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What we have lost: Mastitis resistance in Holstein Friesians and in a local cattle breed

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Abstract

In Holstein Friesian dairy cows, selective pressure for increased milk production has led to a higher propensity to disease, including mastitis, when compared to less selected and lower producing dairy breeds. The biology underpinning the higher resistance to disease of such "local breeds" is not fully understood. With the aim of investigating the factors associated to this phenomenon, we applied a multidisciplinary approach to compare innate immune response patterns, metabolic parameters, milk protein profiles and the milk microbiota in Holstein Friesian and Rendena cows reared in the same farm and under the same management conditions. Quarter milk samples and blood plasma were collected from all cows at dry-off, 1 day after calving, 7–10 days after calving and 30 days after calving. Quarter milk samples were subjected to bacteriological culture, characterization of the milk microbiota by 16S metagenomics, milk protein profiling by electrophoresis and densitometry, somatic cell counting, measurement of the inflammation marker cathelicidin and assessment of different innate immune-related mediators such as lysozyme, CD45, IL-1β, TNF-α, PTX3, IL-1R8. In parallel, the main inflammometabolic parameters were measured in blood plasma samples. Despite having relatively few animals (6 moderate-yielding Holstein Friesian and 4 low-yielding Rendena) some important differences were apparent. Holstein Friesian cows showed a more severe fat mobilization and systemic inflammatory response postpartum in comparison with Rendena cows, which had a greater postpartum muscle mass and an increased amino acid mobilization compared to Holstein Friesians. Upon bacteriological analysis, contagious bacteria such as Staphylococcus aureus and Streptococcus agalactiae were absent, but significant differences were seen in the general composition of the milk microbiota of the two breeds. Concerning the milk protein abundance profile,

pronounced differences were seen in colostrum, with significantly higher amounts of immunoglobulins and other immune-related proteins in Rendena. Added to this, the expression of innate immune related genes such as PTX3, IL-1β, TNF-α, and KRT5 expression in milk epithelial and leukocyte cell components, respectively, was lower in Holstein Friesian colostrum compared with Rendena. In conclusion, several differences were observed in the two breeds, in spite of the same farming conditions. The observations reported in this work present numerous pointers to the factors that may provide autochthonous, more rustic breeds with a higher resistance to disease.

Introduction

The achievement of high production levels in dairy farms poses significant challenges which translate into increased culling rates, reduction of life expectancy, more frequent occurrence of variegated and multifactorial diseases and increased use of veterinary drugs. According to the technical report of the Italian Breeders Association (AIA), the average milk yield in 1,095,576 lactating Italian Holstein Friesian (HF) cows amounted to 9325 kg in 2015, with average contents of 3.67% and 3.25% for fat and protein, respectively. The impact of these performances on animal welfare and health has been considerable. The genetic ability to increase milk production seen during the last years has been associated with a higher risk of metabolic and infectious diseases, as well as with reduced fertility. However, less is known about the biological mechanisms behind these relationships (Oltenacu and Broom, 2010). In keeping with this, cows alive in North-Eastern USA at 48 months of age decreased from 80% in 1957 to 13% in 2002; in the same farms and in the same period the mean calving interval went from 13 to 15.5 months (Oltenacu and Broom, 2010). In the period around calving, high-yielding dairy cattle are probably more susceptible to common environmental stressors in terms of housing, hygiene and feeding conditions. This may have crucial repercussions on disease occurrence early in life and on the subsequent milk production levels (Bach, 2011).

In agreement with the above data, a large meta-analysis study (Ingvartsen et al., 2003) demonstrated an unfavorable genetic correlation between milk yield and incidence of mastitis, and to a lesser extent of ketosis, ovarian cysts, and lameness. Therefore, a high correlation can be demonstrated between metabolic stress in high-yielding dairy cattle and mastitis occurrence. The highest peak of new intramammary infections (IMI) is usually recorded in the first 2–3 weeks after calving (Green et al., 2002), which partly accounts for the highest culling rates in the first 2 months of lactation (Pinedo et al., 2010). This is in contrast with the low prevalence of clinical mastitis in some autochthonous cattle breeds, such as Rendena (REN) (Curone et al., 2016; Gandini et al., 2007). This breed is native of the Rendena Valley in Northeastern Italy (Trentino), but it can now be found in many areas of Northern Italy, and on account of its small size is particularly suitable to pasture-based production systems. Rendena is a rustic and doublepurpose animal, mainly used for milk production, that yields around 5000 kg per lactation, with an average content of 3.4% and 3.3% of fat and protein, respectively (ANARE, 2012). The first calving usually occurs around the 30^{th} month of age and the average number of inseminations is 3.3 with approximately 106 calving to pregnancy "open" days (ANARE, 2012; Mazza et al., 2014).

Inevitably, defense against pathogens invading the mammary gland requires a local response. Bovine mammary epithelial cells (bMECs) lining the inner surface of the mammary gland are a crucial part of this. bMECs constitute a physical barrier and produce several antimicrobial substances and inflammatory mediators such as tumor necrosis factoralpha (TNF-α), interleukin-1 beta (IL-1β), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-8 (IL-8), regulated on activation normal T cell expressed and secreted (RANTES), lactoferrin, serum amyloid A, and cyclooxygenase-2 (Zbinden et al., 2014). Whenever bMECs fail to control intramammary infections, granulocytes go into action and typical clinical signs of mastitis can be detected.

The above observations raise several fundamental questions: what underlies mastitis resistance in local breeds? How can we effectively investigate it? Most importantly, can we define markers of innate immune response in the mammary gland discriminating mastitis resistant dairy cattle? These issues make a case for an ad hoc experimental study, in which high and low-yielding dairy cows are reared under the same conditions and carefully compared for fundamental parameters of the innate immune response to mastitis pathogens. To this aim, we identified a farm meeting these requirements, where a balanced number of healthy HF and REN dairy cows were kept under the same environmental and farming conditions, and we applied a multidisciplinary approach to compare their innate immune response patterns, metabolic parameters, milk protein profiles and milk microbiota composition. The study was encouraged by local and regional Italian projects for the valorization of autochthonous breeds.

2. Materials and Methods

2.1. Animals

We collected samples from 6 HF and 4 REN cows housed in the same farm in Pavia (Italy) under the supervision of expert bovine practitioners. Cows were housed with a tie-stall housing system and were milked using a pipeline milking system twice daily. No dry cow therapy was used. Cows were fed ad libitum a total mixed ration without silage using alfalfa hay, straw and concentrated feed with mineral and vitamin supplementation. All the cows remained clinically healthy throughout the study duration, and no signs of disease (mastitis, laminitis, endometritis and metabolic disorders) were observed. The cows were all between 2 and 4 lactations, with an average of 3.6 for HF and 2.7 for REN. The average milk yield was significantly higher in HF compared to REN (HF = 5366 kg vs REN = 3769; $p = 0.0147$). The percentage of milk fat (HF = 3.52% vs REN = 3.37%) and protein (HF = 3.02% vs REN = 3.08%) content was comparable in the two breeds.

This study complied with Italian laws on animal experimentation and ethics (Italian Health Ministry authorization n. 628/2016-PR).

2.2. Samples

Quarter milk samples and venous blood were collected from each animal at the following time points: dry-off (T1), 1 day after calving (T2), 7–10 days after calving (T3) and 30 days after calving (T4). Blood (10 ml) was collected from the tail vein in an EDTA-vacutainer at each time point and centrifuged for 25 min at 840g at 4 °C. Plasma was collected and immediately stored at −20 °C until use. Before milk sampling, teat ends were carefully cleaned. First streams of foremilk were discharged, and then approximately 150 ml of milk was collected aseptically from each quarter into sterile vials. Samples were delivered to the laboratory at 4 °C and immediately processed. Milk (50 ml) was centrifuged for 10 min at 840g at 4 °C. The fat layer (milk fat globules, MFG) was collected in 2 ml sterile tubes with 1.5 ml TRI Reagent and vortexed for 2 min. Fat samples were stored at −80 °C until RNA extraction. When the remaining fat layer was completely removed, 1 ml of skim milk was transferred to a 1.5 ml sterile tube and immediately stored at −20 °C for lysozyme determination. The cell pellet was washed in PBS with 0.25 mM EDTA, centrifuged for 10 min at 470g at 4 °C and finally lysed in 2–4 ml of TRI Reagent and stored at −80 °C until use. The rest of the milk was stored in 3 aliquots for proteomic analysis (50 ml), metagenomics analysis (15 ml) at −80 °C and for bacteriological analysis and somatic cell count (SCC; 10 ml) at −20 °C.

2.3. Plasma metabolites

Plasma metabolites were analyzed at 37 °C by an automated clinical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA), using the methodologies previously reported (Calamari et al., 2016). Commercial kits were used to measure glucose, total cholesterol, urea, inorganic phosphorus, total protein, albumin, total bilirubin, aspartate aminotransferase (GOT), γ-glutamyltransferase (GGT), and creatinine (Instrumentation Laboratory SpA, Werfen, Monza, Milan,

Italy), NEFA (Wako, Chemicals GmbH, Neuss, Germany), β-OH-butyric acid (BHBA, kit Ranbut, Randox Laboratories Limited, Crumlin, County Antrim, UK), thiol groups (SHp, Kit from Diacron srl, Grosseto, Italy). The ferric reducing antioxidant power (FRAP) assay was assessed by adapting the colorimetric method of Benzie and Strain (Benzie and Strain, 1996) to the clinical auto-analyzer ILAB 650 (Instrumentation Laboratory, Lexington, MA).

2.4. Bacteriological analysis and somatic cell counts

For bacteriological analysis, quarter milk samples were allowed to thaw at room temperature. 10 μl of milk was plated onto blood agar plates containing 5% defibrinated bovine blood. Plates were incubated aerobically at 37 °C and evaluated after 24 and 48 h. Bacteria were identified according to the guidelines of National Mastitis Council (1999). For each quarter, SCC was determined by an automated fluorescent microscopic somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN).

2.5. Metagenomic analysis

Five-ml milk samples from each quarter were centrifuged at 500g for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended with one ml of saline solution (NaCl 0.9%) and centrifuged at 500g for 5 min at 4 °C. The supernatant was discarded and the bacterial DNA was extracted from the samples as described previously (Cremonesi et al., 2006). DNA quality and quantity was analyzed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was stored at −20 °C until use.

Bacterial DNA was amplified using the primers described in literature (Caporaso et al., 2011), which target the V3–V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25-μl volumes per sample. A total of 12.5 μl of Thermo Master Mix 2× (Thermo Scientific) and 0.2 μl of each primer (100 μM) were added to 2 μl of genomic DNA (5 ng/μl). A first amplification step was performed in an Applied Biosystem 2700 thermal cycler, as follows: samples

were denatured at 98 °C for 30 s, followed by 25 cycles with a denaturing step at 98 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplicons were cleaned-up with Agencourt® AMPure® XP (Beckman, Coulter Brea, CA) and the libraries were prepared following the 16S Metagenomic Sequencing Library Preparation protocol (http:// supportres.illumina.com/documents/documentation/chemistry_ documentation/16s/16s-metagenomic-library-prep-guide-15044223-b. pdf, Illumina). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (KapaBiosystems, Inc. MA, United States) pooled in equimolar proportion and sequenced in one Miseq (Illumina) run with 300-base paired-end reads.

After sample loading, the MiSeq system provides on-instrument secondary analysis by the MiSeq Reporter software (MSR) classifying organisms from V3 and V4 amplicon using a database of 16S rRNA data. The classification is based on the Greengenes database (http:// greengenes.lbl.gov/) and the output of this workflow was a classification of reads at several taxonomic levels (kingdom, phylum, class, order, family, genus, and species). Alpha diversities were calculated according to different microbial diversity metrics (i.e.: Shannon index, observed species).

2.6. Cathelicidin and lysozyme determination

Cathelicidin was assessed by a pan-cathelicidin sandwich ELISA as described previously (Addis et al., 2016a; Addis et al., 2016b). For enabling logarithmic visualization, a correction factor of 0.1 was added to OD450 values to obtain the adjusted OD450 values (AOD450). Lysozyme in samples of fat and cell-free bovine milk was assessed by the lyso-plate assay as previously described (Osserman and Lawlor, 1966), but in this case the reaction was carried out at 37 °C, for 18 h, in a humidified incubator.

2.7. Electrophoretic and densitometric analysis of milk proteins

The proteins corresponding to 0.1 μl of each milk sample were separated by reducing SDS PAGE using precast Any kD polyacrylamide gels (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Gels were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA) and digitalized with an ImageScanner III (GE Healthcare, Little Chalfont, UK). Band intensities were compared with the Quantity One 1-D analysis software (Bio-Rad) as described previously (Ghisaura et al., 2016; Salvatore et al., 2014). For defining peaks and calculating relative peak abundances, band areas were estimated using Origin-Pro 8 SR0 software (OriginLab Corporation, One Roundhouse Plaza, Northampton, MA, USA). In order to specifically assess physiological differences between breeds, only samples having negative bacteriological culture, < 1000,000 cells/ml in colostrum (T2) and < 500,000 cells/ml in milk (T3) were included in the evaluation.

2.8. RNA extraction, reverse transcription and real-time PCR from milk fat globules and milk cells

Total RNA was isolated from MFG and milk cells by the TRIreagent (Sigma-Aldrich, St. Louis, MO, USA) protocol; total RNA was extracted according to the manufacturer's directions. The concentration of RNA was determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm wavelength. One μg of total RNA from all the samples was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's instructions. The cDNA obtained from each sample was used as a template for Real Time PCR in an optimized 25 μl reaction volume using Sybr Green chemicals, as previously described (Riva et al., 2010). The primer pairs were designed using the Primer Express Software (Applied Biosystem, Foster City,CA, USA) and purchased from Invitrogen (Carlsbad, CA, USA). Their sequences are listed in Table 1. Specific primers were also employed for bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene.

IL-1β, TNF-α, CD45 and KRT5 genes were investigated in milk cells, whereas PTX3 and IL-1R8 genes in MFG. A duplicate, no-template control (NTC) was included in each plate. Real-time

quantitative PCR was carried out in the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) as previously described (Riva et al., 2010). The expression of bovine target genes was normalized using the calculated GAPDH cDNA expression (mean) of the same sample and run. The relative quantification of each gene was calculated with the method of the "delta Ct" (Schmittgen and Livak, 2008). The value obtained was multiplied by "10,000" in order to obtain the Arbitrary Units.

2.9. Statistical analysis

Statistical analyses were performed using SPSS 23.0 for Windows (IBM, Armonk, NY, USA), GraphPad Prism 6 (La Jolla, CA, USA) and PROC MIXED of SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). For the statistical analysis of the bacteriological data, all quarter samples were included. For the statistical analysis of the metabolic parameters, all plasma samples were included. In order to analyze the milk parameters (SCC, cathelicidin, lysozyme, microbiota, gene expression of immune related genes) under physiological conditions, only healthy quarter samples were evaluated. The healthy quarter samples were defined as follows. For T1 and T2: negative bacteriological culture growth (udder pathogens) (given that at T1 and T2 an increase in SCC is typically observed, the SCC threshold was not applied to the dry off milk and colostrum samples) (McDonald and Anderson, 1981a, 1981b); for T3 and T4: SCC < 200,000 cells/ml (threshold used in high yielding HF cows as a reliable indicator of mastitis) (Bradley and Green, 2005) and negative bacteriological culture growth. Quarter samples showing microbial growth at T1 and T2 and SCC > 200,000 cells/ml and/or positive bacteriological culture growth at T3 and T4, were identified as suspected IMI or subclinical mastitis samples, and were analyzed separately for the gene expression of immune related genes. Student's t-test was used to compare HF and REN gene expression and cathelicidin at each time point. A P value ≤ 0.05 was considered statistically significant. Plasma inflammometabolic parameters and lysozyme were analyzed using a pairwise comparison. The model for the time-course data analysis

contained breed, day, and their interactions as fixed effects, and cow nested within breed as random effect. A spatial power (SP = POW) covariance structure was used. The pairwise comparison was made using least significant difference (LSD) test. Differences were considered significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

3. Results

3.1. Different metabolic pathways are evident in Holstein Friesian and Rendena cows

We investigated different metabolic parameters in plasma samples. Results are plotted in Fig. 1. No differences were observed between HF and REN at any time point in the following parameters: glucose, cholesterol, urea, phosphorus, ceruloplasmin, albumin, AST/GOT and GGT (data not shown). In both breeds NEFA increased after calving, but in HF the peak was more marked at T2 and T3 ($P < 0.01$), suggesting a more severe fat mobilization. The BOHB increased after calving only in HF, and the difference with REN reached the maximum at T3 ($P < 0.01$). Conversely, REN showed a higher concentration of plasma creatinine in comparison with HF ($P < 0.05$) for the whole period. HF showed a more severe systemic inflammatory response in comparison with REN in terms of positive acute phase markers (haptoglobin, total proteins, and globulins) mainly at the beginning of lactation at T2 and T3. Also total bilirubin was higher in HF vs. REN after calving ($P < 0.10$ at T2), but in this case the difference between the breeds was already evident before parturition and remained so during lactation. REN showed a higher concentration of thiol groups (index of the sulfhydryl compounds, SHp) in comparison to HF for the whole period, except for T3. On the other hand, HF showed a significant increase of FRAP (index of total antioxidant capacity) at T3 ($P < 0.05$ vs REN).

3.2. Healthy Holstein Friesian milk has higher levels of cathelicidin and higher SCC than healthy Rendena milk

Udder health was defined at the quarter level. Contagious pathogens (Staphylococcus aureus and Streptococcus agalactiae) were never detected. At T1 and T2 HF cows presented a lower percentage of positive bacterial culture results (33.3% and 33.3%, respectively) when compared to REN (40.0% and 41.7%, respectively), while at T3 and T4 we observed the opposite, with a higher percentage of samples with positive bacterial culture in HF (20.8% and 29.2%, respectively) vs REN (12.5% and 12.5%, respectively). The most common microorganisms isolated in milk were environmental streptococci and Coagulase-negative staphylococci (CNS).

All healthy quarter milk samples from both breeds were evaluated for SCC and cathelicidin abundance as mastitis markers. An increase in SCC is typically considered as a trustworthy indication of mastitis, with a threshold of 200,000 cells/ml (Bradley and Green, 2005), and we have recently demonstrated that the measurement of milk cathelicidin can reliably indicate mastitis with better diagnostic performances than SCC (Addis et al., 2016a; Addis et al., 2016b). Therefore, both markers were evaluated in this work. Results were assessed separately for all lactation time points (Fig. 2). Table 2 reports the respective median and interquartile range (IQR) values. The median and IQR values of both SCC and cathelicidin were higher in HF vs REN milk at all times. As expected, the values of both markers were lowest in T3 and T4 and highest in T2. In the case of SCC, only the HF T2 median was above the 200,000 cells/ml diagnostic threshold (Fig. 2). In the case of cathelicidin, the T2 of both breeds had median values above the threshold, again with higher values for HF. In T3 and T4, 75% of the result distribution was always below threshold for both markers (Fig. 2).

3.3. The microbiota biodiversity in Rendena milk is lower than in Holstein Friesian milk

A more detailed knowledge of the healthy milk microbial communities and their interactions in physiological conditions might provide useful information on the factors influencing milk quality and udder health. Therefore, a NGS approach based on 16 S metagenomics was applied to the milk of HF

and REN during the peripartum period. Results were assessed by pooling all lactation time points together.

Based on the alpha-diversity (Fig. 3) according to the Shannon Index and observed species, a lower biodiversity was present in REN milk when compared to HF milk. At the phylum level, REN milk was dominated by Firmicutes (94%, relative abundance), while HF milk contained Firmicutes (65%), Proteobacteria (15%), Actinobacteria (11%) and Bacteriodetes (6%) (Fig. 4A). At the genus level, REN milk showed the predominance of Streptococcus (71%), followed by Lactobacillus (10%) and Pediococcus (6%), while HF milk was dominated by Streptococcus (29%), followed by Lactobacillus (6%), Corynebacterium and Staphylococcus (4%) (Fig. 4B and C). Among streptococci, Streptococcus thermophilus was the most prevalent (48%) in REN milk, in comparison with only 2% in HF (Fig. 4D and E).

3.4. The protein profile of colostrum is different in the two breeds

For estimating milk protein profiles at T2, T3 and T4, the same volume of each sample (0.1 μl) was subjected to SDS-PAGE and densitometric analysis. In the case of T2 (Fig. 5A), the mean pixel density value was 5 Mpx for FH and 7.3 Mpx for REN, respectively, indicating a higher protein concentration in milk of the latter breed. The SDS-PAGE profile (Fig. 5A) showed numerous differences according to the breed, as reflected in the corresponding densitogram analysis (Table 3). In some cases, these differences were quite relevant, such as for peaks a, e and g, all having molecular weight ranges corresponding to immunoglobulin components. Specifically, in the MW range of band a (200 kDa) we have previously identified a component of IgM (Pisanu et al., 2012), while bands e and g migrate in the MW range of the Ig heavy and light chain (55 and 26 kDa), respectively (Pisanu et al., 2012; Salvatore et al., 2014; Thomas et al., 2016). A higher abundance of band c, corresponding to the MW range of bovine lactoferrin, was also seen in REN milk, together with other minor bands. On the other hand, the protein profiles of T3 and T4 (Fig. 5B and Table 3) were more similar in the

two breeds. At these time points, the mean pixel density values were of 4.3 Mpx for HF and 4.6 Mpx for REN, respectively, indicating similar milk protein concentrations in the two breeds (Fig. 5B). Peak area ratios were also comparable. Nevertheless, differences in the banding pattern at the MW range of caseins (Fig. 5B, group f) could still be observed, as well as differences in the shape, and therefore in relative composition, of the four peaks included in this group.

3.5. Mammary innate immune response patterns are different in the two breeds

In order to gain a more detailed picture on the differences between the two breeds in terms of innate immune response, lysozyme concentration and gene expression pattern of some key regulators were evaluated in milk. HF showed a significant increase in lysozyme at T2 when compared to T1 ($P = 0.0014$). In addition, lysozyme showed a peak at T2 in REN. Interestingly, such an increase at T2 was on average much higher in REN cows, albeit with higher internal variability (Fig. 6). At T1 we observed a significant difference between the two breeds in terms of lysozyme concentration in skim milk ($P = 0.0141$; Fig. 6).

By real time PCR, the expression pattern of PTX3 and IL-1R8 was assessed in the MFG, representative of the mammary epithelium (Addis et al., 2011; McLellan et al., 2011), whereas the expression pattern of IL-1β, TNFα, CD45 and KRT5 was assessed in milk cells. As shown in Fig. 7(A) PTX3 showed no differences in expression levels between the two breeds for all time points, except for the colostrum (T2), at which REN showed a trend ($P < 0.1$) of increased levels. Although the cows remained clinically healthy throughout the experiment, there were occasions when bacterial challenge was suspected on the basis of high SCC milk samples (IMI/subclinical mastitis). Interestingly, HF seemed to be unable to up-regulate PTX3 during such presumed infection, whereas REN significantly up-regulated PTX3 ($P < 0.0001$; Fig. 7B).

Moreover, PTX3 expression level was significantly lower in suspected IMI/subclinical mastitis samples from HF compared to REN ($P = 0.0283$; Fig. 7B). Non-significant (NS), but consistently

higher expression was seen for IL-1R8 in REN at all times when compared to HF. The same NS trend was evident in suspected IMI/subclinical mastitis samples in which HF presented lower expression of IL-1R8 (Fig. 7D). This receptor, as expected, was slightly down-modulated in suspected IMI/subclinical mastitis samples when compared to healthy milk samples, in both breeds (Fig. 7D) (Riva et al., 2012). Proinflammatory cytokines (IL-1β and TNFα) presented lower expression levels in REN milk cells compared to HF at all time points apart from colostrum (T2), where REN samples showed higher cytokine gene expression levels (TNF $P = 0.005$; Fig. 8A). In suspected IMI/subclinical mastitis samples, proinflammatory cytokines were more expressed in HF than in REN (Fig. 8B).

In order to investigate the cell types present in milk samples, we analyzed the gene expression of CD45 (leukocytes) and KTR5 (epithelial cells). CD45 did not present any difference in expression levels between the two breeds except for the dry off period (T1; $P = 0.0037$), when HF showed higher levels than REN (Fig. 8A and B). KRT5 seemed to be slightly up-regulated in REN vs HF milk cells (except at dry off, T1), whereas the opposite was observed in suspected IMI/subclinical mastitis samples, as shown in Fig. 8B. Interestingly, the CD45/KRT5 expression ratio was always lower in REN vs HF cows at all times (Fig. 8A). During infection (in suspected IMI/subclinical mastitis samples), the CD45/KRT5 ratio was higher in REN vs HF (Fig. 8B).

4. Discussion

Autochthonous and lower-yielding dairy cattle breeds are known to possess higher resistance to disease and resilience to intensive farming conditions when compared to high-yielding, highly selected dairy breeds such as HF, especially concerning udder health and metabolic disorders in the peripartum period (Curone et al., 2016; Gandini et al., 2007). The availability of a small group of HF and REN cows reared in the same farm and under the same conditions prompted us to carry out a

multidisciplinary study aimed to investigate the traits that may underline these differences in disease susceptibility. Taken together, the results presented here suggest that HF cows seem to develop a greater systemic and local (in the mammary gland) inflammatory condition compared to the autochthonous REN breed, although our findings will require validation on larger animal cohorts to allow for final conclusions.

Cows of both breeds showed the typical variations of inflammometabolic profile around calving previously described in healthy periparturient dairy cows (Bionaz et al., 2007; Trevisi et al., 2012; Trevisi et al., 2011). In particular, HF and REN cows showed a significant mobilization of body reserves, from the adipose tissue (peak of NEFA at T2) and from muscle tissue (peak of creatinine at T2) and the characteristic inflammatory response immediately after calving, as confirmed by the peak of haptoglobin at T2. The post-calving increase of globulin, total bilirubin and the similar variations of proteins produced by the liver (i.e. albumin, paraoxonase) around calving, suggest that both breeds elicited the same responses at the liver level (Bertoni et al., 2008; Bionaz et al., 2007). Nevertheless, all the above phenomena were more pronounced and prolonged in HF cows, confirming that cows with a high genetic merit for milk yield have a more severe lipomobilization and suffer a more marked inflammation after calving. Interestingly, REN cows at calving time (T2) showed also three other important differences in comparison to HF: absence of ketosis (the BOHB remained unchanged in the first week of lactation), higher muscle mass (the creatinine concentration was constantly higher in the whole period) and lower risk of oxidative stress (the concentration of thiol groups, SHp, was higher in late pregnancy and the total antioxidant concentration after calving, assessed as FRAP, was unchanged, for a lower endogenous production of these molecules). Thus REN cows are less susceptible to oxidative damage probably due to the lower production of free radicals consequent to a lower mobilization of body reserves, a better oxidation of the fatty acids in the liver, a shorter and less severe inflammatory response (Bertoni and Trevisi, 2013; Trevisi et al., 2010). Combining these

data with the lower milk production, we can conclude that REN cows do not exhibit marked negative energy status in the days immediately after calving.

Moreover, REN cows demonstrated the ability to maintain lower levels of the mastitis markers cathelicidin and SCC, and therefore of mammary tissue inflammation; this ability is of significant interest especially in the post-partum period and it appears to be combined with the capability of REN cows to release in colostrum a higher amount of other immune-related proteins, such as lysozyme, that efficiently protect the mammary gland against pathogen infections. Based on electrophoretic and densitometric analysis of colostrum, several bands associated to immunoglobulin components were present in consistently higher amounts in REN when compared to HF cows, but by 10 days after calving these differences had disappeared. In addition, the differences seen in the peak group around 30–35 kDa suggest that the relative abundance of caseins between the two breeds may also vary, prompting further investigations about its implications on cheesemaking properties (Perna et al., 2016).

On the basis of our strict inclusion criteria, fundamental and significant differences in the baseline profile of local innate immunity could be detected mainly in the colostral phase. With respect to HF cows, REN cows showed stronger innate immune responses in the mammary gland (in terms of proinflammatory cytokines, PTX3 and leukocyte/epithelial cell ratio) shortly after calving. These differences in colostrum samples were never accounted for by lower concentrations of SCC in HF cows. On the contrary, these frequently outnumbered the corresponding counts of DIM (days in milk)-matched REN breed cows. Most importantly, SCC in colostrum was shown to be significantly different, leukocytes being more predominant in HF compared with REN cows. These findings outline a new scenario, whereby leukocytes of HF cows are probably less efficient in the colostral phase compared with those of a mastitis-resistant, low-yielding cattle breed. The latter displayed a much stronger expression of inflammatory cytokine genes (IL1β and TNFα) and concentrations of

antimicrobial substances in colostrum (lysozyme and PTX3), despite an overwhelming majority of epithelial cells. This was in sharp contrast to HF cows, which showed very high leukocyte infiltrations in colostrum SCC. The observed hypofunctionality of colostrum leukocytes in HF cows had an important confirmation in a previous study, in which a dramatic up-regulation of innate immunity parameters could be demonstrated in colostrum of HF following the parenteral injection of a very low interleukin-2 dose (Zecconi et al., 2009). The cytokine apparently restored the previously suppressed innate immune response in the udder and caused a significant reduction of new cases of intramammary infections in the first 2 weeks after calving compared with control, untreated cows. These results outline a potential important role of immunomodulators early after calving, to raise local immunity to the level required to effectively face environmental and transmissible mastitis agents.

Among the parameters of innate immunity under study, the possible role of lysozyme should be properly highlighted. Beyond being a fundamental and ubiquitous antibacterial compound, lysozyme is involved in the regulation of the inflammatory response (Lee et al., 2009; Liu et al., 2006). HF cows consistently show much lower serum lysozyme levels compared with other cattle breeds (Trevisi et al., 2011), and concentrations outside the reference interval (1–3 μg/ml) are associated with an increased risk of disease occurrence (Amadori et al., 2015; Trevisi et al., 2012). Therefore, the much lower lysozyme response in the colostrum of HF cows should be considered as an important indicator of susceptibility to new intramammary infections, having in mind the crucial role of the early post calving period for the establishment of new intramammary infections in the herd (Fleischer et al., 2001). The above differences were shown to be restricted to the colostral (T2) phase and were no longer detectable later on.

Moreover, the milk microbiota of REN cows showed a lower biodiversity in terms of bacterial phyla, genera and species. This is likely to have important consequences on innate immunity in the mammary gland,

of pathogen colonization, degradation of xenobiotics and stimulation of both development and maintenance of the immune system (Addis et al., 2016). As previously described (Quigley et al., 2013), typical cow's milk contains a significant lactic acid bacterium population (LAB) that includes Lactococcus, Streptococcus, Leuconostoc and Enterococcus. Other microorganisms, such as Pseudomonas, Acinetobacter and Aeromonas spp., can be present with significantly different proportions. Microbiota can prevent pathogen colonization by competing for space and nutrients and exerting an outright amensalism, i.e. the production of substances (like lactic and short-chain fatty acids, hydrogen peroxide, orbacteriocins) toxic for other microbial species. This underlies the use of lactobacilli and other probiotics in the prophylaxis of farm animal diseases, including bovine mastitis (Espeche et al., 2012; Nader-Macías et al., 2008). Interestingly, REN milk samples showed a much higher prevalence of Streptococcus thermophilus. This bacterium is a thermophilic LAB prevalently used as a starter during the manufacture of dairy products and the second most important industrial dairy starter in the production of fermented milks, yogurts and many cheese types (Quigley et al., 2013). High concentration of Streptococcus thermophilus in REN milk therefore makes it more suitable to dairy processing. Moreover Streptococcus thermophilus might exert a protective function against mammary pathogens (Rigobelo et al., 2015).

The above findings can be viewed in a conceptual framework that includes the different data sets in coherent cause/effect relationships. The greater metabolic stress of HF cows and the related higher NEFA peak early after calving, as well as the higher inflammation and oxidative stress, could be the foundation of some crucial downstream processes affecting the profile of innate immunity in the mammary gland. In particular, high NEFA levels can affect the proliferation of lymphocytes and the

release of interferon-gamma and IgM after treatment with mitogens (Lacetera et al., 2004). Also, they severely depress both vitality and oxidative burst of bovine neutrophils (Scalia et al., 2006). NEFA can directly signal through Toll-like receptor (TLR) 4, being thus an important component of metabolic stress. This can be sensed by the innate immune system following tissue acidosis, osmolarity changes, hypoxia, reactive oxygen species (ROS) accumulation, altered ATP/AMP ratio, and shortage of amino acids (Amadori, 2016). Moreover, also the more severe inflammation in the weeks after having in mind that the microbiota is associated with inhibition calving, measured with higher concentration of positive acute phase proteins and mainly with lower concentration of negative acute phase proteins, has been linked to impairment of the immune system and to a reduction of performance of dairy cows (Bertoni et al., 2008; Trevisi et al., 2016; Trevisi et al., 2010). Therefore, it is conceivable that high NEFA levels and other poorly defined metabolic products after calving could inhibit in HF cows a badly needed local innate immune response in the colostral phase. This response is likely to prime the epithelia of the mammary gland to an effective and time-limited response to mastitis agents. In agreement with a previous study on innate immunity in the dry period, environmental stressors after dry-off could amplify such biological effects after calving by affecting the “memory” of the innate immune system (Amadori et al., 2015; Quintin et al., 2014; Trevisi et al., 2016). In the framework of such a response to metabolic stress, innate immunity in the mammary gland of HF cows would be less pronounced and effective in the colostral period and, probably, also poorly controlled later on, after exposure to mastitis pathogens. As a result, bMEC could fail to control the colonization of pathogenic bacteria; leukocytes would then go into action underlying the typical signs of subclinical and clinical mastitis cases. On the other hand, the lower expression in MFGs of IL-1R8, a negative regulator of TLRs and ILRs proinflammatory signaling, could explain the increased expression of proinflammatory cytokines in HF cows under resting condition at T1 and T4 (Riva et al., 2012). This steady proinflammatory response could exhaust the cells (leukocytes and

mammary epithelial cells), that would no longer be able to respond to a subsequent infectious challenge.

The above findings probably make a case for heterosis, or hybrid vigor, in “problem” herds, to be reappraised within rational crossbreeding systems with local, autochthonous breeds. Also, our findings indicate that fundamental effector activities of innate immunity in the mammary gland should be included in the breeding programs of HF cows, and given adequate priority by the scientific community. Therefore, immunostimulation in the colostral period could be a badly needed choice to effectively face mastitis pathogens in a period in which prevalence of new IMI is highest. This conclusion is strengthened by the contradictory results of the current S. aureus vaccines for dairy cattle, which question the very foundation of a vaccination strategy against mastitis agents (Scali et al., 2015). Immunostimulation shouldbe inserted in a wider strategy, aimed at minimizing the impact of the metabolic stress in the first DIM (Carbonneau et al., 2012), keeping high hygiene conditions and proper BCS levels in the dry period, providing satisfactory housing and feeding conditions during the lactation period and adopting protocols of early and predictive diagnosis of production diseases (Amadori et al., 2015; Bertoni and Trevisi, 2013; Trevisi et al., 2012). This kind of integrated approach could be conducive to a substantial reduction of antibiotic usage in dairy farms, based on active involvement of farmers, veterinary practitioners, dairy extension specialists and veterinary authorities towards better qualification of the food chains and improved consumers' awareness.

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Table 1. Oligonucleotide primer sequences for SYBR Green quantitative RT-polymerase chain reaction amplification.

Gene	Protein	Sequence	Gene bank gi-number
CD45 Trevisi et al. (2014)	Bovine membrane tyrosine phosphatase (CD45)	F: CTCGATGTTAAGCGAGAGGAAT R: TCTTCATCTTCCACGCAGTCTA	GI:9944227
GAPDH Trevisi et al. (2014)	Glyceraldehyde-3-phosphate dehydrogenase	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	GI:89573946
PTX3	Pentraxin 3	F: TCCATCCCACTGAGGACCC R:TCTCCAGCATGGTGAAGAGCT	GI:402691645
IL-1R8	Interleukin-1 receptor 8	F: TCCGGAACATCAGCTCCTCT R: CCGCCAGCCCAGCTC	GI:982972365
TNFα	Tumor necrosis factor alpha	F: TCTTCTCAAGCCTCAAGTAACAAGT R: CCATGAGGGCATTGGCATAC	GI:402693442
IL-1β	Interleukin-1 beta	F: GAGGAGCATCCTTTCATTCATC R:TTCCTCTCCTTGCACAAAGCTC	GI:27806570
KRT5	Bovine keratin 5	F: CAAGGTCCTGGACACCAAGT R: TCCAGCTGTCTCCTGAGGTT	GI: 56710316

Table 2. Medians and interquartile ranges (in parentheses) of SCC and cathelicidin in milk of the two breeds.

Milk sampling	HF			REN		
	N	SCC (cells/ ml)	Cathelicidin (AOD450)	N	SCC (cells/ml)	Cathelicidin (AOD450)
Whole lactation	88	78,000 (9000/ 290,500)	0.150 (0.100/ 0.380)	60	34,500 (1000/ 83,000)	0.110 (0.080/ 0.180)
Dry (T1)	24	184,000 (58,500/ 418,800)	0.215 (0.120/ 0.340)	20	71,000 (6500/ 148,500)	0.125 (0.100/ 0.207)
Colostrum (T2)	25	382,000 (130,000/ 1,173,000)	0.515 (0.263/ 1.258)	12	147,000 (57,250/ 394,300)	0.240 (0.152/ 0.722)
Mature (T3)	17	147,000 (5500/ 108,500)	0.100 (0.085/ 0.150)	14	41,000 (1000/ 48,000)	0.090 (0.067/ 0.140)
Mature (T4)	22	19,000 (4000/ 24000)	0.105 (0.080/ 0.130)	14	9000 (1000/ 15500)	0.080 (0.070/ 0.115)

Table 3. Densitogram results. Estimated MW, areas and area ratios for peaks/bands reported in Fig. 2. Areas are expressed as pixel intensity values.

Peak/band	Est. MW	Area - HF	Area - REN	REN/HF	HF/REN
			T2		
A	200	299.8	866.3	2.89	0.35
B	130	200.2	360.9	1.80	0.55
C	80	514.6	866.2	1.68	0.59
D	70	383.7	379.8	0.99	1.01
E	52	1084.5	2955.0	2.72	0.37
F	30–35	4711.8	3252.8	0.69	1.45
G	24	–	2172.5	–	–
H	16	1004.9	1312.3	1.31	0.77
I	12	575.4	473.9	0.82	1.21
J	4	292.7	367.7	0.80	1.26
			T3		
C	77	193.1	260.6	1.35	0.74
D	70	372.5	298.3	0.80	1.25
E	53	397.4	532.7	1.34	0.75
F	25–35	3727.0	3877.9	1.04	0.96
H	16	974.9	1006.8	1.03	0.97

I	12	500.9	511.7	1.02	0.98
J	4	261.6	344.9	1.32	0.76

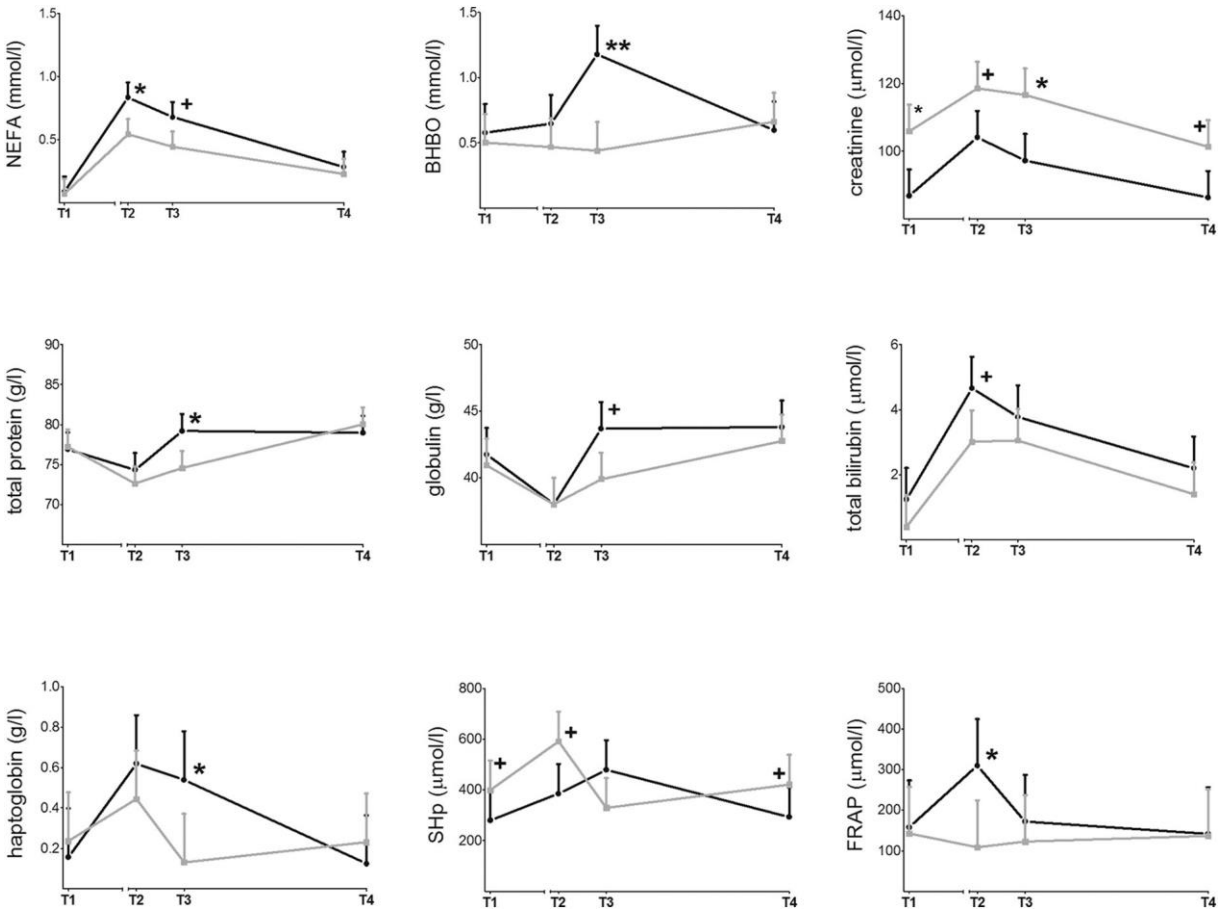

Fig. 1. Plasma concentration of metabolic markers. Plasma concentration of non-esterifiend fatty acid (NEFA), β-idrossi butirric acid (BHBO), creatinine, total protein, globulin, total bilirubin, haptoglobin, thiols groups (SHP) and total antioxidant (ferric reducing antioxidant power = FRAP) in Holstein Friesian (black line) and Rendena (grey line) cows during the dry period (T1), and at 1 (T2), 7–10 (T3) and 30 days (T4) after calving. Significance of differences between groups at each time point is indicated with: + for non-significant but $P < 0.1$, * for $P < 0.05$ and ** for $P < 0.01$.

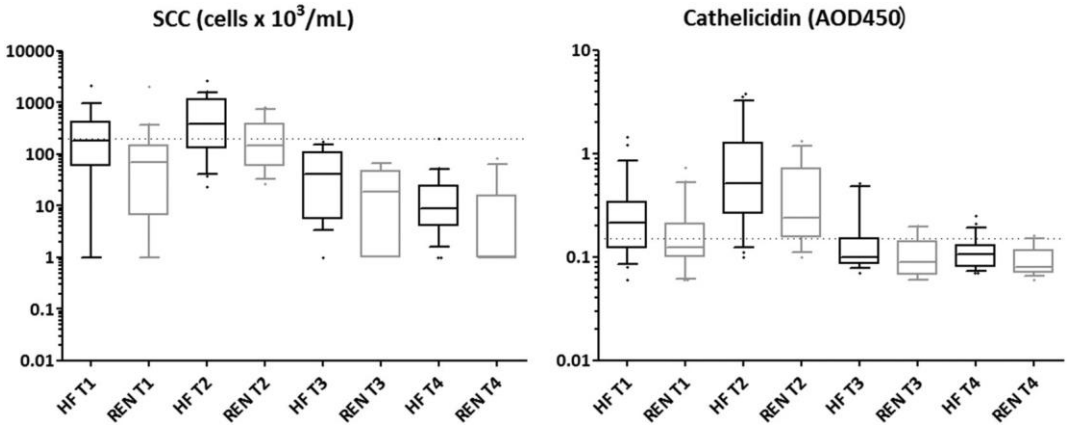

Fig. 2. Cathelicidin and SCC evaluation in colostrum and milk. Cathelicidin was measured by sandwich ELISA in colostral and milk quarter samples from 6 Holstein Friesian (HF) and 4 Rendena (REN) cows during the dry period (T1), and at 1 (T2), 7–10 (T3) and 30 days (T4) after calving; it is expressed as AOD 450. SCC was determined by an automated fluorescent microscopic somatic cell counter; it is expressed as 10^3 cells/ml.

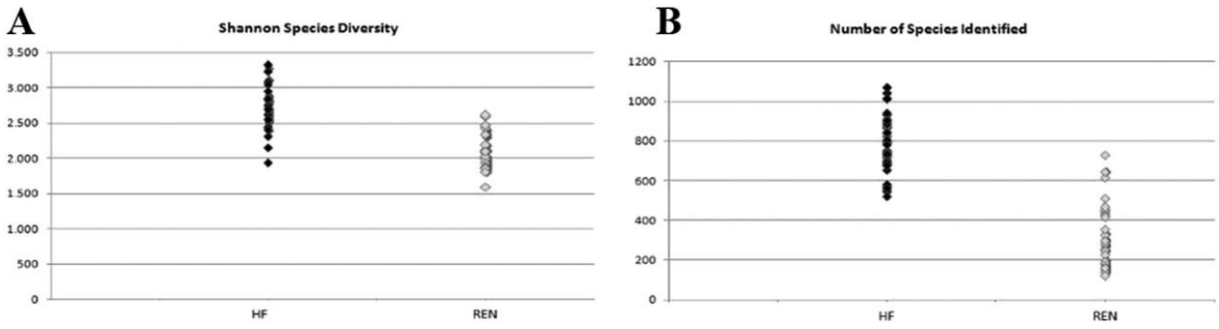

Fig. 3. Alpha diversities of the milk microbiome. Alpha diversities of the milk microbiome collected from Holstein Friesian (HF) and Rendena (REN) cows was calculated by Shannon index (A) and observed species (B).

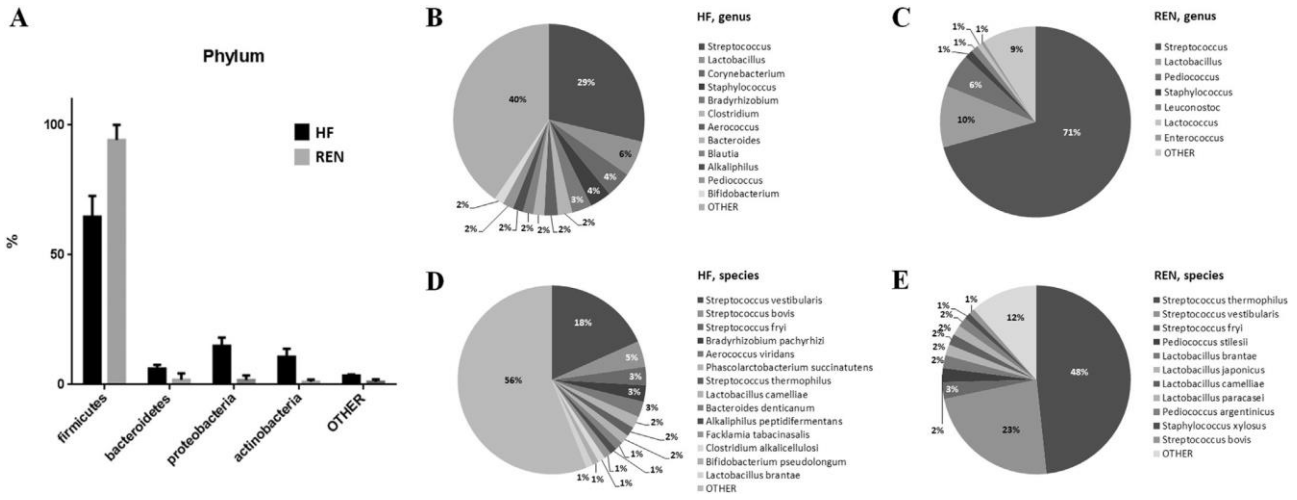

Fig. 4. Milk microbiome composition in Holstein Friesian (HF) and Rendena (REN). Relative abundance of the milk bacterial phyla (A). Pie charts summarizing taxonomy at the genus level (B: HF, C: REN) and at the species level (D: HF, E: REN).

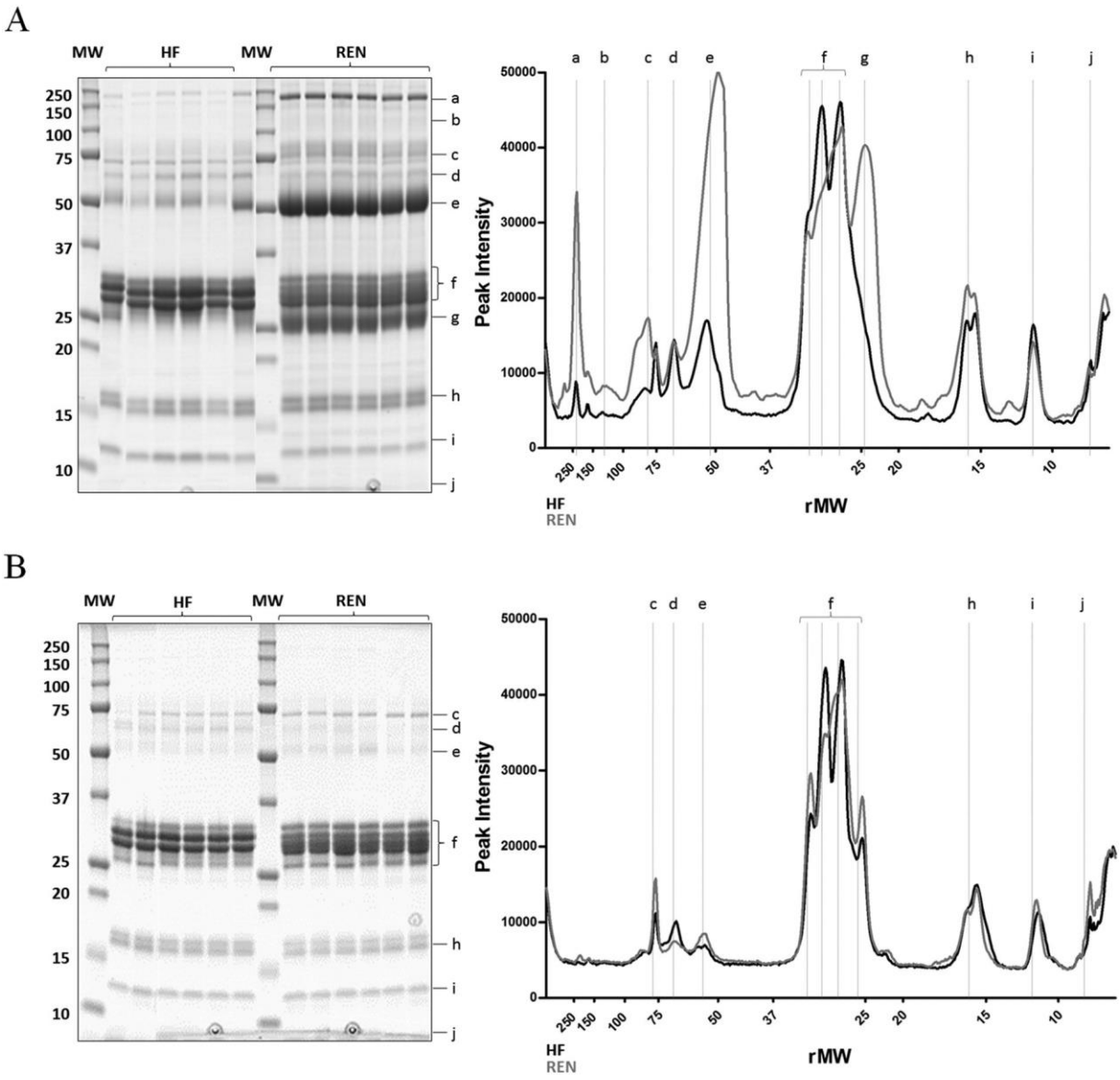

Fig. 5. SDS-PAGE and densitometry of milk samples at 1 day postpartum (T2:A) and 7–10 days postpartum (T3:B). Left: SDS-PAGE profile of milk samples; right: corresponding densitometric profile. The letters indicate the main electrophoretic bands and the corresponding peaks in the densitogram. MW: molecular weight. rMW: relative molecular weight. Black:

Holstein-Friesian; Grey: Rendena. HF: Holstein Friesian. REN: Rendena.

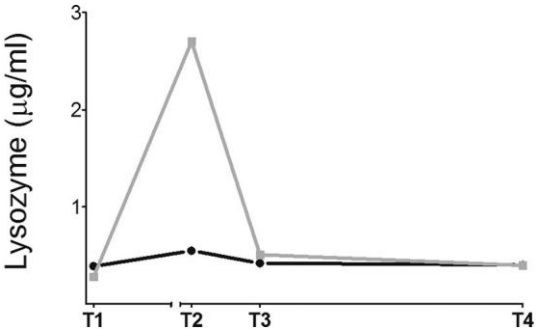

Fig. 6. Lysozyme concentration in colostrum and milk. Lysozyme concentration (µg/ml) was measured in colostral and milk quarter samples from 6 Holstein Friesian (black line) and 4 Rendena (grey line) cows during the dry period (T1), and at 1 (T2), 7–10 (T3) and 30 days (T4) after calving. *P < 0.05, **P < 0.01, ***P < 0.001.

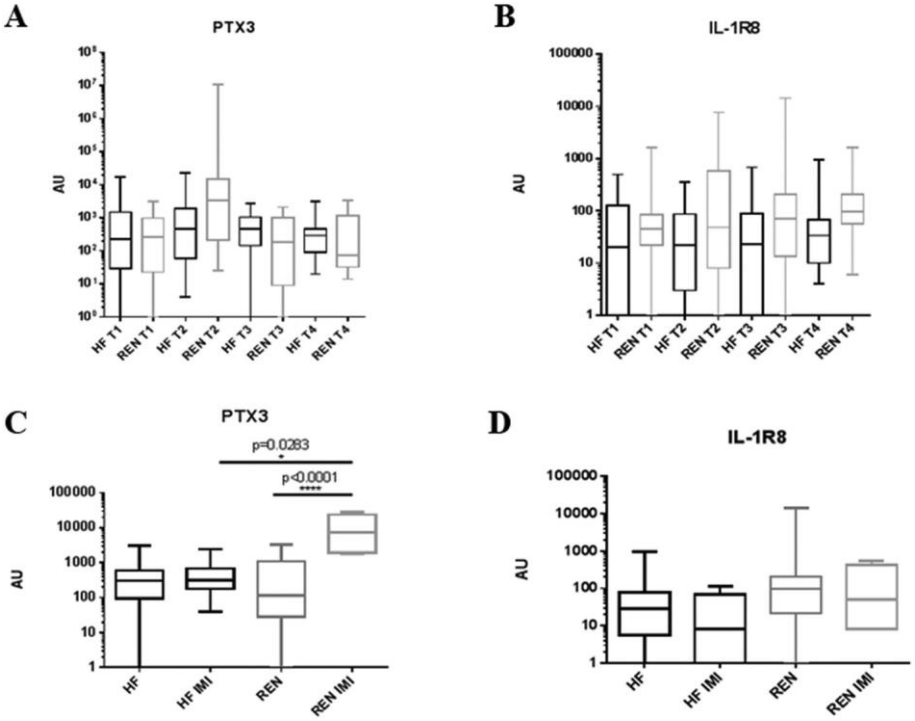

Fig. 7. Expression of IL-1R8 and PTX3 in MFG. PTX3 (A) and IL-1R8 (C) mRNA expression was analyzed by real time PCR in MFG of healthy quarter samples from 6 Holstein Friesian (HF) and 4 Rendena (REN) cows during the dry period (T1), and at 1 (T2), 7–10 (T3) and 30 days (T4) after calving. The gene expression

level of each target gene was normalized to GAPDH and the results are presented as arbitrary units. PTX3 (B) and IL-1R8 (D) mRNA expression was analyzed by Real Time PCR in MFG of suspected IMI/ subclinical mastitis quarter samples from HF and REN cows at T3 and T4. The gene expression level of each target gene was normalized to GAPDH and the results are presented as arbitrary units. *P < 0.05, **P < 0.01, ***P < 0.001.

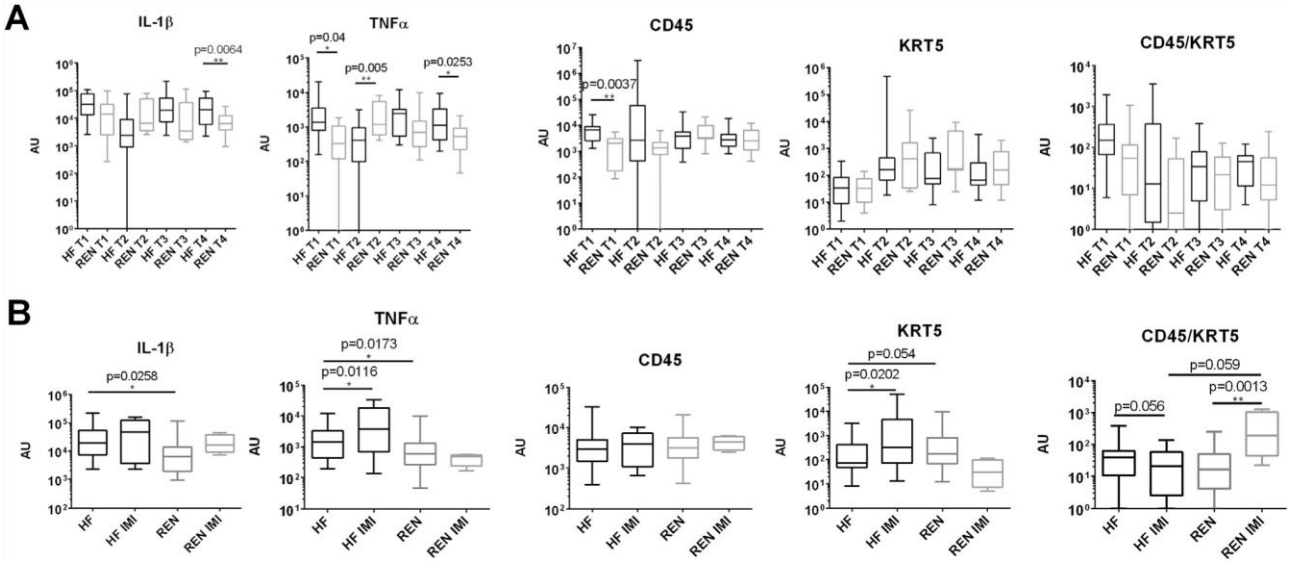

Fig. 8. Expression of IL-1β, TNFα, CD45 and KRT5 in milk cells. A) IL-1β, TNFα, CD45 and KRT5 mRNA expression was analyzed by Real Time PCR in isolated milk cells of healthy quarter samples from 6 HF and 4 REN cows at T1, T2, T3 and T4. The gene expression level of each target gene was normalized to GAPDH and the results are presented as Arbitrary Unit. CD45/KRT5 report the ratio of expression of the two messengers. B) IL-1β, TNFα, CD45 and KRT5 mRNA expression was analyzed by Real Time PCR in isolated milk cells of suspected IMI/subclinical mastitis quarter samples from HF and REN cows at T3 and T4 and compared to the expression of the healthy quarters at the same time points. The gene expression level of each target gene was normalized to GAPDH and the results are presented as Arbitrary Units. *P < 0.05, **P < 0.01, ***P < 0.001.