosystem plays a pivotal role in dairy cows health and productivity, and the recent issue of methane emissions/energy losses from ruminants has attributed to it even more importance.

Direct comparison of whole rumen content microbiome among healthy dairy cows of different breeds from commercial farms has not been performed before. Similarly, there are not information about the relation between rumen microbiome and genetic merit for productive traits of milk-specialized or milk-meat dual purpose cows.

Considerable individual variability, independent from the diet and environment, has been widely shown in rumen microbial profile [18,20,37]. On the other hand, Ross et al (2013)[23] after repeated samples from the same cows taken several weeks apart, observed a high repeatability of microbiome composition within the animal.

The same authors suggested the existence of some stability of rumen microbial profile over the time, overall confirming on the whole the hypothesis of a substantial host specificity[7] and a host-rumen microbiome exclusive interaction. Despite of this, the details and the mechanisms that regulate this mutual relationship remain mostly unknown.

In this contest, it is reasonable to think that also a breed-specificity exists, legitimate by the common genetics within breed and the rumen-host genomes relation. This hypothesis can support the higher amount of *Ruminococcus* and lower amount of *Methanobrevibacter* and *Bifidobacteria* showed in IH cows compared to IS, independently from the farm. Moreover, for *Bifidobacteria* genus the statistical analysis reported significant effect only for the breed, while the farm resulted not significant.

Actinobacteria, the phylum to which *Bifidobacteria* belongs that also resulted significant for breeds, is one of the largest phylum of bacteria. This bacterial group possesses highly variable physiological and metabolic capabilities. Its presence in rumen contents has been widely reported but its role in the rumen ecology has not been fully clarified yet. Some evidences indicate the ability of the genus *Bifidobacteria* in particular to ferment a large variety of oligosaccharides in the human gastro intestinal trait[38]. In a recent study on the association between residual methane production and rumen microbiome profile in Holstein cows, *Actinobacteria* resulted the only one significantly over-represented in the contigs associated

with methane production [23]. We did not measure methane production in our study, and there are not information about GHG emission from IS, but it is interesting to underline how *Actinobacteria*, as well as *Euryarchaeota*, were over-represented in a dual purpose cattle breed, compared with a milk specialized breed. This finding do not allow to draw clear conclusions, but may represents one of the effect of a breeding program with a not exclusive productive orientation. In fact, the selection of a dual purpose breed aims to improve contemporary two metabolic profiles that are divergent for many aspects. Hence, it is reasonable to think that this could result in a residual inefficiency of energy utilization due more to the main selective goals of the breed, than to the real genetic improvement already achieved within the breed.

This seems to be confirmed by comparing rumen profiles grouped only for breed or only for EBVp value independently from the breed (Figure 10). Observing the radial plot, in fact, it can be noted how the rumen profile of all cows (IH and IS) plus variant for EBVp follows the trend of IH cows, while that of the minus variant resembles more the profile of IS cows.

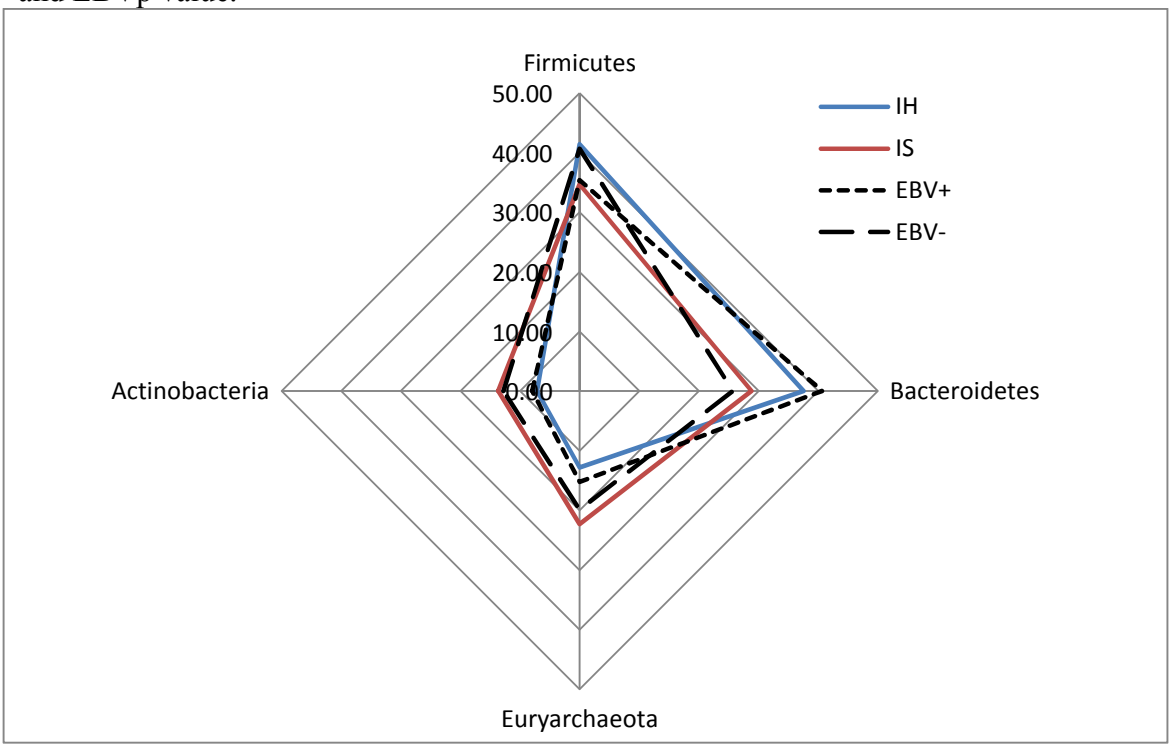
This is explained by the significant effect of EBVp covariate on the abundance of the *Euryarchaeota* and *Bacteroidetes* phyla ($P<0.05$ the former, $P<0.001$ the latter). The EBVp is an estimation of the genetic merit relative to a phenotypic trait. In our cows these values were within breed significantly related to the protein milk yield standardized to 305d lactation (See Table 5). The protein content in milk is known to be largely determined by the genetics of the animals but, due to the high requirement of energy for protein synthesis, it can be deeply affected also by energy content of the diet [39]. The diets fed to the cows in our study had similar energy content, and the differences were certainly not enough to result in the

productive differences observed (30.05, 27.40, 28.50 and 27.27 Mcal/d of NEl in farms A, B, C and D, respectively).

Prevotella spp., the major component in our study of rumen *Bacteroidetes* population, degrade starch, dextrin, pectin and xylan[24] and were positively correlated with EBVp. Hence, considering the limited difference in starch content of the diets (26.63, 27.93, 25.34 and 25.84 of starch%/DM in farms A, B, C and D, respectively), the significant lower presence of *Euryarchaeota* in rumen of cows with higher EBVp might be explained with a major propionate synthesis, which utilize more H+ ions than acetate [24].

Taken together, these results seem to indicate a selection within the rumen for more efficient starch and oligosaccharides fermenting bacteria in animals with higher EBVp and production, that in our study appears to be few dependent from the breed.

Figure 4. Distribution of the main microbial phyla in rumen samples grouped by breed and EBVp value.

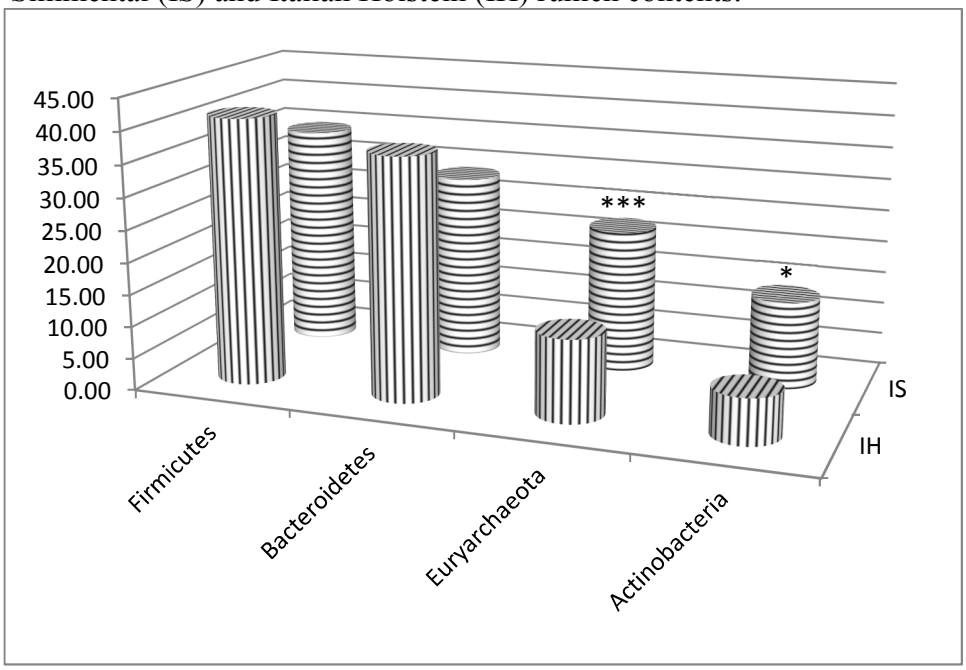


IH: Italian Holstein cows; IS: Italian Simmental cows; EBV+: cows with positive values for EBVp; EBV-: cows with negative values for EBVp.

Similarity clustering of bacterial and archaeal rRNA taxonomic populations

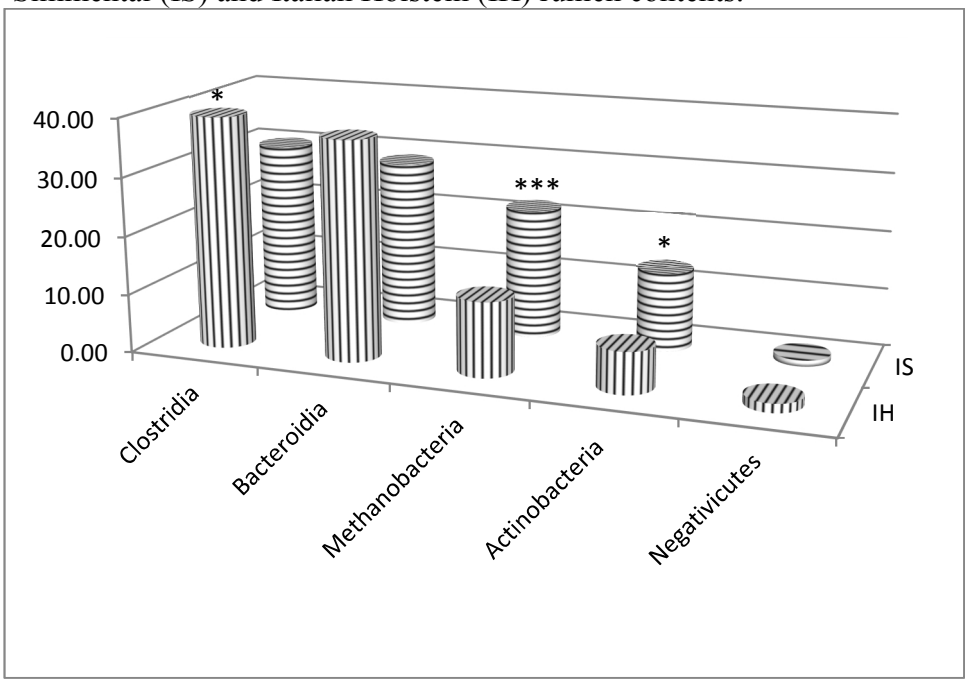
On the whole, IS cows rumen contents appear to be richer in *Euryarchaeota* and *Actinobacteria* than IH (Figure 5). This is confirmed also at the class taxonomic level (Figure 6), where *Clostridia*, belonging to *Firmicutes* phylum, are also shown to be significantly higher in IH than in IS. Considering the mean phyla composition in all the samples grouped for positive or negative EBVp (Figure 7), the starch fermenting bacteria *Bacteroidetes* result more abundant in cows positive for EBVp than in those with negative genetic merit, while the opposite trend is shown for *Euryarchaeota*, the phylum that include the methanogenic species of the rumen.

Figure 5. Distribution of the main microbial phyla in Italian Simmental (IS) and Italian Holstein (IH) rumen contents.



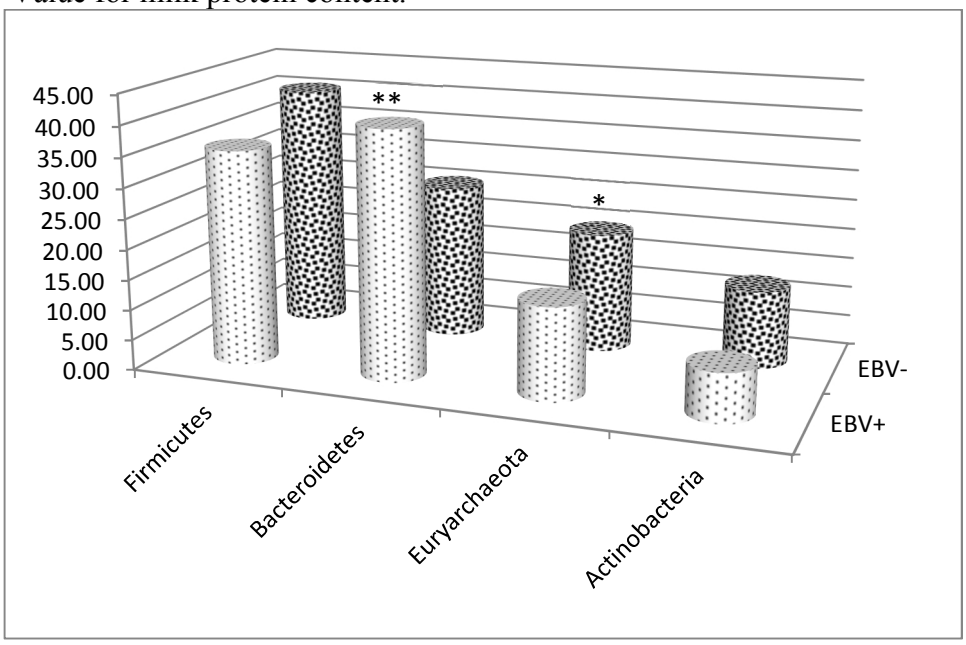
* indicates a significant effect for P<0.05;
*** indicates a significant effect for P<0.001

Figure 6. Distribution of the main microbial classes in Italian Simmental (IS) and Italian Holstein (IH) rumen contents.



* indicates a significant effect for P<0.05;
*** indicates a significant effect for P<0.001

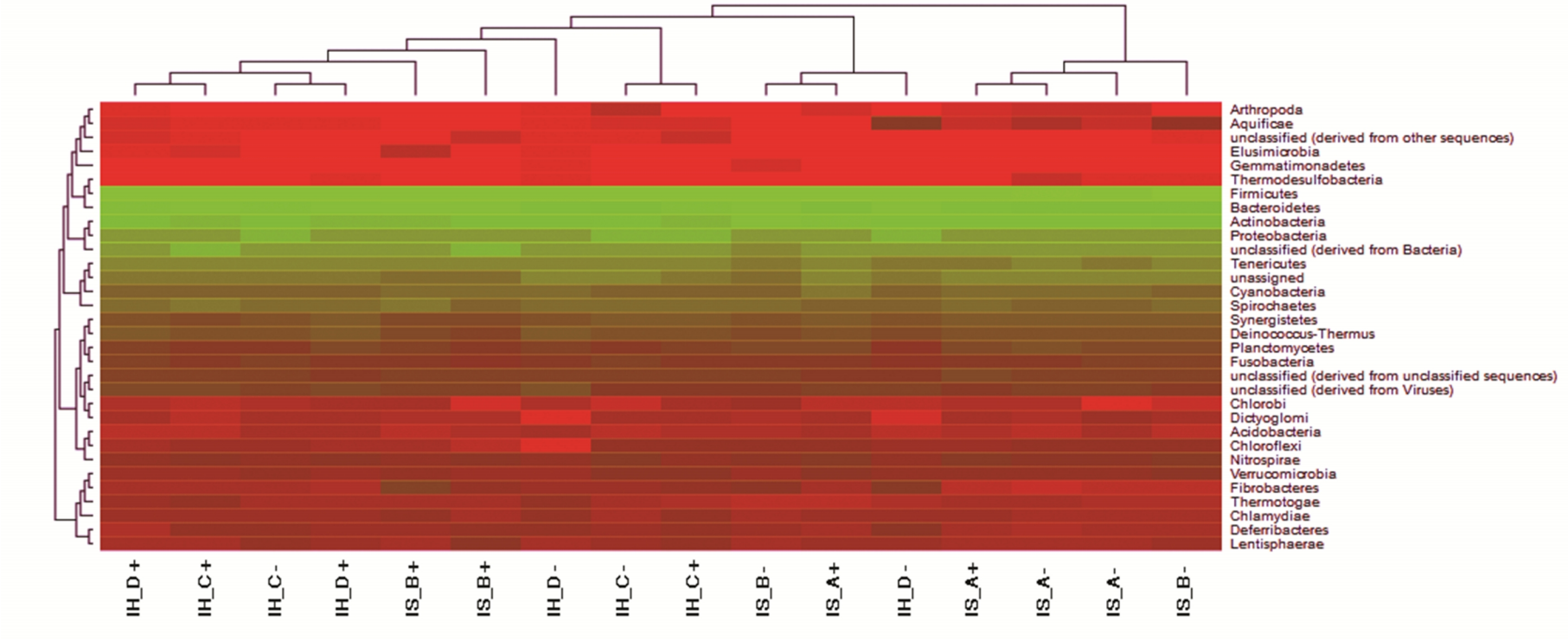
Figure 7. Distribution of the principal microbial phyla in rumen content of cows with negative (EBV-) or positive (EBV+) Estimated Breeding Value for milk protein content.



* indicates a significant effect for P<0.05;
** indicates a significant effect for P<0.01

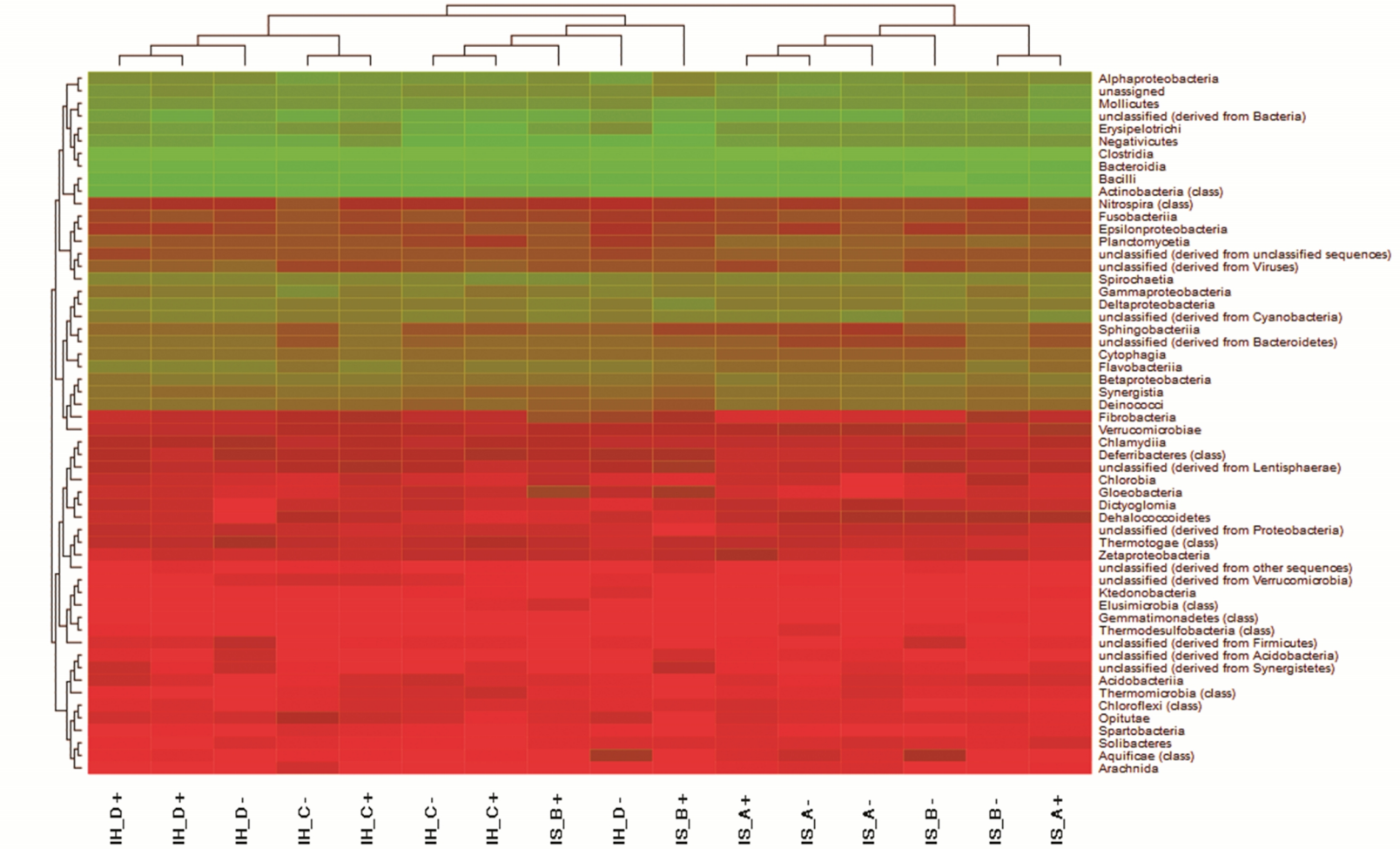
Figures 8 and 9 show the similarity heatmaps of the bacterial clustering of all 16 rumen samples, annotated at the phylum and class taxonomic levels, respectively. At the phylum level a clear branch can be noted grouping four IS cows, three of farm A and one of farm B, three out of four negative for EBVp. At the opposite side of the figure two sub-clusters with two IH each are present, three out of four positive for EBVp. In the middle, the clustering appears not so clear and four of the remaining samples are not coupled with others. The trend of breed clustering and in lower measure of EBVp clustering is more evident at the class taxonomic level (Figure 9). The branch on the right side of the figure is still present, being added of other two IS samples and gathering four negative and two positive for EBVp. At this level two more branches are showed on the left side of the image, comprising five IH samples. In the middle another branch gathers the remaining samples, not showing many similarities. These samples clustering data, according with bacterial rumen microbiome composition at the phylum and class taxonomic level, shows a tendency to follow the grouping criteria of breed and of genetic merit of the cows, for the latest in a way not fully depend from the breed. This is more evident observing the radial plot shown in Figure 4. The grouping according to the EBVp values in fact shows how the positive EBVp microbiome composition is more similar to the profile of IH cows, while the negative is close to the IS profile, both for *Bacteroidetes* and *Euryachaeota* abundances.

Figure 8. Heatmap for similarity clustering of microbial composition of the 16 samples of rumen contents at the phylum taxonomic level.



Full red indicates total dissimilarity while full green total similarity. Different color gradations indicate different levels of similarity between the samples for the relative phylum.
IH: Italian Holstein; IS: Italian Simmental; A, B, C, D indicates the farm to which the sample belongs; +/- indicates the positive or negative value for Estimated breeding value for kg of milk protein.

Figure 9. Heatmap for similarity clustering of microbial composition of the 16 samples of rumen contents at the class taxonomic level.



Full red indicates total dissimilarity while full green total similarity. Different color gradations indicate different levels of similarity between the samples for the relative class.
IH: Italian Holstein; IS: Italian Simmental; A, B, C, D indicates the farm to which the sample belongs; +/- indicates the positive or negative value for Estimated breeding value for kg of milk protein.

Chapter 2

Gene expression profile of peripheral leukocytes in lactating cows with different genetic merit for milk protein production

2.1. Introduction

Phenotypic selection through estimated breeding values has improved milk yield over past decades. This means animals underwent a great tuning of their digestive and metabolic systems, but many of the genetic and genomic variations responsible for this improvement are not fully uncovered.

In the high yielding dairy cows, a deterioration in reproductive performance and higher disease susceptibility has been observed and this represents the negative consequences of the selection programs that had as priority only productive traits [40-43].

Several studies on genetics and immunology are currently ongoing to gather information to readdress breeding programs in order to recover the health and reproductive performances of cows.

In different lineages of Holstein cattles there are some evidences that milk yield is not the only factor strictly responsible for reproductive failure [41] and that the selection for high immune response without lowering productivity is feasible [44]. Some studies are focusing on the effects of DNA polymorphisms associated to milk traits and to immune response on reproductive performance [45] or milk production [46]. According to the data provided by the Italian Breeder Association (AIA, see Figure 1), also for the Italian Simmental dual purpose cattles, the

selection for milk traits has lead to a decrease of reproductive performances, confirming the need to better understand the biological mechanisms that link the productive and reproductive performances of dairy cows.

As a first approach, the investigation of gene expression of healthy lactating cows can provide basic information on the biological response of animal differing for genetic make up. In the present study, we used a 44K bovine microarray to compare gene expression profile in peripheral blood leukocytes of healthy Italian Simmental (IS) and Italian Holstein (IH) lactating cows diverging for estimated breeding values for milk protein (EBVp).

2.2. Aim of the study

The aim of this study is to compare the expression profiles of lactating dairy cows reared in intensive farm system with divergent genetic merit for a productive trait as milk protein yield.

In particular, we tried to depict at the molecular level the overall effect of phenotypic selection in healthy Italian Holstein and Italian Simmental cows in the mid stage of lactation, a period expected to be stationery from a physiological and metabolic point of view [47,48].

The biological material we analyzed belongs to peripheral blood cells, mainly leucocytes, which allows to observe differences directly related to the immune system but also useful to indirectly speculate about metabolic differences between the experimental groups.

Blood is the carrier for all the organism compartments of oxygen, nutrients, hormones, cytokines and immune systems cellular components. Having the advantage of being easy to sample and reaching all body compartments, the blood is going to become a preferential surrogate tissue for numerous analysis taking the place of inaccessible target tissues [49]. This because genes previously believed to be restricted to non-blood tissues are expressed in peripheral blood cells [50].

Liew et al., (2006)[50], showed as the 80% of the genes expressed in a range of different tissues overlapped with gene expression in blood. The author observed that many of these genes could be considered housekeeping, but such a large number of genes shared between blood cells and other tissues cannot be only explained by housekeeping function. Furthermore, it has been reported that tissue-specific genes may be expressed in a non-tissue-specific manner [51,52], and that expression profiles of circulating blood cells do contain specific expression signatures in response to various physiological, pathological, and environmental changes [53,54].

Abbreviations

IH	Italian Holstein
IS	Italian Simmental
EBVp	Estimated Breeding Value for milk protein (kg)
H/SQ1	Cows (IH or IS) belonging to the minus variant group of EBVp
H/SQ2	Cows (IH or IS) belonging to the intermediate group of EBVp
H/SQ3	Cows (IH or IS) belonging to the plus variant group of EBVp
DEG	Differentially expressed genes
ACOX3	Acetyl-CoA oxidase 3
DGAT1	Diacylglycerol O-acyltransferase-1
DGAT2	Diacylglycerol acyltransferase-2
AGPAT1	1-acylglycerol-3-phosphate-O-acyltransferase 1
TAGs	Triacylglycerols
GSTM1	Glutathione-S-transferase M1
GSTM3	Glutathione S-transferase M3
GSS	Glutathione synthetase
G6PD	Glucose-6-phosphate dehydrogenase

GPx	Glutathione peroxidase
TAS	Total antioxidant status
AA	Amino acid
CD4	CD4 antigen
BoLA-DQB	Bovine lymphocyte antigen DQB
BoLA-DQA5	Bovine lymphocyte antigen DQA5
TNFSF13B	B-cell activating factor of the tumor necrosis factor superfamily
TGFB1	Transforming growth factor B1
RelA	subunit p65 of the NFkB proteins family
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1
BHOB	β-hydroxy-butirrate
ACSL1	Acyl-CoA synthetase long-chain family member 1
POMC	Pro-opiomelanocortin gene
PPARγ	Peroxisome proliferator-activated receptor
GUCY2C	Guanylate cyclase 2C (heat stable enterotoxin receptor)
ALDH3B1	Aldehyde dehydrogenase 3 family, member B1
ACSL1	Acyl-CoA synthetase long-chain family member 1

FAs	Fatty acids
PLA2G4B	Phospholipase A2, group IVB
CLDN11	Claudin 11
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
PGDFRA	Platelet-Derived Growth Factor Receptor A

2.3. Materials and Methods

Animals and sampling

One subgroup of 24 Italian Simmental cows belonging to the farm B and one of 24 Italian Holstein cows belonging to the farm C, were selected from the group of 135 animals enrolled for the study (see overview) based on the EBVs values. Hence, 3 groups of cows per breed were identified (8 cows per group), according to the EBV quartile they belonged to: SQ1, SQ2, SQ3 and HQ1, HQ2 and HQ3 for the IS and the IH cows belonging respectively to the first (SQ1 and HQ1), the second (and third) (SQ2 and HQ2) and the fourth (SQ3 and HQ3) EBV quartile within each breed.

The blood samples for gene expression analysis were collected as described in the overview. A more detailed description about cows EBVs, EBV quartiles and productive traits is presented in Table 11.

Table11. Average characteristics and productive traits of cows involved in study and divided according to the Estimated Breeding Values for milk protein (eight cows for each group).

Breed	**It. Simmental (Farm B)**			**It. Holstein (Farm C)**		
EBV group	**SQ1**	**SQ2**	**SQ3**	**HQ1**	**HQ2**	**HQ3**
Cows characteristics						
Parity	3±1	3±1	4±2	2±1	3±1	3±1
DIM at sampling	132±28	142±40	152±27	159±32	152±29	142±39
BCS at sampling	3.00±0.3	3.00±0.3	2.75±0.15	3±0.20	2.84±0.1	2.81±0.3
EBV for milk protein (kg)	-2±2	7±2	21±6	-2±6	25±7	46±3
Average Productive traits (305d)						
Milk yield (t)	8.1±0.3[a]	8.7±0.7[a]	10.0±1.2[b]	9.1±0.6[a]	9.5±1.3[a]	10.6±0.7[b]
Milk protein (kg)	288±20[a]	309±22[b]	355±23[c]	278±22[a]	295±39[b]	344±21[c]
Milk fat (kg)	318±20[a]	326±34[a]	380±45[c]	333±32[a]	366±44[a]	453±45[c]

Milk yield and milk fat a, c: indicate a significant difference for $P<0.01$
Milk protein a, b: indicate a significant difference for $P<0.05$
Milk protein a, c: indicate a significant difference for $P<0.01$
DIM : days in milk; BCS : Body Condition Score; EBV : Estimated breeding value;

RNA extraction

Total RNA from blood was extracted with the PAXgene Blood RNA System (Preanalytix, Hombrechtikon, Switzerland) following the manufacturer's instructions for genes expression evaluation

RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) while its integrity was checked by using an Agilent 2100 Bioanalyzer and the test chip kit (Agilent, Santa Clara, CA, USA). All samples were deemed of good/excellent integrity, showing a RIN (RNA Integrity Number) > 6.5.

Production of labeled cDNA

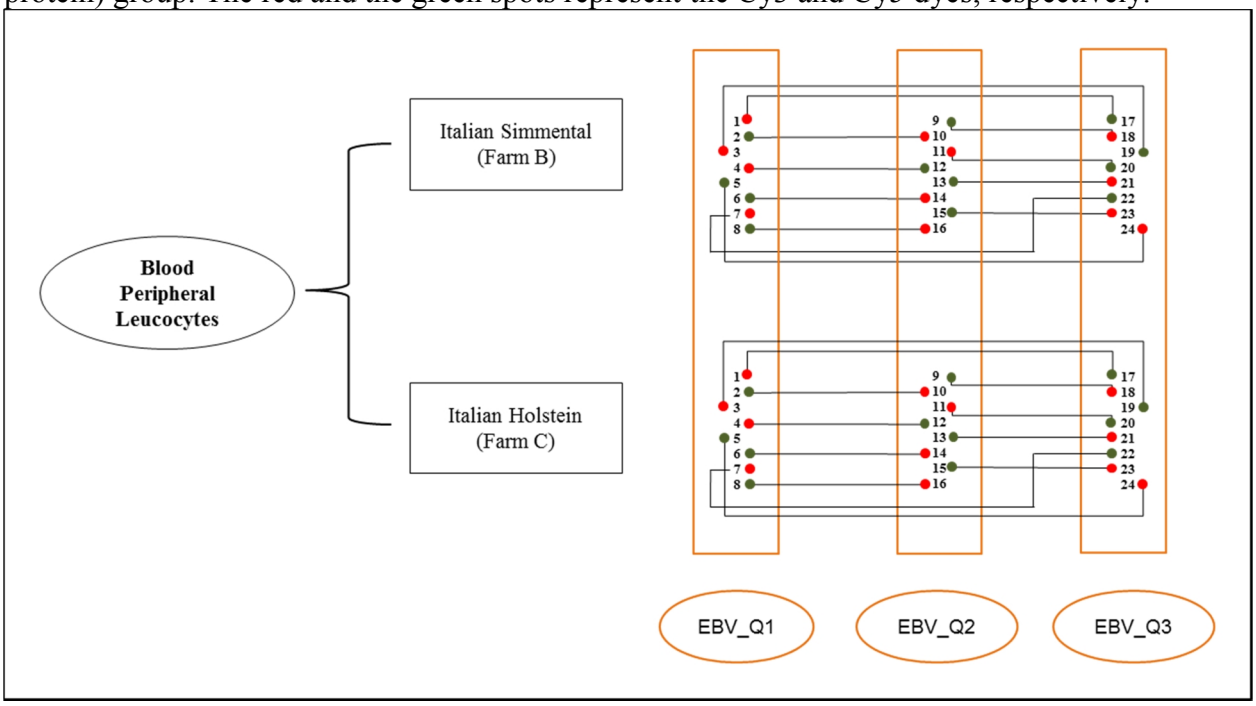
RNA samples were labeled using a 2-dyes Low Input Quick Amp Labeling Kit (Agilent, Santa Clara, CA, USA). The cDNA was obtained for each sample by a reverse transcriptase reaction adding 200ng RNA, 0.8µL T7 Promotor and DNase-RNase-free water to a volume of 5.3µL. The mixture was incubated at 65°C for 10 min and kept on ice for 5 min. To the mixture were added 4.7µL solution composed of 2µL 5X FS buffer, 1µL 0.1 M DTT, 0.5 µL 10mM dNTP mix and 1.2µL AffinityScript RNase block mix. The new mixture was incubated at 40°C for 2 hours then moved at 75°C for 15 min and then the tubes were kept on ice for 5 min. Incorporation of Cy dyes was obtained incubating at 40°C for 2 hours the previous mixture added 6 µL solution composed of 0.75µL DNase-RNase-free water, 3.2µL 5X Transcription buffer, 0.6µL 0.1 M DTT, 0.5µL NTP mix, 0.21µL T7 RNA polymerase blend and 0.24µL Cyanine 3 or cyanine 5-CTP. The unbound dyes were removed using a Qiagen RNase purification Kit (Qiagen, Germantown MD, USA) and clean labelled cDNA was measured by means of a NanoDrop ND-1000 spectrophotometer. An average of 2.4 ± 0.7 µg

of cDNA with 24 ± 7 pmol/ng cDNA of Cy dyes were obtained for each reaction. The labelled samples were then frozen at -80°C until the hybridization.

Microarray protocol

Six annotated Bovine (V2) Gene Expression Microarrays, 4x44K (Agilent, Santa Clara, CA, USA) were used for the gene expression analysis, three for each farm. Hybridizations were performed according to the scheme shown in Figure 10, balancing across arrays and slides the samples from each EBVp quartile. Every slide had four Cy3 and four Cy5 labeled samples.

Figure 10. Hybridization scheme of the labeled cDNA samples. The black full lines represent the arrays for 2 samples, basing on the EBVp (Estimated Breeding Value for kg of milk protein) group. The red and the green spots represent the Cy3 and Cy5 dyes, respectively.



Microarray hybridization, image acquisition and intensity signal extraction

For each microarray, a mixture composed of 825ng labelled sample per each dye, 11 µL 10X Blocking agent, 2.2µL 25X Fragmentation buffer and DNase-RNase-free water to a volume of 55µL was incubate in a 60°C water bath for 30 min to fragment the cDNA, and immediately moved in ice. To stop the fragmentation reaction 55µL of 2X GEX Hybridization buffer were added to each tube.

The microarray mixtures were dropped, according to the hybridization pattern, on the Hybridization Gasket Slide (Agilent, Santa Clara, CA, USA) used with the recorded slides and the Hybridization Chamber Kit - SureHyb (Agilent, Santa Clara, CA, USA) to prepare the hybridization assemblies. The assemblies were then placed in a rotating hybridization oven at 65°C for 17 hours.

After the hybridization the chambers were disassembled and the slides washed with the Agilent Gene Expression Wash Buffer 1 and 2, each for 1 min by agitation.

Soon after the washing step the 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 an Agilent Features Extraction Software.

Real time PCR analysis

The differentially expressed genes GUCY2C (guanylate cyclase 2C (heat stable enterotoxin receptor)), ALDH3B1 (aldehyde dehydrogenase 3 family, member B1), GSTM3 (glutathione S-transferase M3), ACSL1 (acyl-CoA synthetase long-chain family member 1), PLA2G4B (Phospholipase A2, group IVB), CD4 (CD4 antigen) for IH cows group and BOLA-DQA5

(bovine lymphocyte antigen DQA5), CLDN11 (claudin 11), DGAT2 (diacylglycerol acyltransferase-2), GSTM1 (glutathione S-transferase M1), CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4) and PGDFRA (Platelet-Derived Growth Factor Receptor A) for IS cows group were analyzed for real time quantitative PCR (RT-qPCR). The selection was made according to the presence and the relevance of these genes among the significant differentially expressed genes resulted from the statistical analysis of microarray data.

For each gene, an aliquot of cDNA samples was pooled and standard curves with serial dilution of pool were used to optimize PCR conditions and to calculate the efficiency, fluorescence baseline and threshold. Real time PCRs were performed in triplicate using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Milan Italy). A master mix with the following components was prepared to the indicated end concentration: 1 µL of cDNA, 9.5 µL water, 1 µL of each primer and 12.5 µL of 2X Platinum SYBR Green qPCR SuperMix-UDG for a total volume of 25 µL. cDNA concentrations and primer molarities were different for each gene and determined with standard curves analyses performed before Real time PCR reactions. Amplifications were conducted in a 96-well spectrofluorometric thermal cycler (DNA Engine Opticon 2; MJ Research, Inc. Waltham, MA USA). The melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

The expression of target genes was normalized using as housekeeping genes ACTB (beta-actin) and MRPS7 (mitochondrial ribosomal protein S7) and ΔCts were calculated by the difference between Ct of genes target and the geometric mean of the two internal control genes.

Microarray data statistical analysis and data mining

For the statistical analysis, data of 24 microarrays from the two breeds were imported to the GeneSpring GX software (Agilent, Santa Clara, CA, USA), applying to the signals intensity values a quantile normalization in order to correct potential bias due to not biological effects [55]. Afterward, the intensity data of the samples were split for each breed into 3 columns referring to the same EBVp quartile group. The gene annotations from the gal file were then uploaded according to the probes identifier. The intermediate EBVp quartile in Italian Simmental (SQ2) and Italian Holstein (HQ2) cows was taken as reference to evaluate the up-regulated and down-regulated genes of the extreme groups (Q1, lowest EBVp and Q3, highest EBVp). A permutation matrix was created by calculating for each gene of each cow of the Q1 and Q3 the ratios with the corresponding gene of the Q2 group. The resulting linear expression ratios were then $\log_2$ transformed ($\log_2$_ratios).

Finally, a tab-delimited file was imported to MeV software (version 4.9.0 http://mev-tm4.sourceforge.net/, [56] and used to perform a t-test of the expression $\log_2$_ratios between the Q1/Q2 and Q3/Q2 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 differently expressed genes was performed for the KEGG pathways (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) with DAVID tool (http://david.abcc.ncifcrf.gov) [57].

Statistical analysis of biochemical parameters and milk

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 the SPSS

(1997). Normality of data was tested by the Kolmogorov-Smirnov non parametric test. Somatic cell count (SCC) was not normal distributed and a log(2) transformation was used before statistical analysis.

2.4. Results and discussion

Statistical analysis of the differentially expressed genes

Statistical analysis with MeV allowed to identify 443 and 281 differentially expressed genes for $P<0.05$ after Bonferroni correction between the Q1 and the Q3 EBVp quartiles in the IS and IH group, respectively. Details of the number of up and down-regulated gene and of the genes with opposite regulation between the groups are shown in Table 12. Overall, the results indicated that there is a slightly greater number of down than up-regulated DEG in the peripheral blood cells of cows minus variant for EBVp relative to the intermediate group. The cows belonging to plus variant EBVp group showed an opposite trend with a greater number of up than down-regulated DEG both in IS (Farm B) and in IH (Farm C) cows.

The results obtained from MeV were validated with real time PCR analysis for the list of genes reported in Material and Methods, using ACTB (beta-actin) and MRPS7 (mitochondrial ribosomal protein S7) as housekeeping genes.

Table 12. Summary of the differentially expressed genes (DEG) (P<0.05) in the Italian Simmental (IS) and Italian Holstein (IH) groups. The differentially regulated genes are calculated as ratios of Q1 to Q2 and Q3 to Q2 of EBVp groups.

Breed	**IS (Farm B)**		**IH (Farm C)**	
EBV group	**SQ1/SQ2**	**SQ3/SQ2**	**HQ1/HQ2**	**HQ3/HQ2**
Up-regulated	200	252	154	168
Down-regulated	243	191	127	113
Genes with opposite expression	398		241	
Total DEG	443		281	

Q1: cows belonging to the 25% of the lowest EBV values in the population (breed) included in the study; Q2: cows between the 25% of the lowest and the 25% of the highest EBV values in the population (breed) included in the study; Q3: cows belonging to the 25% of the highest EBV values in the population (breed) included in the study

Impact of differentially expressed genes on KEGG pathways

Data mining with DAVID tool was performed in order to identify KEGG pathways affected in cows minus and plus variant for milk protein EBVp (Q1 and Q3 groups, respectively). Although the expression level of genes uploaded in the DAVID tool were significantly (P<0.05) different in the experimental groups, some of the involved pathways did not result significant, likely due either to the limited number of genes present in the pathway or to the low entity of the fold-changes. However, all the pathways affected are reported in the tables and the most relevant for our study are discussed.

KEGG pathways in the Italian Simmental cows group (Farm B)

The most impacted pathways and the significant genes involved in the Q1 and Q3 groups of IS cows, compared with the Q2, are shown in Table 13.

For the "Metabolism" and "Human diseases" categories the largest number of pathways was showed. Our study deals with cattle, and the "Human diseases" pathways are likely to be present since KEGG pathways are mainly based on human information and numerous genes across the KEGG pathways can be included also in the this category.

Within the "Metabolism" category, "Energy metabolism", "Lipid metabolism" and "Metabolism of cofactors and vitamins" sub-categories had the highest number of genes, that were involved in one pathway for each sub-category. Instead, for both "Amino acids metabolism" and "Xenobiotics biodegradation and metabolism" sub-categories, two pathways were shown.

Both "Cellular processes" and "Organismal systems" had six pathways represented, the first with "Regulation of actin cytoskeleton", "Endocytosis" and the second with "Vascular smooth muscle contraction" and "Progesterone-mediated oocyte maturation" as the pathways within the relative categories containing the highest number of genes (10, 10 and 6, 5 respectively).

Others KEGG pathways showed to be impacted in IS cows belonged to the category "Environmental information processing", in particular "Calcium signaling pathway" and "Cell adhesion molecules" (7 and 5 genes represented, respectively).

Further considerations on the pathways, regardless the number of genes represented, will be reported in the discussion, based on the role that genes are expected to play in relation to differences in the EBVp values.

Table 13: KEGG pathways represented in Italian Simmental (Farm B) cows plus (SQ3) and minus (SQ1) variant for milk protein EBV in comparison with the cows belonging to the intermediate EBVp values quartile (SQ2).

Category	Sub-category	Pathway	Term	n of genes	Genes	Fold Enrichment
Metabolism	Energy metabolism	Oxidative phosphorylation	bta00190	5	CYTB, SDHA, ND4L, ATP5G2, ATP6V1A	1.7
	Metabolism of cofactors and vitamins	Retinol metabolism	bta00830	4	ADH5, DGAT2, CYP3A4, RDH16	3.1
	Lipid metabolism	Glycerolipid metabolism	bta00561	4	AGPAT1, DGAT2, LIPG, ALDH1B1	3.1
	Amino acid metabolism	Alanine, aspartate and glutamate metabolism	bta00250	3	ALDH5A1, GOT2, ABCB7	3.4
	Xenobiotics biodegradation and metabolism	Drug metabolism	bta00983	3	CYP3A4, UPP1, TPMT	2.9
	Amino acid metabolism	Tyrosine metabolism	bta00350	3	MIF, ADH5, GOT2	2.9
	Xenobiotics biodegradation and metabolism	Metabolism of xenobiotics by cytochrome P450	bta00980	3	GSTM1, ADH5, CYP3A4	2.6
	Metabolism of other amino acids	Glutathione metabolism	bta00480	3	GSTM1,GSS, G6PD	2.2
	Carbohydrate metabolism	Glycolysis / Gluconeogenesis	bta00010	3	ADH5, PFKP, ALDH1B1	1.8
Environ. information processing	Signal transduction	Calcium signaling pathway	bta04020	7	PDGFRA, SLC4A2, VDAC3, TAF6, PLCG1, TAF6L, ITPR3	1.6
	Signaling molecules and interaction	Cell adhesion molecules (CAMs)	bta04514	5	CLDN2, NLGN2, BOLA-DQA5, CLDN11, BOLA-DQB	1.6
	Signal transduction	ErbB signaling pathway	bta04012	4	CRK, KRAS, PLCG1, AGL	1.9
	Signal transduction	Notch signaling pathway	bta04330	3	DTX3, PSENEN, DTX3L	2.5

Cellular processes	Cell motility	Regulation of actin cytoskeleton	bta04810	10	PGFFRA, CRK, KRAS, MRAS, CFL1, F2, ARHGEF6, ITGAD, PFN1, ACTN1	2.1
	Transport and catabolism	Endocytosis	bta04144	10	PDGFRA, CHMP2A, NR1HA, IL2RG, TAS2R42, ACAP3, SH3GLB2, VPS37B, ACAP1, PARD6G	2.0
	Cell communication	Tight junction	bta04530	8	LLGL1, KRAS, CSDA, MRAS, CLDN2, CLDN11, ACTN1, PARD6G	2.7
	Cell communication	Gap junction	bta04540	7	PDGFRA, PPM1B, KRAS, CDK1, TAF6L, ITPR3, TUBA1C	3.3
	Cell growth and death	Oocyte meiosis	bta04114	6	PPM1B, YWHAH, CDK1, TAF6L, ITPR3, MAD2	2.3
	Transport and catabolism	Regulation of autophagy	bta04140	3	GABARAPL1, ULK1, BECN1	4.1
Organismal systems	Circulatory system	Vascular smooth muscle contraction	bta04270	6	ARHGEF12, PPM1B, SLC4A2, TAF6, TAF6L, ITPR3	2.2
	Endocrine system	Progesterone-mediated oocyte maturation	bta04914	5	MAD2, PPM1B, KRAS, CDK1, TAF6L	2.2
	Development	Axon guidance	bta04360	5	SEMA4A, ARHGEF12, KRAS, NRP1, CFL1	1.7
	Nervous system	Neurotrophin signaling pathway	bta04722	5	ARHGDIA, CRK, KRAS, YWHAH, PLCG1	1.6
	Immune system	Intestinal immune network for IgA production	bta04672	4	TNFSF13B, BOLA-DQA5, TGFB1, BOLA-DQB	3.2
	Endocrine system	GnRH signaling pathway	bta04912	4	PPM1B, KRAS, TAF6L, ITPR3	1.6
Human diseases	Neurodegenerative diseases	Alzheimer's disease	bta05010	7	CAPN1, CYTB, SDHA, CYCS, ATP5G2, PSENEN, ITPR3	1.9
	Neurodegenerative diseases	Parkinson's disease	bta05012	6	CYTB, SDHA, ND4L, CYCS, ATP5G2,	2.0

				VDAC3	
Cancers: Specific types	Chronic myeloid leukemia	bta05220	5	CRK, BCR, NR1H2, KRAS, TGFB1	2.4
Cardiovascular diseases	Viral myocarditis	bta05416	4	CYCS, BOLA-DQA5, AGL, BOLA-DQB	2.6
Cardiovascular diseases	Dilated cardiomyopathy	bta05414	4	TPM4, PPM1B, TGFB1, TAF6L	2.4
Cancers: Specific types	Pancreatic cancer	bta05212	4	NR1H2, KRAS, ARHGEF6, TGFB1	2.1
Immune diseases	Systemic lupus erythematosus	bta05322	4	H3F3B, BOLA-DQA5, BOLA-DQB, ACTN1	1.6
Cancers: Specific types	Colorectal cancer	bta05210	6	PDGFRA, NR1H2, CYCS, KRAS, TGFB1, MSH6	2.6
Cancers: Specific types	Glioma	bta05214	3	PDGFRA, KRAS, PLCG1	1.9

Interpretation of KEGG pathways in the Italian Simmental cows group (Farm B)

The breeding program of a dual purpose cattle breed, as the IS cows, aims at contemporary improving two metabolic profiles that can diverge for goal and related aspects. On the contrary, in milk specialized animals fat deposition and mobilization are very relevant and largely contribute to support the milk output at the onset of lactation and to store energy for the next productive cycle at the end of lactation and during the dryoff. Dietary protein is furthermore directed to the mammary gland for milk components synthesis rather than to muscle deposition.

The phenotype-based selection schemes for dual purpose breeds assign relative weight to productive traits for both milk yield and carcass traits, as well as the respective qualitative characteristics. In this contest, the impact of selection at the genomic level, as appreciated by trasncriptome analysis, appears to be unidirectional in IH specialized dairy animals and not univocal in dual purpose IS, particularly in the animals with high total genetic merit (e.g. having high values for both productive purposes).

Glycerolipid metabolism in dairy cows plays a fundamental role in milk fat synthesis and is affected by diet composition and genetic make-up. DGAT1, a diglyceride-O acyltransferase-1, an enzyme responsible for the last step in triglycerides synthesis, is widely known to carry a polymorphism that is responsible for a major QTL, underlying several milk production traits in Holstein cows[58,59]. Many other cattle breeds [58,59], also derived from Simmental lineage [60], were reported to bear this QTL. However, DGAT1 segregation at the QTL level has been reported to have a very low frequency in the IS breed [61], indicating that the dual purpose breeding program has probably selected against the positive allele. This speculation is further

supported by the differential expression in the IS of the DGAT2 (diacylglycerol-O acyltransferase-2), an isoform of the diglyceride-O acyltransferase that was up and down-regulated in SQ1 and SQ3, respectively. The DGAT2 and the DGAT1 are represented in the "Glycerolipid metabolism" KEGG pathway. DGAT2 has not been deeply investigated yet, especially in dairy cows. There are some recent evidences of the association of this isoform with fat carcass traits in commercial feedlot steers [62] and in one Nyniang Chinese cattle breed, in which different allelic variants for DGAT2 resulted associated with increased body height and weight, body length and heart girth [63]. The role of DGAT2 in dairy cows remains mostly unknown, but in laboratory animals there are emerging studies showing that its activity is related with glucose and triglyceride blood levels. Zammit [64] assigned to DGAT2 a central role in the carbohydrate-induced hypertriglyceridaemia and hepatic steatosis, due to its specialized role (diacylglycerol-O acyltransferase-2) in catalysing the de novo synthesis of triacylglycerols from newly synthesized fatty acids and nascent diacylglycerols. The same author underlined that these observations have a particularly relevance for the peripheral tissues not normally expected to be lipogenic. In these tissues, the synthesis of triacylglycerols may largely bypass DGAT2 except in hyperglycaemic/hyperinsulinaemic conditions, when the induction of de novo fatty acid synthesis may contribute towards increased triacylglycerol secretion (intestine) or insulin resistance (adipose tissue, and cardiac and skeletal muscle). Consistent results are reported by Choi et al., [65] in DGAT2 mRNA expression suppressed mice with diet-induced nonalcoholic fatty liver disease. The effects of the treatment were an increase of plasma fatty acids with the hepatic lysophosphatidic acid and diacylglycerol concentrations similar to those of control rats. The changes of plasma fatty acids were associated with a decreased lipogenesis and an increased hepatic oxidation of fatty acids.

Overall, these results could indicate that the DGAT2 over-expression in SQ1 is associated to hepatic insulin resistance and to a subsequent alteration of the glycaemia control mechanism. Interestingly, in our study we found a negative correlation ($P<0.05$) between plasma glucose and EBVp in IS cows (Table 14), and so we can speculate an effect of DGAT2 on the response to insulin regulation in SQ1 cows.

Table 14. Effects of breeds, DIM and genetic merit for milk protein (EBVp) on plasma and blood constituents in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 4 commercial farms in North-East of Italy.

Items		Breeds		Effects					SEM
		IH	IS	Breed	Farm	Covariates			
						DIM	EBVpIH	EBVpIS	
Total proteins	g/l	83.60	80.56	0.084	0.000	-0.004	+0.018	+0.043	5.990
Albumin	g/l	37.46	37.23	0.699	0.000	-0.004	+0.032	+0.038	2.474
Globulin	g/l	46.14	43.34	0.226	0.000	-0.003	-0.014	+0.003	7.203
Urea	mmol/l	4.79	5.01	0.345	0.000	-0.005*	-0.008	-0.001	0.070
Creatinine	µmol/l	63.23	89.99	0.000	0.000	+0.015	+0.011	-0.030	8.312
AST	UI/l	84.38	77.61	0.039	0.000	-0.078*	+0.066	+0.232	1.372
Zinc	µmol/l	12.92	11.21	0.000	0.004	+0.004	-0.003	+0.000	0.180
TAS	mmol/l	1.19	1.14	0.001	0.570	+0.000	+0.001	+0.001	0.005
Hemoglobin	g/dl	10.81	10.42	0.275	0.005	+0.007*	-0.001	-0.012	0.094
GPx	U/gHb	326.00	243.00	0.000	0.000	+0.084	-0.087	+0.564	52.296
Cortisol	pmol/ml	9.64	8.66	0.061	0.000	+0.011	-0.069	+0.035	0.581
Glucose	mmol/l	3.40	3.40	0.067	0.000	+0.001	+0.000	-0.007*	0.023
FFA	meq/l	0.12	0.13	0.725	0.005	+0.000	+0.001	-0.001	0.006
BOHB	mmol/ml	0.58	0.55	0.059	0.000	+0.000	-0.003*	-0.002	0.012

IH : Italian Holstein; IS : Italian Simmental; DIM : Days In Milk; EBVpIF : Estimated Breeding Values for milk protein of Italian Frisian cows; EBVpIS : Estimated Breeding Values for milk protein of Italian Simmental cows; TAS : Total Antioxidants Status; GPx : Glutathione peroxidase; FFA : Free Fatty Acids; BOHB : beta-hydroxy-butyrate.

* indicates a significant effect for $P<0.05$; ** indicates a significant effect for $P<0.001$.

Signs indicate the relationship, positive or negative, between the parameter and the effect.

AGPAT1 (LPAATα) (1-acylglycerol-3-phosphate-O-acyltransferase 1) represented, as DGAT2, in the glycerolipid metabolism pathway, is a gene coding for the transferase enzyme in charge of the second acylation in the glycerol phosphate pathway, adding an acyl group to the monoglyceride lysophosphatidic acid in order to obtain the diglyceride phosphatidic acid. Phosphatidic acid has been shown to increase mTOR activity, which is known to down regulate insulin signaling.

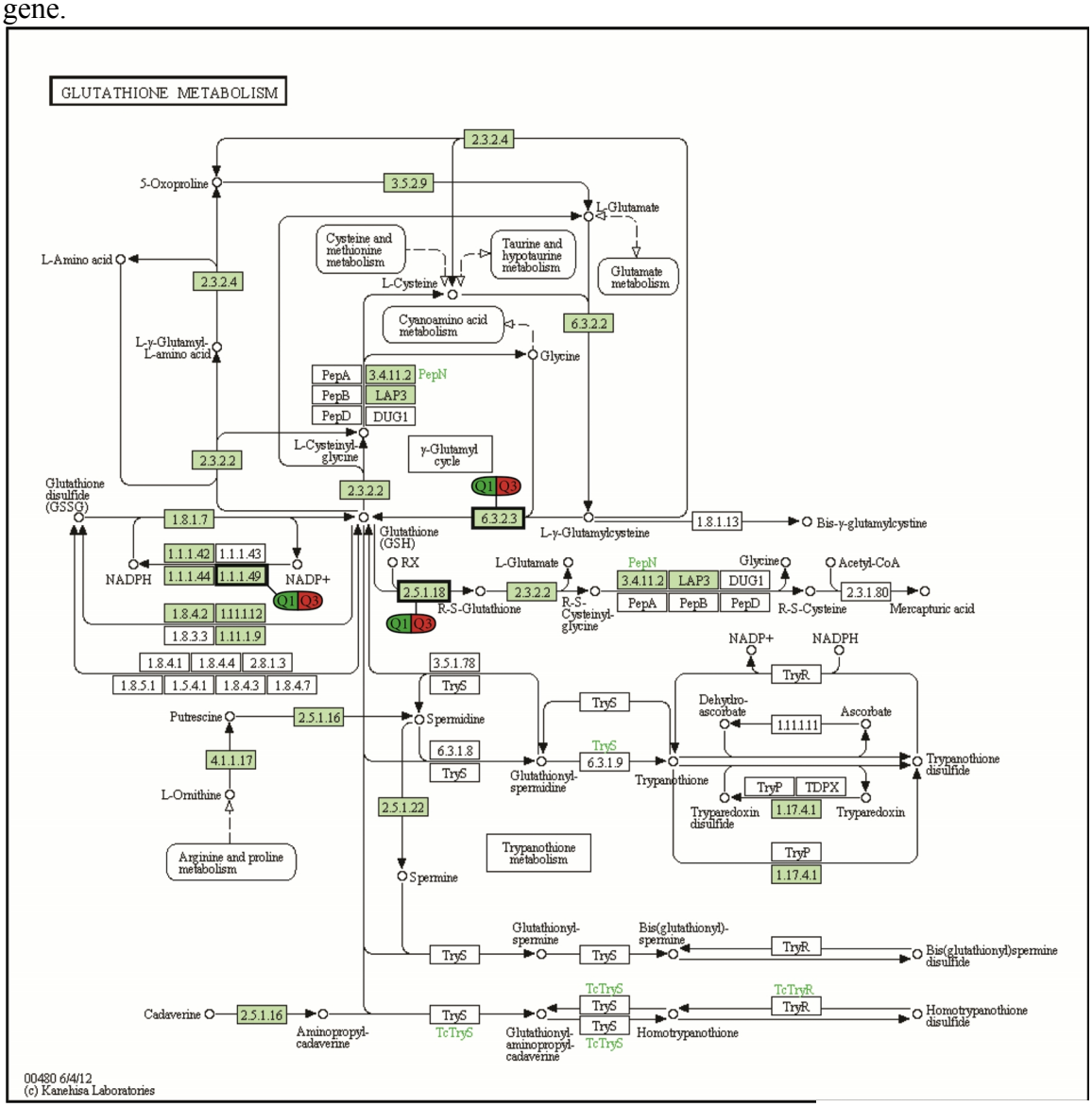
In our study, AGPAT1 was differentially expressed with opposite regulation compared with DGAT1 (i.e. down and up regulated in SQ1 and SQ3, respectively compared with SQ2). This could be explained with the similar, but different role that AGPAT1 appears to have in energy handling in different tissues. In human overnight lipid infused, Cornford et al., (2009) [66] reported reduced insulin sensitivity and up-regulation in AGPAT1 mRNA in skeletal muscle. The same author in liver samples from insulin resistant human subjects with NAFLD (non alcoholic fatty liver disease) observed an increase in AGPAT1 mRNA levels, which directly correlated with BMI. Recently Yamashita et al., [67] reported that the AGPAT1 overexpression in 3T3-L1 adipocytes increased oleate uptake and its incorporation in TAG and insulin-stimulated glucose uptake by >50% and its conversion to lipids. Instead, in myotubes glucose uptake was not altered even though its metabolic fate changed, with a decreasing glycogen synthesis by 30% and increasing lipid synthesis by 33% [68]. Furthermore, AGPAT1 is likely to be involved in skeletal muscle development, conversely to its isoform AGPAT2 [69]. In this sense, the overexpression of AGPAT1 in SQ3 could be explained as an overall better metabolic efficiency of these dual purpose cows, combining the metabolic profiles of milk production with muscle development.

For the IS cows, interesting results have been shown also for the "Glutathione metabolism" pathway. All three genes represented, GSTM1 (an enzyme of the glutathione-S-transferase family), GSS (glutathione synthetase) and G6PD (glucose-6-phosphate dehydrogenase), were up-regulated in Q3 group (Figure 11). GSS and G6PD in particular indicate both a greater *de novo* GSH synthesis and an active role helping indirectly to reduce the oxidized GSSG (glutathione disulfide) to the original active form, then avoiding GSH depletion from the cell. The tripeptide glutathione GSH is important for the antioxidant defense, the xenobiotic and eicosanoid metabolism, and for the regulation of cell cycle and gene expression [70-72]. GSH participates in many cellular reactions, as scavenging free radicals and other reactive oxygen species directly or acting as a cosubstrate in the GPx (glutathione peroxidase) - catalyzed reduction of H_2O_2 and lipid hydroperoxides [73,74]. Furthermore, GSH plays an important role in the generation of biologically reactive intermediates for some compounds as various electrophilic compounds, xenobiotics, and physiological metabolites, through the formation of conjugates catalyzed by the GSTs protein family (a family of Phase II detoxification enzymes) [73,75,76]. GSH serves also as a substrate for the removal of formaldehyde produced from the metabolism of methionine, choline, methanol (alcohol dehyderogenase) and xenobiotics (via the cytochrome P450-dependent monooxygenase system of the endoplasmatic reticulum). In human immune system, GSH is essential for the activation of T-lymphocytes polymorphonuclear leukocytes as well as for cytokines production, and therefore for mounting successful immune response when the host is challenged [77]. Although there is much evidence that GSH is increased in response to chronic oxidative stress, for the SQ3 group no biochemical evidences of this condition (blood TAS or GPx) were found (Table 14), supporting the hypothesis that an increase of GSH actually provides just a benefit to

antioxidant defenses [73]. The up-regulation of GSS and G6DP in SQ3 can be interpreted then as an enhanced antioxidant response to the increased metabolism and to the presence of free oxygen radicals due to the higher milk production. A further explanation, related to cell metabolism, can be the nutrient uptake from the diet, in particular cysteine and methionine, precursors of GSH. Cysteine is generally considered the limiting amino acid for GSH synthesis in rat, in pigs and in chickens [78,79] and factors (e.g. insulin and growth factor) that stimulate cysteine uptake by cells generally increase intracellular GSH concentration. Thereof, considering the effect of EBVp on glycaemia in IS (Table 14) and the potential involvement of insulin regulation, we can propose the hypothesis that the up-regulation of GSS gene is related to the higher availability of amino acids in response to a more efficient insulin sensitivity of SQ3 cows, compared with SQ2 and SQ1 cows.

The high involvement of protein metabolism in IS cows with high genetic merit during lactation is confirmed by the negative effect ($P<0.05$) of EBVp on the proportion of urea N and PDN on total urinary N, reported in Table 15. In fact, urinary urea N losses in animal fed the same diet mostly depend from the hepatic urea synthesis from the surplus of rumen ammonia and from the salvage of urea by the kidney. As far as the first event is concerned, the rate of ammonia absorption is known to reflects - at least in part - the nature of the proteins fed, but also the extent to which rumen ammonia is utilized for microbial protein synthesis and the catabolism of urea by microbial urease [80]. On the other side, the increase in the salvage of urea by kidney is not limited to low protein diets, but it also present when the requirements for N increases [81], as in lactation [82], allowing more urea to be recycled into the GI tract

Figure 11. Glutathione metabolism pathway (bta00480, KEGG). Highlighted are the genes differentially expressed in Italian Simmental SQ1 (Q1) and SQ3 (Q3) compared with the Q2 quartile. Red and green boxes indicate the over or under expression of the gene.



6.3.2.3 molecule: GSS, glutathione synthetase; 2.5.1.18 molecule: GSTM1, glutathione-S-transferase; 1.1.1.49 molecule: G6PD, glucose-6-phosphate dehydrogenase

Table 15. Effects of breeds, DIM and genetic merit (EBV) on urinary constituents in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 4 commercial farms in North-East of Italy.

Items		Breeds		Effects					SEM
		IH	IS	Breed	Farm	Covariates			
						DIM	EBVpIH	EBVpIS	
Total N	g/l	6.52	7.94	0.008	0.000	-0.003	-0.003	+0.013	3.545
Urea N	g/l	3.96	3.44	0.668	0.886	-0.007	-0.011	-0.031	4.298
Creatinine N	g/l	0.90	1.35	0.000	0.007	+0.001	+0.001	+0.002	0.031
Allantoin N	g/l	0.80	0.78	0.961	0.117	+0.000	+0.000	-0.002	0.019
Uric N	g/l	0.06	0.06	0.704	0.704	+0.196	+0.513	+0.121	0.003
PDN	g/l	0.87	0.83	0.987	0.220	+0.000	+0.000	-0.002	0.022
Crea N:Total N	ratio	0.15	0.17	0.001	0.000	+0.000*	+0.000	-0.000	0.004
Urea N:Total N	ratio	0.65	0.45	0.141	0.001	+0.000	-0.001	-0.007*	0.031
PDN:Total N	ratio	0.14	0.11	0.027	0.000	+0.001	+0.002	-0.000*	0.003
PDN:Creatinine	ratio	0.97	0.61	0.001	0.065	-0.000	-0.011*	-0.006	0.065

IH : Italian Holstein; IS : Italian Simmental; DIM : Days In Milk; EBVpIH : Estimated Breeding Values for milk protein of Italian Holstein cows; EBVpIS : Estimated Breeding Values for milk protein of Italian Simmental cows; * indicates a significant effect for $P<0.05$; ** indicates a significant effect for $P<0.001$.
Signs indicate the relationship, positive or negative, between the parameter and the effect.

The “Organismal systems” category, subcategory “Immune system”, showed DEG of IS cows involved in the “Intestinal immune network for IgA production” pathway. In particular, two BoLA-DQ antigens (BoLA-DQB and BoLA-DQA5) and the B-cell activating factor of the TNF superfamily (TNFSF13B) resulted overexpressed and under expressed in SQ1 and SQ3 group, respectively. On the contrary, the transforming growing factor B1 (TGFB1) showed an opposite regulation in both the groups (Figure 12).

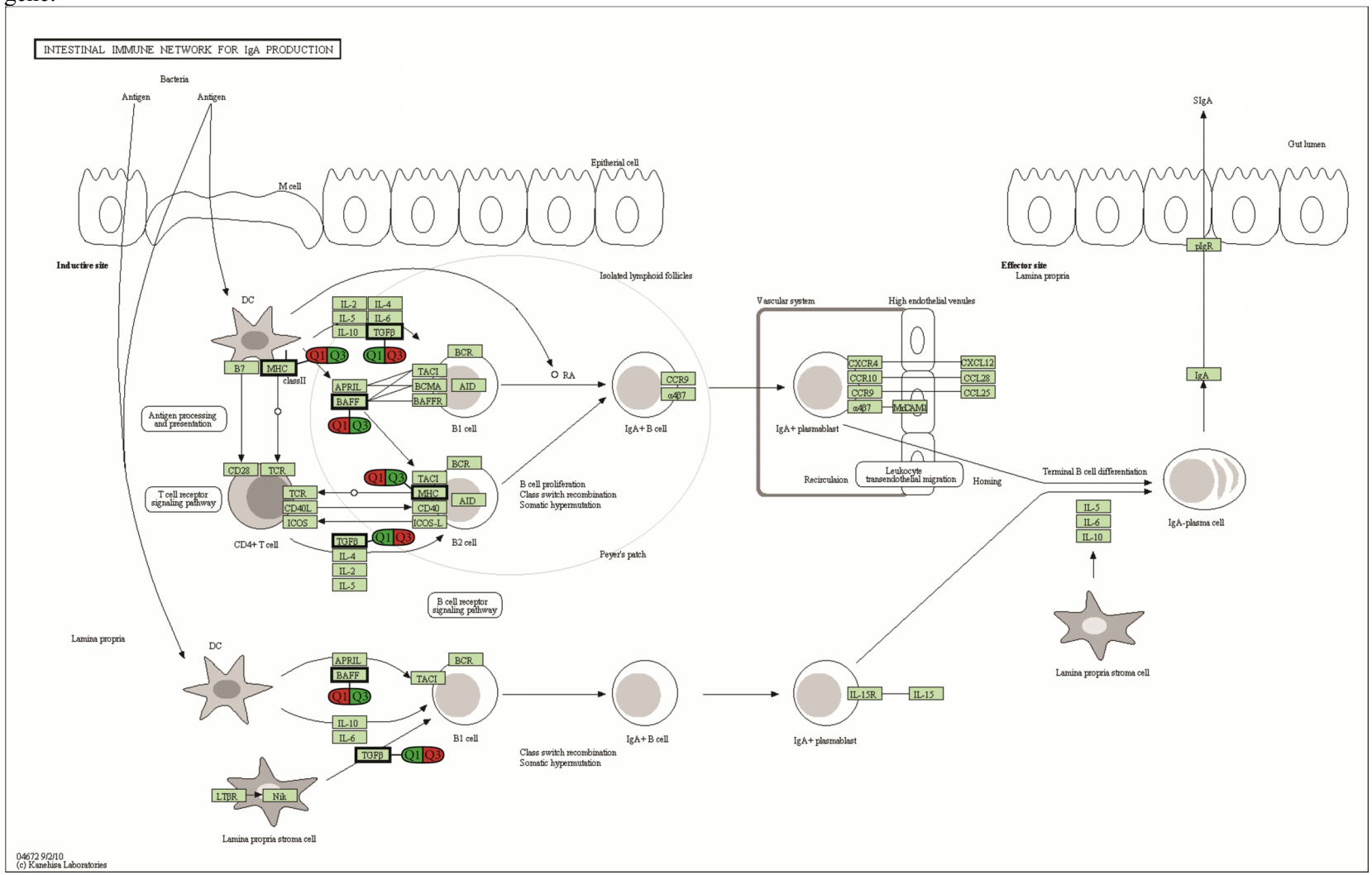
BoLA-DQB and BoLA-DQA5 belong to the II class of histocompatibility antigens of the MHC (Major histocompatibility complex), one of the most important genetic system for infectious disease resistance in vertebrates [83,84]. Differently form Class I, Class II molecules are expressed on “professional” antigen-presenting cells (APCs), such as dendritic cells and macrophages. APCs present peptides derived from extracellular pathogens to CD4+ T cells, which once stimulated activate macrophages and B cells to generate inflammatory and antibody responses, respectively [85]. The importance of these molecules is underlined by the fact that DQA and DQB gene clusters have been observed to be duplicated along the genome and the duplicated clusters has been observed to be tightly linked in most haplotypes [86]. Moreover, the MHC class II genomic sub region in ruminants includes DQ and DR gene clusters, and the later has been extensively associated, in one or more of DRB3.2 alleles, with susceptibility/resistance to some infectious diseases (i.e. severe mastitis and persistent lymphocytosis caused by Bovine Leukemia Virus) in cattle [85]. Park et al., [87] studied the association between mastitis and MHC haplotypes, in particular DR and DQ antigens, in Holstein cows, showing that mammary gland mononuclear cells from mastitis-resistant cows expressed MHC class II DR+DQ, DQ and DR more frequently ($P<0.05$) than the corresponding cells from mastitis-susceptible cows. The author concluded that the high

proportion of mononuclear cells expressing MHC class II in the mastitis-resistant cows indicates a substantial level of lymphocyte and macrophage activation. Conversely, the low proportion of cells expressing MHC class II in the chronically infected, mastitis-susceptible cows suggests a relatively low state of cell activation. In the same study, the association between mastitis and the MHC haplotypes that have only a single set of DQ genes was also found. Given the high polymorphism of DQB genes and the paucity of data on the precise function of these antigens in the Simmental breed, a precise meaning to their different expression in our study is difficult to find. Considering the results showed in the literature, we can argue an effect of the selection for productive traits, such as milk protein, on the lymphocytes activation state in cows with different genetic merit. This idea appear to be consistent with the TNFSF13B expression pattern in ISQ1 (higher expressed) and ISQ3 (lower expressed) cows. Regulation of IgA production in cattle is not completely understood but comparison with human and mice highly conserved factors, such as TNF-super family members, have been done. A proliferation-inducing ligand (APRIL) and TNFSF13B have been shown to play a role in regulating IgA responses [88,89]. The primary role of these two cytokines is in the area of B cell maturation and survival. In fact, dendritic cells, responsible for TNFSF13B and APRIL expression, modulate antigen class switching by activating B cells through both T cell-dependent and T cell-independent pathways [89]. Furthermore, in TNFSF13A knock-out mice B cell maturation is halted at the T2 stage, and few mature B cells are present [90].

In “Intestinal immune network for IgA production” TGFB1 was the last gene resulted differentially expressed, showing opposite expression compared with the genes previously discussed. In fact, it was over and under expressed in ISQ3 and ISQ1 cows, respectively. The

transforming growth factor (TGFB1) family of proteins are a set of pleiotropic secreted signaling molecules with unique and potent immunoregulatory properties [91]. Within the concerned pathway, TGFB1 appears to influence B cell response, directly from dendritic cells or through CD4+ T cells. TGFB1 controls several aspects of the normal maturation and differentiated functions of B cells, in addition to its effects on B cells proliferation. Typically, TGFB1 has been shown to have an anti-proliferative and apoptosis-stimulating activity on B cell, inhibiting also immunoglobulin synthesis and the secretion of Igs of all classes [91]. Hence, the overall inhibitory effect of the EBVp on B cells involved in the intestinal network for IgA production seems to be confirmed also by the overexpression of the TGFB1 in ISQ3 cows.

Figure 12. Intestinal immune network for IgA production pathway (bta04672, KEGG). Highlighted are the genes differentially expressed in Italian Simmental SQ1 (Q1) and SQ3 (Q3) compared with the Q2 quartile. Red and green boxes indicate the over or under expression of the gene.



MHC gene: BoLA –DQB or BoLA-DQA5: bovine lymphocyte antigen DQB or DQA5; BAFF: TNFSF13B : B-cell activating factor of the tumor necrosis factor superfamily.

KEGG pathways in the Italian Holstein cows group (Farm C)

The pathways most impacted and the significant genes involved for the IH cows of the Q1 and Q3 groups, compared with the Q2, are shown in Table 16.

The "Organismal systems", "Human diseases" and "Metabolism" categories reported the highest number of pathways. The presence of "Human diseases" pathways is likely to be the result of the presence of both genes included also in other pathways and a group of isolated genes in different expected pathways and subsequently included in humans.

The sub-categories "Purine metabolism" and "Xenobiotics biodegradation and metabolism" showed the highest number of represented genes within the "Metabolism" category, while within the "Organismal systems" category "T cell receptor signaling pathway" and "Vascular smooth muscle contraction" were reported. Regarding this category has to be noted that 5 out of 10 sub-categories belong to the "Immune system", with "T cell receptor signaling" and "B cell receptor signaling" and "Hematopoietic cell lineage" pathways being represented.

The "Cellular processes" category showed three pathways, with "Regulation of actin cytoskeleton" and "Focal adhesion" as the richest of represented genes, with 8 and 6 genes, respectively.

Table 15: KEGG pathways represented in Italian Holstein (Farm C) cows plus (HQ3) and minus (HQ1) variant for milk protein EBV in comparison with the cows belonging to the intermediate EBVp values quartile (HQ2)

Category	Sub-category	Pathway	Term	n of genes	Genes	Fold Enrichment
Metabolism	Nucleotide metabolism	Purine metabolism	bta00230	7	ADCY8, NME1, ADK, NME7, GUCY2C, CANT1, ADCY9	2.5
	Xenobiotics biodegradation and metabolism	Metabolism of xenobiotics by cytochrome P450	bta00980	5	ALDH3B1, GSTM3, CYP1A2, ADH4, GSTO1	6.5
	Lipid metabolism	Fatty acid metabolism (degradation)	bta00071	4	ACSL1, ACOX3, CYP4A11, ADH4	5.1
	Metabolism of cofactors and vitamins	Retinol metabolism	bta00830	4	CYP4A11, CYP1A2, ADH4, LRAT	4.8
	Lipid metabolism	Linoleic acid metabolism	bta00591	3	PLA2G4B, CYP1A2, ALOX15	7.6
	Amino acid metabolism	Tryptophan metabolism	bta00380	3	ACMSD, CYP1A2, RPS6KA4	3.8
	Lipid metabolism	Arachidonic acid metabolism	bta00590	3	PLA2G4B, CYP4A11, ALOX15	3.0
Cellular processes	Cell motility	Regulation of actin cytoskeleton	bta04810	8	PDGFRA, ITGA1, VAV1, PFN2, NDUFA9, BAIAP2, FGF16, ITGB6	2.5
	Focal adhesion	Focal adhesion	bta04510	6	PDGFRA, ITGA1, VAV1, NDUFA9, CCND1, ITGB6	2.1
	Cell communication	Gap junction	bta04540	3	ADCY8, PDGFRA, ADCY9	2.2
Organismal systems	Immune system	T cell receptor signaling pathway	bta04660	5	VAV1, CD3G, RELA, MALT1, CD4	2.8
	Circulatory system	Vascular smooth muscle contraction	bta04270	5	PLA2G4B, RPLP0, CYP4A11, ADCY9, MYL6	2.8

	Immune system	Hematopoietic cell lineage	bta04640	4	ITGA1, CD1D, CD3G, CD4	3.1
	Endocrine system	Melanogenesis	bta04916	4	ADCY8, GNAO1, POMC, ADCY9	2.2
	Immune system	Cytosolic DNA-sensing pathway	bta04623	3	IRF7, TMEM173, RELA	3.4
	Immune system	B cell receptor signaling pathway	bta04662	3	VAV1, RELA, MALT1	2.7
	Endocrine system	Adipocytokine signaling pathway	bta04920	3	ACSL1, POMC, RELA	2.7
	Immune system	RIG-I-like receptor signaling pathway	bta04622	3	IRF7, TMEM173, RELA	2.6
	Endocrine system	PPAR signaling pathway	bta03320	3	ACSL1, ACOX3, CYP4A11	2.2
	Endocrine system	GnRH signaling pathway	bta04912	3	PLAG4B, ADCY8, ADCY9	1.9
Human diseases	Cancer: overview	Pathways in cancer	bta05200	7	PDGFRA, CASP3, TRAF5, JUP, FGF16, RELA, CCND1	1.2
	Cardiovascular diseases	Arrhythmogenic right ventricular cardiomyopathy	bta05412	4	ITGA1, NDUFA9, JUP, ITGB6	5.1
	Cancer: specific types	Small cell lung cancer	bta05222	3	TRAF5, RELA, CCND1	2.2
	Cancer: specific types	Colorectal cancer	bta05210	3	PDGFRA, CASP3, CCND1	2.0
	Cancer: specific types	Prostate cancer	bta05215	3	PDGFRA, RELA, CCND1	2.0
	Cancer: specific types	Acute myeloid leukemia	bta05221	3	JUP, RELA, CCND1	3.0
	Cancer: specific types	Melanoma	bta05218	3	PDGFRA, FGF16, CCND1	2.5
	Cardiovascular diseases	Dilated cardiomyopathy	bta05414	4	ADCY8, ITGA1, ADCY9, ITGB6	3.7

Interpretation of KEGG pathways in the Italian Holstein cows group (Farm C)

The analysis of peripheral blood cells of healthy cows during the plateau of the lactation and diverging for EBVp represents an attempt to characterize from a genomic perspective the additive genetic effect obtained by the phenotypic selection.

Purine metabolism in ruminants is known to be the preferential way to utilize the rumen microbial source of nucleic acids and nitrogen. In this study some genes involved also in recycling products of purine metabolism were up-regulated in HQ3 and down-regulated in HQ1 group. In particular, the HQ3 group showed the stimulation of some enzymes that catalyze the production of 3',5'-cyclic AMP, an important second messenger in the cells, through the up-regulation of adenilate cyclase 8 (ADCY8), nucleoside diphosfate kinase 1 and 7 (NME1, NME7) and adenosine kinase (ADK) genes. Furthermore, the same genes resulted under expressed in HQ1 cows, where the guanidine metabolism appeared to be stimulated toward the synthesis of purine derivative xanthine and subsequently to the urinary excretion of purines, with the up-regulation of a nucleoside diphosphate phosphatase (CANT1).

These findings seem to be consistent with the results of urinary purine derivatives nitrogen (Table 15), where for the IH a significant negative correlation with genetic merit for milk protein was shown, and with previous results reporting the better capability of highly productive cows to utilize purine and purine nitrogen [92,93]. Furthermore, due to the their role in the regulation of the hormone-mediated cellular network, 3',5'-cyclic AMP and GMP are present in many different cell types. An active role of these molecules on immune cells response and as intracellular mediator of inflammation has also been reported [94].

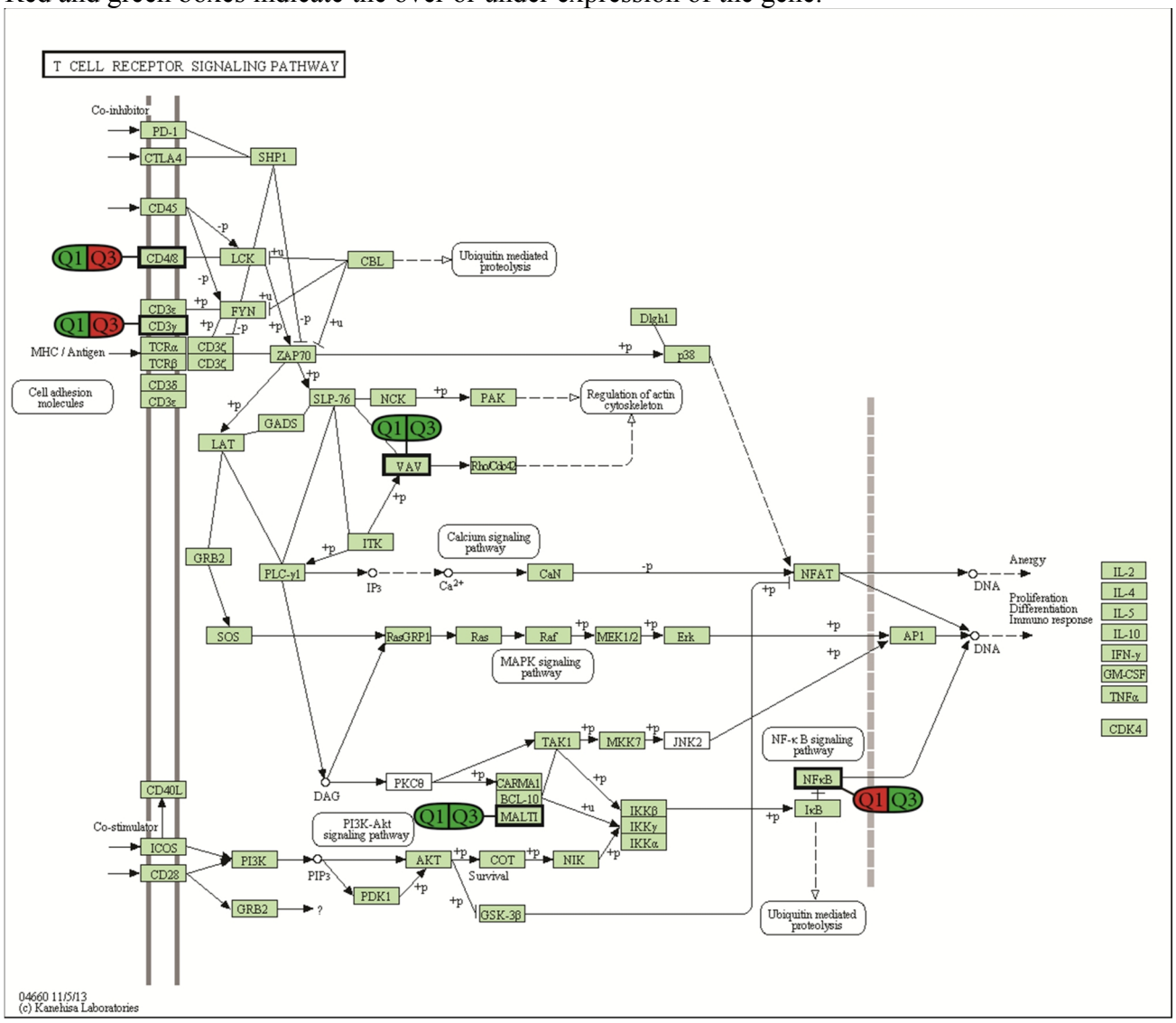
"Immune system" sub-category reports three pathway represented by the DEG of IF cows, "Hematopoietic cell lineage", "T cell receptor signaling pathway" and "B cell receptor signaling pathway". The Common lymphoid progenitors branch of the first pathway reported in the HQ3 group the over-expression of the CD1d, CD4 and CD3 antigens, all presented by Major Histocompatibility Complex to T cells in specific set of surface markers. Interestingly, CD3 and CD4 antigens have been reported to be expressed during *Staphilococcus Aureus* mastitis [95,96].

Both T and B cell receptor signaling pathways indicated also the presence of MALT1 and RelA genes, the first down-regulated either in HQ1 than in HQ3 group, while the latest showing a positive direction in HQ1 and negative in HQ3. Furthermore, CD4 and CD3 antigens were again present in T cell receptor pathway (Figure 13). In animal with no clinical or biochemical evidences of inflammation is rather difficult to find an exhaustive explanation to these data, but some considerations can be done. The genetic antagonism between milk yields and mastitis is well known [97-99], as well as the phenotypic association between *Staphylococcus Aureus* with high SCC and especially with subclinical mastitis through chronic infections [100]. Since at the sampling time there were no evidences of mastitis or high SSC (SSC<200,000/ml), the over-expression of CD3 and CD4 antigens in the high production healthy cows could be explained as a previous exposure or a more efficient reaction, to a previous exposure to the pathogen agent.

RelA, coding for the p65 member of the NFkB transcription factor and MALT1, coding for paracaspase, are both involved in NFkB activation, particularly in T cells [101]. The discrepancy showed in the regulation of these genes is difficult to interpret, especially considering the high complexity of NFkB regulation and the clinical and physiological condition of these animals.

We can suppose that in the HQ1 the increase of RelA expression, downstream to MALT1 in the “T cell signaling pathway”, indicates that a different inflammatory condition was present at the time of sampling. However, any further conclusion would be misleading.

Figure 13. T cells receptor signaling pathway (bta04660, KEGG). Highlighted are the genes differentially expressed in Italian Holstein SQ1 (Q1) and SQ3 (Q3) compared with the Q2 quartile. Red and green boxes indicate the over or under expression of the gene.



NFkB = RelA: p65 NFkB subunit

In our study ACSL1, member of the acyl-CoA synthetase long-chain fatty acids family involved in milk TGs synthesis, was present in "Adipocytokine", "PPAR" and "Fatty acids metabolism" pathways. Higher rate of lipolysis can provide essential nutrients, fatty acids and glycerol, for milk fat synthesis in the mammary gland or for energy supplying via β-oxidation in hepatocyte mitochondria.

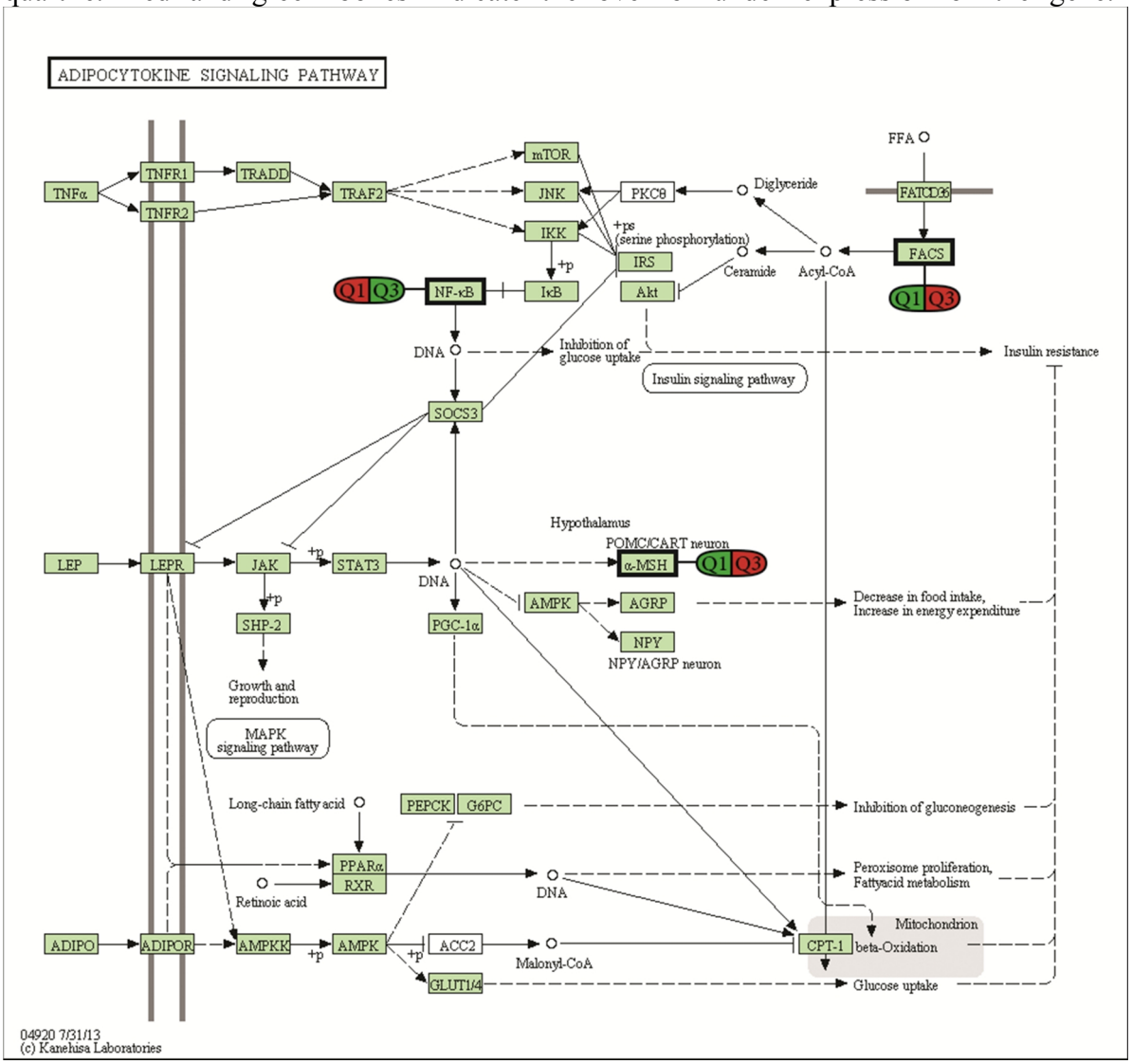
The expression of this gene was greater in HQ3 and lower in HQ1 compared with HQ2 group (Figure 14). Bionaz and Loor (2008) [102] have previously reported isoform 1 of ACSL as predominantly expressed in mammary tissue at the onset and during lactation, confirming the active role of this enzyme in fatty acids metabolism. This result is consistent considering the productive performances of IH, where a significant and positive effect of EBVp on milk fat yield was observed (Table 17). In addition, it is to note that B-BOH resulted negatively correlated with EBVp in IH ($P<0.01$), indicating that in high genetic merit cows FAs β-oxidation is predominant. This may confirm the better energy efficiency in FAs utilization of high genetic merit cows.

The differences in POMC expression ($P<0.05$) across the EBVp groups (Figure 14) could be explained as the different regulation of appetite stimulus in animal with different metabolic efficiency. Is well recognized the anorexigenic effect POMC-derived α-MSH peptide (α-melanocyte stimulating hormone). This effect has been reported for both free-feeding and fasted rodents [103,104] as well as for postpartum lactating cows fed a high energy diet compared to a control diet [105]. In our study, blood was sampled around 1 hour after the morning meal, so we could interpret the down-regulation of POMC in HQ1 as a "delayed" activation of the hormonal-metabolic mechanism of feed intake regulation. Hence, it is reasonable to consider that one of the main effect of the higher EBVp can be an "early" satiety signaling, due a

better utilization of feed nutrients. This can be in part confirmed by the negative effect ($P<0.05$) of EBVp on blood BOHB in IH (Table 14).

PPAR signaling pathway is related to the expression of ACOX3 and CYP4A11 genes, beyond to ACSL1. Again, the lipid metabolism is the target of these genes, in particular for fatty acids transport and oxidation. CYPA4A11 oxidoreductase regulation was the same as ACSL1, while ACOX3 unexpectedly showed an opposite regulation, with over expression in HQ1 and under expression in HQ3 (Figure 14). Nonetheless, we can conclude that HQ3 gene expression regulation shows an overall better utilization of nutrients, in particular of lipids and fatty acids, and a catabolic asset of the metabolism when compared to the animals with lower EBVp.

Figure 14. Adipocytokine signaling pathway (bta04920, KEGG). Highlighted are the genes differentially expressed in Italian Holstein SQ1 (Q1) and SQ3 (Q3) compared with the Q2 quartile. Red and green boxes indicate the over or under expression of the gene.



FACS = ACSL1 : acyl-CoA synthetase long-chain family member 1; NFkB = RelA : v-rel reticuloendotheliosis viral oncogene homolog A, avian gene; α-MSH = POMC : pro-opiomelanocortin gene

Table 17. Effects of breeds, DIM and genetic merit (EBVp) on BCS score, milk yield and its composition, somatic cell count, urea and cortisol contents in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 5 commercial farms in North-East of Italy

Items		Breeds		Effects					SEM
		IF	IS	Breed	Farm	Covariates			
						DIM	EBVpIH	EBVpIS	
BCS	Score	2.75	3.14	0.000	0.000	+0.002*	-0.003	-0.008*	0.029
Milk yield	kg/d	35.34	30.98	0.000	0.000	-0.058**	+0.101**	+0.229**	4.984
Fat	%	3.79	3.78	0.601	0.048	+0.003	+0.002	-0.008	0.064
Fat	kg/d	1.33	1.16	0.007		-0.001*	+0.004*	+0.006*	0.024
Protein	%	3.12	3.49	0.000	0.000	+0.002**	+0.005**	+0.002	0.020
Protein	kg/d	1.10	1.08	0.685	0.000	-0.001*	+0.005**	+0.008**	0.016
SCC	Count	4.22	5.05	0.000	0.000	+0.012**	+0.001	-0.017	0.130
Urea	mmol/l	17.10	23.30	0.000	0.000	-0.014	-0.060	+0.053	3.669
BOHB	mmol/l	0.20	0.23	0.000	0.000	+0.003	+0.000	+0.000	0.002
Cortisol	pmol/l	1.01	0.88	0.548	0.034	-0.002	+0.003	+0.000	0.042

IH : Italian Holstein; IS : Italian Simmental; BCS : Body Condition Score; DIM : Days In Milk; EBVpIH : Estimated Breeding Values for milk protein of Italian Holstein cows; EBVpIS : Estimated Breeding Values for protein of Italian Simmental cows; BOHB : beta-hydroxy-butyrate. * indicates a significant effect for $P<0.05$; ** indicates a significant effect for $P<0.001$. Signs indicate the relationship, positive or negative, between the parameter and the effect.

CONCLUSIONS

The study aimed at investigating the gene expression in blood cells and the metagenome composition in the rumen content of dairy cows of Italian Simmental and Italian Holstein breeds differing for estimated breeding value for milk protein.

At the best of our knowledge, this approach has never been used and a direct comparison with published studies is not straight full, but some preliminary considerations from the data gathered from our study can be drawn.

As expected, the blood and urinary constituents, obtained from a larger investigation on 4 commercial farms on 135 cows, indicates a different biological response between the cows of the two breeds and the driving role that genetic merit for milk protein can exert on energy and protein utilizations.

Despite of the limited number of cows included in the study, the rumen metagenomic data obtained from a massive parallel HTS indicate that microbial population is significantly affected by genetic merit other than breed. This support the hypothesis that rumen microbiome is an integrated component of the whole organism, interplaying with the host to support the nutrients required to satisfy the different metabolic efficiency related to the genetic merit.

The relationship between EBVp and biological response was further investigated by analyzing the differential gene expression in blood sample of a subset of cows by means of microarray. The number and the entity of up and down-expressed genes was not as large as when as the effect of a treatment is studied. In our research, the EBVp class was the only inferred variables and cows were healthy and in the middle stage of lactation. Nonetheless, the results suggest that specific metabolic and immunitary pathways are directly related to the genetic merit, and can be considered a signature of the quantitative selection.

Starting from this preliminary observations, the approach is promising since it can identified phenotypic biomarkers than can be used for genomic selection, base on differentially expressed genes more than on DNA variants.

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