Accepted Manuscript

Innate immune responses to metabolic stress can be detected in rumen fluids

Erminio Trevisi, Federica Riva, Joel Fernando Soares Filipe, Matteo Massara, Andrea Minuti, Paolo Bani, Massimo Amadori

PII: S0034-5288(17)30686-0
DOI: doi:10.1016/j.rvsc.2017.11.008
Reference: YRVSC 3460

To appear in: *Research in Veterinary Science*

Received date: 22 June 2017
Revised date: 10 November 2017
Accepted date: 18 November 2017

Please cite this article as: Erminio Trevisi, Federica Riva, Joel Fernando Soares Filipe, Matteo Massara, Andrea Minuti, Paolo Bani, Massimo Amadori , Innate immune responses to metabolic stress can be detected in rumen fluids. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Yrvsc(2017), doi:10.1016/j.rvsc.2017.11.008

Innate immune responses to metabolic stress can be detected in rumen fluids

Erminio Trevisi[a], Federica Riva[b], Joel Fernando Soares Filipe[b], Matteo Massara[b,d], Andrea Minuti[a], Paolo Bani[a], Massimo Amadori[c]

a Istituto di Zootecnica, Facoltà di Scienze Agrarie, Alimentari ed Ambientali, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29122, Piacenza, Italy.

b Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, via Celoria 10, 20133, Milano, Italy.

c Laboratory of Cellular Immunology, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, via A. Bianchi 9, 25124, Brescia, Italy.

d Current affiliation of Dr. Massara: Laboratory of leukocytes biology, Humanitas Clinical and Research Center, via Manzoni 113, 20089, Rozzano (MI), Italy.

Corresponding Author: Federica Riva

Dipartimento di Medicina Veterinaria, Università degli Studi di Milano

Via Celoria 10, 20133, Milano, Italy

federica.riva@unimi.it

tel. +39-02-50318086

fax +39-02-50318089

Authors' emails: Erminio Trevisi (erminio.trevisi@unicatt.it), Joel Fernando Soares Filipe (joel.soares@unimi.it), Matteo Massara (matteo.massara@humanitasresearch.it), Andrea Minuti (andrea.minuti@unicatt.it), Paolo Bani (paolo.bani@unicatt.it), Massimo Amadori (massimo.amadori@izsler.it)

ABSTRACT

Many production diseases of dairy cows are related to digestive troubles. The rumen subacute acidosis is the most relevant one, albeit not easily recognized. Recent studies suggest that forestomachs can perform regulatory actions at both regional and systemic levels, since forestomach walls express immune receptors and cytokines, and the rumen liquor is infiltrated by leukocytes. Therefore, the rumen fluid could be conveniently collected for investigating metabolic production diseases. Thus, we investigated the origin of the leukocytes of the rumen fluid and demonstrated that they partly derive from saliva. Next, we carried out a field survey of innate immunity in rumen fluids of 128 cows from 12 dairy farms, along with clinical inspections, assessment of milk yield, rumen pH, volatile fatty acids (VFA) and major inflammo-metabolic parameters. Significant statistical correlations were found between immune markers in rumen fluids and biochemical parameters. A significant negative correlation was found in rumen between CD45 gene expression (leukocyte infiltration) and pH level. B cells were the most frequent mononuclear leukocyte population in the rumen liquor and their infiltration was negatively affected by low ruminal pH and high concentrations of VFA. Moreover, total Ig and IgM in rumen fluids were negatively correlated with ruminal pH and positively correlated with uremia.

Our data suggest that forestomach immune responses could be directed to "dangers" arising within the forestomach environment. The immune markers could integrate consolidated diagnostic parameters (e.g. rumen pH) and contribute to robust, early diagnosis of tricky digestive troubles of cattle.

Keywords: Dairy cow, forestomach, immune response, metabolic stress, rumen fluid, production disease.

INTRODUCTION

Production diseases of dairy cows include several pathologies, which mainly occur in the pregnancy to lactation transition period. This phase is characterized by a condition of negative energy balance, since high-yielding dairy cows are unable to achieve nutrient intake matching their high production requirements (Drackley, 1999). During this period also the rumen milieu is markedly challenged by the drastic changes of the diet necessary to cover the increased requirements for milk production, which alters the microbiota, the profile of fermentations and the epithelial permeability (Minuti e al., 2015). This is particularly relevant to genetically selected cattle breeds, which have to manage high production levels underlying an increased prevalence of diseases, as observed e.g. for ketosis, mastitis and lameness in Holstein Friesian cows (Ingvartsen et al., 2003). These findings should be viewed in the framework of a global, high susceptibility to infectious and non-infectious stressors (Trevisi et al., 2011), among which the environmental ones undoubtedly play a relevant negative role (Van Knegsel et al., 2014). Among factors that trigger metabolic diseases, the changes of feed quality and feed intake during the transition period are very important. In particular, the diet markedly affects saliva production (Davis et al., 1964), rumen functionality, extent and profile of rumen fermentations (Murphy et al., 2000), feed digestion (Knowlton et al., 1998) and feed passage rates (Allen, 1997). An excessive increase of fermentable carbohydrates in the diet induces a condition of acidosis, subacute (SARA) or chronic, in accordance with the duration of rumen pH values below 5.5-5.8 (Oetzel and Smith, 2000). Nevertheless, regardless of the cause, these conditions are definitely dangerous. Interestingly, although cows experiencing SARA do not often exhibit overt clinical symptoms, the affected animals show reduced/erratic feed intake, reduced rumination, mild diarrhea, undigested grain in the feces (Plaizier et al., 2008). Furthermore, SARA is implicated in the occurrence of laminitis (Enemark et al., 2002), poor body condition (Oetzel and Smith, 2000), ruminitis (Enemark, 2008), low milk fat syndrome and abomasal displacement (Olson, 1991). The diagnosis of SARA in the field is complicated as the clinical signs are neither evident (subclinical cases) nor pathognomonic

and the reliability of ruminal pH measures remains controversial (Penner et al., 2010; Plaizier et al., 2008; Trevisi et al., 2014b). Moreover, some of the signs attributed to SARA are dysfunctions common also to other digestive and metabolic disorders. These may affect the complex perception of satiety (Sartin et al., 2011), cause the release and absorption of immune-stimulating molecules such as lipopolysaccharides or histamine (Eckel and Ametaj, 2016; Minuti et al., 2014; Minuti et al., 2015; Nocek, 1997) and determine the modification of the microbiota (Minuti et al., 2015; Weimer, 2015; Yáñez-Ruiz et al., 2015). Because of this complexity, we have investigated the possible role of other factors involved in the forestomach functions. In a previous research (Trevisi et al., 2014a), we found that bovine forestomachs can potentially receive and process signals for the immune cells infiltrating the rumen content, suggesting that forestomachs are involved in a cross-talk with the lymphoid tissues in the oral cavity. Furthermore, Trevisi et al. (2009) observed that the inclusion of small amounts of human alpha interferon (1 - 10 IU/kg b.w.) in the diet increased the typical inflammatory response in periparturient cows. Thus, forestomachs can promote regulatory actions of the immune and endocrine systems at both regional and systemic levels. In this conceptual framework, an improved diagnostic approach is badly needed and could be actually based on assays for ruminal dismicrobism and related metabolic changes. These could be associated with local and systemic inflammatory conditions (Fernando et al., 2010; Tajima et al., 2000), caused by innate immune responses to metabolic stress (Amadori, 2016).

Owing to the above, our working hypothesis implied that ruminal fluids could be an important source of information related to abnormal fermentations and metabolites thereof, conveyed to the immune system during clinical and subclinical disorders, in agreement with our previous findings (Trevisi et al., 2014a). For this purpose, we investigated first the content of leukocytes in the saliva of dairy cows, to assess its contribution to leukocyte infiltration into the rumen fluid. A large field survey of ruminal, fecal and blood parameters was then performed in 12 dairy farms of similar genetic merit to verify the variability and the profile of leukocytes and immunoglobulins in the rumen and their relationship with other rumen characteristics, such as pH and volatile fatty acids.

MATERIALS AND METHODS

Saliva sampling and isolation of cells

Saliva was collected from 7 multiparous cows (4.0±1.7 calvings), including 4 lactating cows (324±78 days in milk) and 3 dry cows (about 30 days before expected calving), housed in an Experiment Station (Università Cattolica del Sacro Cuore, Piacenza Italy). Saliva samples were collected by inserting sterile gauze compresses into the mouth. After withdrawal, compresses were placed into a 5 ml pipet tip in a sterile 15 ml collection tube and centrifuged for 2 min at 1,000 x *g*. Saliva was then processed for flow cytometry and molecular analyses.

Samples were diluted 1:3 in cold and sterile PBS with 10% FCS. After centrifugation for 16 minutes at 500 x *g* at 5°C, the cell pellet was used for flow cytometry analyses or RNA extraction.

Animals and field sampling

This study was realized in 2013 and complied with Italian laws on animal experimentation and ethics enforced at that time (Legislative Decree 116/1992 enforcing EU Directive 86/609/EEC). Accordingly, the study was ratified by the Research Ethics Committee of Università Cattolica del Sacro Cuore, Piacenza Italy (Act 25906/13 of 22 November 2013).

We collected samples in different farms located in Lombardy and Emilia-Romagna Regions (Italy), under the supervision of the Italian National Veterinary Services. All the animals under study were clinically inspected at sampling times and in the month before the survey. All the samples analyzed in this study were processed in the lab within 4 hours after collection.

Rumen fluids were collected by rumenocentesis as previously described (Trevisi et al., 2014b) about 6 hours after total mixed ration (TMR) feeding, from 128 dairy cows of 12 herds, half of them being in the 30-90 and half in the 150-250 days in milk (DIM) period. Due to the limited amount of rumen fluid collected with this technique, it was not possible to analyze all the parameters described

hereunder in the samples. The pH of the rumen fluids was measured immediately after withdrawal by a pH meter (GLP 21; Crison Instruments SA, Alella, Barcelona, Spain). An aliquot of the remaining fluid was immediately cooled in ice water and centrifuged at 3,000 × g for 10 min at 10°C; 2-mL aliquots of the supernatant were transferred into tubes with 1 mL of 0.12 M oxalic acid and frozen at -20°C for later gas-chromatographic analysis of volatile fatty acids (VFA), according to methods described in Ahmed et al. (Ahmed et al., 2013) and Minuti et al. (Minuti et al., 2014). An additional aliquot of ruminal fluids was deep-frozen at -80°C after addition of 10% dimethyl sulfoxide (DMSO) and 40% (final) Fetal Calf Serum (FCS) for flow cytometry and molecular assays.

At the same time, blood samples were collected from the jugular vein in vacutainer tubes containing lithium heparin (Vacutainer; Becton Dickinson, Plymouth, UK), and immediately cooled in ice water. A small amount of blood was used for packed cell volume determination (Centrifugette 4203; ALC International Srl, Cologno Monzese, Italy); the remainder was centrifuged at 3,500 × g for 15 min at 6°C, and the plasma fraction was frozen (-20°C) for subsequent tests.

Fecal samples were collected from rectal ampulla for pH and VFA analyses. Milk Somatic Cell Counts, body condition score according to a 5-points scale (Agricultural Development and Advisory Service, Alnwick, UK), milk yield and composition were evaluated throughout the field study.

Processing of ruminal fluids for flow cytometry and molecular assays

Ruminal fluid leukocytes were isolated as previously described (Trevisi et al., 2014b). Briefly, frozen samples in 10% DMSO and 40% FCS (see above) were thawed at 38 °C and immediately placed on ice. Samples were diluted 1:3 with sterile PBS (without Ca++ and Mg++). Then, non-bacterial mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (density 1.083; Sigma-Aldrich Co, St. Louis, MO, USA) at 1,500 x *g* for 20 minutes at 20 °C. The harvested cells

on top of the separating medium were diluted 1:2 with sterile PBS and placed on ice. Cells were then pelleted (441 x g, 10 min, 5 °C), resuspended in 2 ml of PBS and centrifuged again through a 1- ml FCS layer at 300 x *g* for 10 minutes at 5 °C. The supernatant, containing bacteria, was discarded and the pellet (containing an overwhelming majority of protozoa and a minor part of leukocytes and detached rumen wall cells) was used for flow cytometry experiments or RNA extraction.

Flow cytometry

Ex vivo cells (from saliva and rumen fluid), obtained as described above, were resuspended in PBS with 2% FCS and 0.1% sodium azide (PBS-FCS-A) and reacted in aliquots (30 min, 4 °C) with monoclonal antibodies recognizing different bovine leukocyte populations.

Saliva cells were reacted with PBS-FCS-A (negative control) and anti-bovine CD45 (mAb 151, pan-leukocyte marker, kindly donated by Dr. Ch. Mackay, former member of Basel Institute for Immunology), respectively; mAb 151 has not been published, however its reactivity is identical to previously published anti-CD45 mAbs such as 1-11-32 (Maddox et al., 1985). Ruminal cells were reacted with PBS-FCS-A (negative control) and the following panel of monoclonal antibodies to leukocyte surface markers: anti-bovine CD45 mAb 151; anti-MHC I mAb W6/32 monomorphic epitope (Fayen et al., 1998); IL-A30, anti-bovine sIgM (B cell-specific) (Naessens et al., 1988); IL-A24 (myeloid lineage-specific, granulocytes and monocytes) (Ellis et al., 1987); IL-A12, anti-bovine CD4 (Baldwin et al., 1986); IL-A29 (WC1 marker of gamma delta T cells) (Howard et al., 1991); IL-A51, anti-bovine CD8 (Howard et al., 1991). After 30 minutes at 4°C, cells were washed and reacted with a fluorescein isothiocyanate-conjugated goat $F(Ab)_2$ anti-mouse IgG (ThermoFisher, code A24513, heavy + light chains) and analyzed in a GUAVA Easy Cyte HT flow cytometer (Merck Millipore, Darmstadt, Germany) using Incyte software. After setting a gate including viable leukocyte cells in a forward scatter/side scatter cytogram, the prevalence of the different leukocyte populations in 10,000 events (including the large majority of protozoa and

rumen wall cells) was established on the basis of the threshold of non-specific fluorescence defined by the negative control. The intra-assay coefficient of variation (CV) values amounted to 1.3, 0.4 and 1.6% for green fluorescence, FSC intensity and SSC intensity, respectively. The inter-assay CV values amounted to 0.9, 0.3 and 2.6% for green fluorescence, FSC intensity and SSC intensity, respectively.

RNA extraction from ruminal fluid cells

The cell pellets obtained from saliva and ruminal fluids were lysed in 0.5 ml TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA); total RNA was extracted according to the manufacturer's directions. The concentration and quality of RNA (integrity) was determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 and 280 nm wavelengths.

Reverse transcription and Real Time PCR

Total RNA from saliva and ruminal fluid cells 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). Saliva samples were analyzed for gene expression of CD45 (Leukocyte Common Antigen), IGLC (immunoglobulin light chain, lambda gene cluster; B cells) and KRT5 (keratin 5; rumen epithelial cells). Ruminal samples were analyzed only for the expression of CD45 gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was investigated as housekeeping gene. Primer pair sequences are listed in Table I. The primers were purchased from Invitrogen (Carlsbad, CA, USA).

A duplicate, no-template control (NTC) and a positive control (rumen wall cDNA), were also included in each plate. Real Time quantitative PCR was carried out in the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), as previously described (Riva et al., 2010).

Each sample was amplified by Real Time PCR in duplicate. 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 using the formula ΔCt=Ct (target gene) – Ct (housekeeping), where Ct (cycle of threshold) values were the mean of two test replicates. The obtained values were multiplied by 10,000 in order to obtain the test Arbitrary Units. Gene expression levels thereby obtained are not directly proportional to the number of cells in the samples, but mainly depend on the level of expression of the target gene in a specific cell population, which may widely vary. In order to check the specificity of the primer pairs, representative amplicons from some rumen fluid and rumen wall cDNAs were purified and sequenced. The sequences obtained were 97-99% homologue to the respective GeneBank sequences (Table I). Moreover, efficiency of the PCR assay was shown to be 94.7% for the CD45 (slope = -3.456), 97.8% for the IGLC (slope=-3.375), 81 % for the KRT5 (slope=-3.88) and 94.4% for the GAPDH (slope = -3,463) primer pairs.

Assays for total Ig and IgM in ruminal fluids

IgM and total Ig were measured in ruminal fluids by means of two distinct sandwich ELISA reactions, based on mAb 1C11/1C11-HRP to bovine IgM (μ chain-specific) and 6D4/6D4-HRP to bovine IgG light chains (pan), respectively (IZSLER test method 13/035, ISO/IEC 17025 accreditation). Monoclonal antibodies and ELISA tests were developed at IZSLER (Capucci L., Brocchi E., unpublished results). Briefly, distinct wells of NUNC Maxisorb ELISA plates (Thermo Scientific, Waltham, Massachusetts, USA) were coated overnight at 4°C with the two catcher mAb 1C11 and 6D4 at 2 μg/ml, respectively, in 0.05 M carbonate-bicarbonate buffer pH 9,6. Excess mAbs were removed by flicking off and plates were washed thrice for 5 minutes with PBS + Tween 20 0.05%. Clarified ruminal fluid samples (18407 x *g*, 10 minutes, 5°C) were diluted 1:4 with PBS/1% yeast extract/ 0.05% Tween 20, and 60 μl of these dilutions were added to duplicate wells for both IgM and total Ig assays. Plates were incubated at 37 °C for 1 hour and washed thrice for 5

minutes with PBS + Tween 20 0.05%. Next, the tracer mAbs (1C11-HRP and 6D4-HRP for IgM and IgG, respectively) in PBS/1% yeast extract/ 0.05% Tween 20 were added at pre-established optimal dilutions (1:2,000 and 1:500, respectively), and plates were again incubated at 37 °C for 1 hour. After three, 5 minutes-washings with PBS + Tween 20 0.05%, 50 µl/well of color substrate (o-phenylenediamine dihydrochloride 0.5 mg/ml + H_2O_2 0.02%) was added and plates were incubated at room temperature for 15 minutes. The color reaction was stopped by adding 50 µl/well of H_2SO_4 2N and plates were read spectrophotometrically at 492 in an ELISA reader (EL808, BioTek Instruments, Winooski, VT, USA). IgM and total Ig concentrations were determined from a standard curves created with serial dilutions of a bovine reference serum (Bethyl Laboratories, cat. RS10-10) with known concentrations of IgM, IgA and IgG antibody isotypes. The intra-assay CV values amounted to 5.0 and 6.4% for IgM and total Ig assays, respectively. The inter-assay CV values amounted to 6.5 and 5.2% for IgM and total Ig assays, respectively.

Inflammometabolic assays

Plasma samples were analyzed for metabolic indicators (glucose, total cholesterol, creatinine, urea, aspartate aminotransferase or GOT, γ-glutamyl transpeptidase or GGT, alkaline phosphatase or ALP, non-esterified fatty acids or NEFA, β-hydroxybutyrate or BHBA), selected minerals (Ca, P, Mg, K, Na, Cl, Zn) and oxidative-inflammatory indicators: total protein, albumin, globulin, total bilirubin, haptoglobin, ceruloplasmin, reactive oxygen metabolites (ROMs), paraoxonase (PON), nitrates (NO_3), nitrites (NO_2), nitric oxide metabolites (NOx).

Plasma metabolites were analyzed by an automated biochemistry analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA, USA). Total protein, albumin, total cholesterol, total bilirubin, triglycerides, creatinine, urea, Ca, P, Mg, GOT, GGT, ALP were determined using kits purchased from Instrumentation Laboratory (Lexington, MA, USA). Plasma globulin was calculated as the difference between total protein and albumin, thus including fibrinogen, too. Ions (K, Na and Cl) were measured by a potentiometric method (ion-selective electrode connected to

ILAB 650). Zn, NEFA and BHBA were measured by methods previously reported (Calamari et al., 2016). Haptoglobin and ceruloplasmin were analyzed using methods described by Skinner et al., (Skinner et al., 1991) and Sunderman and Nomoto (Sunderman and Nomoto, 1970), respectively, adapted to the ILAB 650 conditions. PON was measured by the method of Ferré et al., (Ferré et al., 2002) adapted to ILAB 650 apparatus, as previously described (Bionaz et al., 2007). ROMs were measured by a commercial kit (Diacron International s.r.l., Grosseto, Italy). NO_3, NO_2, and NOx metabolites were measured using the Griess test according to Gilliam et al. (Gilliam et al., 1993) and Bouchard et al. (Bouchard et al., 1999). The inter- and intra-assay CV were 3.76 and 1.13% for ROM, 4.63 and 0.46% for NO2, and 6.46 and 2.32% for NOx. NO3 was calculated by difference from NOx and NO2.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5, GraphPad Software (La Jolla, CA, USA). Due to the small amount of rumen liquor collected with rumenocentesis, it was not possible to perform flow cytometry, Real Time PCR and immunoglobulin analyses on all the samples. In particular, out of 128 cows sampled in 12 farms, Real Time PCR was performed on 98 animals, flow cytometry analyses on 93 animals and immunoglobulin tests on 77 animals. All the parameters were investigated in 37 animals only. Statistical tests were used to reveal correlations (bivarial linear correlations) between CD45 gene expression and each of the other parameters investigated, as well as correlations between each leukocyte population (flow cytometry analysis) or ruminal immunoglobulin concentration versus each of the other parameters. Given that all the immunological parameters of the rumen liquor did not show a normal distribution (Shapiro-Wilk test), they were computed with the non-parametric two-tails rho test of Spearman with $\alpha=0.05$. Finally, partial correlation analysis was performed on variables showing a strong, statistically significant bivariate correlation. Tendencies were declared with $P< 0.1$

RESULTS

Rumen leukocytes partly derive from saliva

In order to confirm our hypothesis that rumen fluid leukocytes could partly derive from saliva, we collected saliva samples from 7 healthy multiparous cows, including 4 lactating and 3 dry cows. Cells were isolated from saliva and tested for leukocyte prevalence. Three samples were analyzed by flow cytometry, as shown in Figure 1A. The average number of total live cells was 6.9×10^3 cells/ml of saliva, with a range between 2.9×10^3 and 1.1×10^4 cells/ml and a median value of 6.4×10^3 cells/ml. The average prevalence of CD45+ (leukocytes) cells was 7.9 %, corresponding to an average number of 4.28×10^2 cells/ml of saliva, with a range between 3.6×10^2 and 4.8×10^2 cells/ml and a median value of 4.4×10^2 cells/ml. Four other saliva samples were analyzed by Real Time PCR. All of them expressed CD45 (leukocytes), KRT5 (epithelial cells) and IGLC (B cells) genes (Figure 1B), indicating that saliva cells include both epithelial cells and leukocytes. Among leukocytes we demonstrated the presence of B cells, given the expression of the IGLC gene.

Animal health and production levels

All the cows under study did not present detectable health disorders at sampling times and in the month before. On the contrary, there was a high prevalence in some herds of metabolic disorders and reproductive troubles. The latter were not considered because detailed and comprehensive farm records were not available. Instead, somatic cell counts and milk production data were obtained and reciprocal correlations were investigated. According to rumen pH measures, some herds included animals under SARA conditions. Yet, a thorough analysis of our records revealed no significant relationships between ruminal subacute acidosis ($pH < 5.6$); (Gozho et al., 2005) and reduced milk yield and/or high somatic cell counts in the 12 farms under study. On the contrary, herds with lower mean ruminal pH tended to have higher VFA concentrations and higher milk yields in the two lactation periods under study ($P < 0.1$; data partially anticipated in Trevisi et al., 2014b). In addition, farms at risk of SARA had significantly lower Zn and cholesterol plasma concentrations ($P < 0.05$).

CD45 gene expression in rumen fluid samples

CD45 gene expression in ruminal samples was assessed as leukocyte marker in 98 animals from 11 farms by Real Time PCR. CD45 showed peculiar, farm-specific patterns of expression. As shown in Figure 2, the farms could be divided in two groups: farms with a compact pattern of CD45 gene expression, such as farm 7, 8, 9, 11 and 12 (low average values and standard deviations), and farms with a diffuse CD45 gene expression pattern among their animals, such as farm 2, 3, 4, 5 and 6 (high average values and standard deviations). Interestingly, farms with a compact, more homogeneous pattern of CD45 gene expression showed lower levels of rumen pH. Indeed, we observed in the same samples a significant positive correlation between CD45 gene expression levels and ruminal pH ($p=0.007$) (Table II), in agreement with our previous study on rumen-fistulated heifers (Trevisi et al., 2014a). CD45 gene expression was negatively correlated with the prevalence of IL-A24+ myeloid cells ($p=0.042$) and positively with the levels of total immunoglobulins ($p=0.009$) in the rumen liquor (Table II). In order to confirm that the correlations were due to a strong biological link between the two variables and not due to a potential confounder, we performed a partial correlation using fecal pH as control variable. Partial correlation analysis confirmed only the positive correlations (with ruminal pH and total immunoglobulins) (Supplementary table II).

B cells are the most frequent mononuclear leukocyte population in rumen liquor

Rumen leukocytes were investigated by flow cytometry analysis. Different leukocyte populations at low concentrations were identified among the mononuclear cells isolated from the rumen liquor of 93 animals from 12 farms. As shown in Figure 3, the most frequent mononuclear leukocyte population in our rumen fluid samples were B cells (IL-A30+ cells; 1% of samples with zero counts), with a median value of 2.93% of the total number of analyzed cells. All the other

populations showed the following medians: 0.5% (CD8+ T cells; 15% of samples with zero counts), 0.2% (IL-A24+ myeloid cells; 26% of samples with zero counts), 0.1% (both CD4+ and gamma delta T cells; 41% of samples with zero counts of CD4 and 42% of gamma delta T cells). The CD45 mAb was shown to underestimate the real prevalence of leukocytes in rumen liquor. Therefore this set of data was not included in the final analysis. These prevalence figures refer to total, mononuclear, non-bacterial cells in rumen fluid samples, mainly including protozoa and detached rumen wall cells. As expected, no significant correlation was found between prevalence of cells and mRNA expression (IGLC and CD45 genes). Our results do not rule out a possible presence in rumen liquor of neutrophils, which were excluded though by our isolation procedure.

Correlations among rumen liquor leukocyte profile, metabolic and hematologic parameters

In order to define new diagnostic parameters in dairy cows, we searched any possible correlation between the leukocyte profile obtained by flow cytometry analyses and the other sets of parameters analyzed in the present study. Statistical analyses demonstrated a significant correlation between ruminal leukocyte populations, ruminal immunoglobulins and biochemical parameters. Statistical results are recapitulated in Table III. Interestingly, we observed a positive correlation between B cells (IL-A30, marker) in rumen fluid and the pH of feces ($p<0.0001$). Moreover, the prevalence of IL-A29-positive, γδ T cells showed a positive correlation with paraoxonase levels in plasma ($p=0.0005$) whereas IL-A30-positive cells had a negative correlation ($p< 0.0001$). IL-A24 positive leukocytes (myeloid cells) in the rumen liquor also showed a positive correlation with bilirubinemia ($p =0.0002$). The statistical significance of the bivariate correlation could be strongly biased by the high percentage of zero values, overall for IL-A12+, IL-A29+ and IL-A24+ cells. Accordingly, we also did the partial correlation analysis among variables showing a strong statistically significant bivariate correlation, to exclude the influence of a specific parameter on the correlation between two other parameters. Partial correlation analysis confirmed all the bivariate correlations, except for IL-A24 with bilirubinemia (Supplementary table II).

Correlation among rumen liquor immunoglobulins and metabolic parameters

Given that B cells are the major population among rumen mononuclear leukocytes, in 77 animals from 12 farms we also investigated the presence of immunoglobulins in the rumen liquor. IgM were detectable in the rumen fluid in a wide range of concentrations (10-1800 ng/ml), as also shown for total Ig (40-10400 ng/ml). Both IgM and total Ig were positively correlated with ruminal pH ($p=0.0055$ and $p=0.0002$, respectively) and negatively with VFA concentrations (total acids and single acids alike) (Supplementary data and Table IV). IgM, but mainly total Ig presented a strong correlation with some blood ion concentrations (Ca, K, Na) (Table IV). Also, Ig showed a strong positive correlation with urea in blood ($p=0.0001$), a parameter that might reflect protein intake, provided that its dietary percentage and degradability are analogous through the diets (Supplementary data and Table IV). In order to confirm that the correlations were due to a strong biological link between the two variables and not due to a potential confounder, we performed a partial correlation using hematocrit as control variable. Partial correlation analysis confirmed the majority of the bivarial correlations (Supplementary table II).

Discussion

The innate immune responses to non-infectious stressors have been highlighted in a recent multi-author book of ours (Amadori, 2016), which also deals with the metabolic stress of high-yielding dairy cows. The common molecular basis of such responses is represented by the formation of products related to tissue damage (Damage-Associated Molecular Patterns, DAMPs) such as salts (extracellular potassium), ATP, Reactive Oxygen Metabolites (ROMs), uric acid, mitochondrial DNA. DAMPs are recognized by the same Toll-Like Receptors (TLRs) recognizing microbial molecules (Pathogen Associated Molecular Patterns, PAMPs). The activation of TLRs leads to signaling cascades and production of pro-inflammatory mediators, regardless of the activating ligands (Amadori, 2016). In addition to these components, neo-antigens (stress antigens) are expressed on the cell surface, and recognized by lymphoid cells (T cells and Natural Killer cells). In practice, in a state of homeostasis, the healthy subject is constantly exposed to inflammatory stimuli of different nature at very low concentrations (LPS, nucleic acids, NEFA, DAMPs, etc.), which determine a condition of alert of the innate immune system (so-called "homeostatic inflammation") (Amadori, 2016). In this conceptual framework, our previous studies (Trevisi et al., 2009; Trevisi et al., 2014a) had demonstrated the functional capacity of bovine forestomachs to start innate immune responses to abnormal diet profiles, including modulation of cytokines and leukocyte infiltrations in rumen fluids through a continuous cross-talk between forestomachs and oral lymphoid tissues. The present study confirms the findings of the previous one in a field scenario and sets further important milestones.

First, we outlined previously unreported relationships between productive, metabolic and immune parameters, which could have important repercussions on diagnosis and characterization of production diseases and digestive troubles. Thus, our data confirm the difficulties related to the diagnosis of SARA by a single measurement of ruminal pH (Trevisi et al., 2014b), or other traditional diagnostic parameters; this sets the need for more specific indices, ruminal or else, to identify herds at risk. The actual boundaries of SARA and relevant threshold levels of rumen pH

should be re-appraised as well on the basis of our findings. Also, the lower levels of plasma Zn (sequestration in the liver during inflammatory events) and cholesterol (as index of the cholesterol binding protein, a negative acute phase protein, APP) (Bertoni and Trevisi, 2013) in the alleged SARA farms under study are likely to depict an ongoing inflammatory response within the animals' coping ability. Our data agree with the results of a meta-analyses of SARA experiments (Zebeli and Metzler-Zebeli, 2012), in which a decrease of fiber digestibility and an increase in plasma concentrations of positive APPs were observed when rumen pH was below 5.8 for at least 6 hours a day.

In agreement with our working hypothesis, our results show a correlation between inflammatory markers (i.e. paraoxonase, negative acute phase proteins) in blood and some markers in the rumen liquor (e.g. gamma delta T cells and B cells). Also, partial correlation analysis confirmed wide correlations of B cells with some metabolic parameters under study. So, we can conclude that the analysis of rumen fluids could contribute to identification of herds at risk for subclinical metabolic disorders. In particular, these data highlight the role of analyses on rumen fluids as diagnostic support tools in doubtful cases, integrating the traditional analyses on blood and fecal samples, and revealing subclinical rumen dysfunctions. Accordingly, the analysis of the rumen liquor should be adopted whenever traditional diagnostic protocols provide inconclusive results. Importantly, rumen liquor sampling is an easy manual task for a trained veterinary practitioner, that does not cause any harmful side effect to the animals. In our study, hundreds of samplings were performed on farm and not even one side effect was observed in the cows under study. This was also reported by other researchers and veterinary practitioners (Bramley et al., 2008; Garrett et al., 1999; Kleen et al., 2004; Nordlund and Garrett, 1994; Nordlund et al., 1995).

Rumen leukocytes can vary in number and cell subpopulations as a result of the saliva output. Indeed, leukocytes in saliva amounted in our study to some 400 cells/mL. This finding implies the existence of a substantial exchange of information between rumen and oral cavity. On the basis of some 200-250 liters/day of saliva production by dairy cows (Maekawa et al., 2002), 80-100 x 10^6

leukocytes approximately could move back and forth from the rumen cavity and be exposed to cytokines and other mediators released by forestomach walls (Trevisi et al., 2014a). Most importantly, such leukocytes could be the foundation of a cross-talk, liaising potential noxae in the digestive tract with the lymphoid tissues in the oral cavity for subsequent responses and effector activities. Our findings do not rule out the possibility that some leukocytes enter rumen fluids from the lamina propria of the forestomach walls (Sato et al., 1990), where they reside in possible association with dendritic (Langerhans) cells (Gerneke, 1977). Thus, our data make a case for new research efforts concerning the origin and clearance of rumen leukocytes. Regardless of the actual origin of leukocytes in rumen fluids, a powerful amplification of inflammatory signals could take place for local and systemic responses, which may also involve the rumen microbiota (Minuti et al., 2015; Yáñez-Ruiz et al., 2015).

On the whole, our results confirm and widen the scope of our previous study on innate immunity in bovine forestomachs (Trevisi et al., 2014a). First, we could confirm in a field trial that leukocyte infiltration in rumen fluids is inhibited by low, SARA-like pH levels. In this respect, there was a substantial agreement between Real Time PCR data for CD45 gene expression and prevalence data of B cells (i.e. the prevailing mononuclear leukocyte population in rumen fluids). Accordingly, the concentrations of total Ig and IgM in rumen fluids were also regulated by rumen pH levels, as well by VFA concentrations. These findings outline a novel integrated control of the innate immune system over the metabolic activities in bovine forestomachs. This is probably based on the aforementioned cross-talk between forestomachs and lymphoid tissues in the oral cavity (Trevisi et al., 2009), whereby metabolic changes could be sensed following the release of inflammatory mediators by the forestomach walls and/or by the leukocytes in rumen fluids (Trevisi et al., 2014a). In this scenario, the pathogenesis of inflammatory diseases linked to digestive disorders like laminitis (Nagaraja et al., 1978; Nocek, 1997) could be re-appraised and investigated on the basis of new, more convincing working hypotheses, based on the release of second messengers from

lymphoid tissues of the oral cavity. Such a release had been demonstrated *in vitro* in a model of IFN alpha-treated pig tonsil lymphocytes (Razzuoli et al., 2014).

Interestingly, Ig levels in rumen fluids would be positively affected by the levels of feed intake, as indirectly suggested by the correlation with plasma urea levels, and negatively by low ruminal pH levels, as also shown for leukocyte infiltration. It is tempting to speculate that the innate immune system could somehow react to metabolic changes by modulating the balance between orexygenic and anorexigenic signals in the hypothalamus (Perry and Wang, 2012; Sartin et al., 2011), and/or regulating the levels of lipomobilization and plasma NEFA, which are also sensed by TLR4 for further possible feedback regulations (Suganami et al., 2007).

Total Ig in rumen fluids also showed a negative and a positive correlation with plasma K and Na, respectively. This may imply that a pronounced inflammatory condition and production of DAMPs, like extracellular K, give rise to lower dry matter intake, in agreement with our correlation data with urea levels in this study, and with our previous results (Trevisi et al., 2010; Trevisi et al., 2007).

On the basis of our findings, dairy farms could be ranked, using the immunological profile in rumen fluids (rumen leukocyte populations, CD45 gene expression, and immunoglobulin concentrations) and inflammo-metabolic blood parameters. In this scenario, further experiments are badly needed to characterize the immunological profile of the rumen liquor under different pathological conditions, not included in our study. Beyond this caveat, the described markers can represent an important adjunct to established laboratory procedures. In particular, they can reveal fine regulatory activities in cows with normal rumen pH levels, and under SARA-like conditions, respectively. In fact, such SARA-like conditions may underlie a successful coping strategy of high-yielding dairy cows in the framework though of a persistent disease risk. In this scenario, our panel of ruminal immune markers would suit the need for adequate testing of diets and rations during lactation. Most important, an integrated view of clinical and productive data associated with ruminal markers and inflammo-metabolic blood parameters should allow for a better insight into the homeostasis of dairy cattle vis-à-vis the metabolic stress of high milk yield, negative energy balance

and pathological conditions in other organs. This outlines a fascinating model to study the global role of the innate immune system for environmental adaptation and survival under intensive farming conditions.

Acknowledgements

The authors wish to thank Giovanni Linetti and Andrea Vegezzi (NPM TECH srl) for help during sampling and data collection; Cristiano Barisani (bovine practitioner) for performing rumenocentesis; Dr. L. Capucci and E. Brocchi (IZSLER, Brescia, Italy) for providing monoclonal antibodies to bovine Ig and instructions for use in ELISA; Dr. A. Brambilla for her support in the laboratory procedures; Prof. V. Bronzo for his support in the statistical analysis.

Fundings: This work was supported by a Grant provided by Guna SpA (Milan, Italy); the Piano Sviluppo UNIMI Project, linea B of the University of Milan (G42I14000070005); a grant provided by NPM TECH srl, Sant'Angelo di Piove di Sacco, Padua, Italy.

References

Ahmed, S., Minuti, A., Bani, P., 2013. In Vitro Rumen Fermentation Characteristics of Some Naturally Occurring and Synthetic Sugars. Italian Journal of Animal Science 12, e57.

Allen, M.S., 1997. Relationship between fermentation acid production in the rumen and the requirement for physical effective fiber. J. Dairy Sci. 80, 1447–1462.

Amadori, M., 2016. The Innate Immune Response to Non-infectious Stressors: Human and Animal Models. Academic Press.

Baldwin, C.L., Teale, A.J., Naessens, J.G., Goddeeris, B.M., MacHugh, N.D., Morrison, W.I., 1986. Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonal antibodies and function: similarity to lymphocytes defined by human T4 and murine L3T4. J Immunol 136, 4385-4391.

Bertoni, G., Trevisi, E., 2013. Use of the liver activity index and other metabolic variables in the assessment of metabolic health in dairy herds. The Veterinary clinics of North America. Food animal practice 29, 413-431.

Bionaz, M., Trevisi, E., Calamari, L., Librandi, F., Ferrari, A., Bertoni, G., 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. Journal of dairy science 90, 1740-1750.

Bouchard, L., Blais, S., Desrosiers, C., Zhao, X., Lacasse, P., 1999. Nitric oxide production during endotoxin-induced mastitis in the cow. Journal of dairy science 82, 2574-2581.

Bramley, E., Lean, I., Fulkerson, W., Stevenson, M., Rabiee, A., Costa, N., 2008. The definition of acidosis in dairy herds predominantly fed on pasture and concentrates. Journal of dairy science 91, 308-321.

Calamari, L., Ferrari, A., Minuti, A., Trevisi, E., 2016. Assessment of the main plasma parameters included in a metabolic profile of dairy cow based on Fourier Transform mid-infrared spectroscopy: preliminary results. BMC veterinary research 12, 4.

Davis, C.L., Brown, R.E., Beitz, D.C., 1964. Effect of feeding high-grain restricted-roughage rations with and without bicarbonates on the fat content of milk produced and proportions a of volatile fatty acids. J. Dairy Sci. 47, 1217–1226.

Drackley, J.K., 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? Journal of dairy science 82, 2259-2273.

Eckel, E.F., Ametaj, B.N., 2016. Invited review: Role of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. Journal of dairy science 99, 5967-5990.

Ellis, J.A., Morrison, W.I., Goddeeris, B.M., Emery, D.L., 1987. Bovine mononuclear phagocytic cells: Identification by monoclonal antibodies and analysis of functional properties. Veterinary Immunology and Immunopathology 17, 125-134.

Enemark, J.M., 2008. The monitoring, prevention and treatment of sub-acute ruminal acidosis (SARA): a review. Vet J 176, 32-43.

Enemark, J.M.D., Jorgensen, R., Enemark, P.S., 2002. Rumen acidosis with special emphasis on diagnostic aspects of subclinical rumen acidosis: a review. Veterinarija ir zootechnika 20, 16-29.

Fayen, J., Huang, J.H., Ferrone, S., Tykocinski, M.L., 1998. Negative signaling by anti-HLA class I antibodies is dependent upon two triggering events. International immunology 10, 1347-1358.

Fernando, S.C., Purvis, H.T., 2nd, Najar, F.Z., Sukharnikov, L.O., Krehbiel, C.R., Nagaraja, T.G., Roe, B.A., Desilva, U., 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. Applied and environmental microbiology 76, 7482-7490.

Ferré, N., Camps, J., Prats, E., Vilella, E., Paul, A., Figuera, L., Joven, J., 2002. Serum paraoxonase activity: a new additional test for the improved evaluation of chronic liver damage. Clinical chemistry 48, 261-268.

Garrett, E., Pereira, M., Nordlund, K., Armentano, L., Goodger, W., Oetzel, G., 1999. Diagnostic methods for the detection of subacute ruminal acidosis in dairy cows. Journal of dairy science 82, 1170-1178.

Gerneke, W.H., 1977. Langerhans cells in the epithelium of the bovine forestomach: their role in the primary immune response. Journal of the South African Veterinary Association 48, 187-192.

Gilliam, M.B., Sherman, M., Griscavage, J., Ignarro, L., 1993. A spectrophotometric assay for nitrate using NADPH oxidation by Aspergillus nitrate reductase. Analytical biochemistry 212, 359-365.

Gozho, G.N., Plaizier, J.C., Krause, D.O., Kennedy, A.D., Wittenberg, K.M., 2005. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. Journal of dairy science 88, 1399-1403.

Howard, C.J., Morrison, W.I., Bensaid, A., Davis, W., Eskra, L., Gerdes, J., Hadam, M., Hurley, D., Leibold, W., Letesson, J.J., et al., 1991. Summary of workshop findings for leukocyte antigens of cattle. Vet Immunol Immunopathol 27, 21-27.

Knowlton, K.F., Glenn, B.P., Erdman, R.A., 1998. Performance, ruminal fermentation, and site of starch digestion in early lactation cows fed corn grain harvested and processed differently. J. Dairy Sci. 81, 1972–1984.

Ingvartsen, K.L., Dewhurst, R.J., Friggens, N.C., 2003. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. Livestock Production Science 83, 277-308.

Kleen, J., Hooijer, G., Rehage, J., Noordhuizen, J., 2004. Rumenocentesis (rumen puncture): a viable instrument in herd health diagnosis. DTW. Deutsche tierarztliche Wochenschrift 111, 458-462.

Loor, J.J., Bertoni, G., Hosseini, A., Roche, J.R., Trevisi, E., 2013. Functional welfare – using biochemical and molecular technologies to understand better the welfare state of peripartal dairy cattle. Animal Production Science 53, 931-953.

Maddox, J.F., Mackay, C.R., Brandon, M.R., 1985. The sheep analogue of leucocyte common antigen (LCA). Immunology 55, 347-353.

Maekawa, M., Beauchemin, K.A., Christensen, D.A., 2002. Chewing activity, saliva production, and ruminal pH of primiparous and multiparous lactating dairy cows. Journal of dairy science 85, 1176-1182.

Minuti, A., Ahmed, S., Trevisi, E., Piccioli-Cappelli, F., Bertoni, G., Jahan, N., Bani, P., 2014. Experimental acute rumen acidosis in sheep: consequences on clinical, rumen, and gastrointestinal permeability conditions and blood chemistry. Journal of animal science 92, 3966-3977.

Minuti, A., Palladino, A., Khan, M.J., Alqarni, S., Agrawal, A., Piccioli-Capelli, F., Hidalgo, F., Cardoso, F.C., Trevisi, E., Loor, J.J., 2015. Abundance of ruminal bacteria, epithelial gene expression, and systemic biomarkers of metabolism and inflammation are altered during the peripartal period in dairy cows. Journal of dairy science 98, 8940-8951.

Mulligan, F.J., Doherty, M.L., 2008. Production diseases of the transition cow. Vet J 176, 3-9.

Naessens, J., Newson, J., Williams, D.J., Lutje, V., 1988. Identification of isotypes and allotypes of bovine immunoglobulin M with monoclonal antibodies. Immunology 63, 569-574.

Murphy, M., Åkerlind, M., K. Holtenius, K., 2000. Rumen fermentation in lactating cows selected for milk fat content fed two forage to concentrate ratios with hay or silage. J. Dairy Sci. 83, 756–764.

Nagaraja, T.G., Bartley, E.E., Fina, L.R., Anthony, H.D., 1978. Relationship of rumen gram-negative bacteria and free endotoxin to lactic acidosis in cattle. Journal of animal science 47, 1329-1337.

Nocek, J.E., 1997. Bovine acidosis: implications on laminitis. Journal of dairy science 80, 1005-1028.

Nordlund, K., Garrett, E., 1994. Rumenocentesis: A technique for collecting rumen fluid for the diagnosis of subacute rumen acidosis in dairy herds. Bovine Practitioner 28, 109-109.

Nordlund, K.V., Garrett, E.F., Oetzel, G.R., 1995. Herd-based rumenocentesis-a clinical approach to the diagnosis of sub acute rumen acidosis. Compendium on Continuing Education for the Practising Veterinarian. 17(8): S48-56.

Oetzel, G., Smith, R., 2000. Clinical aspects of ruminal acidosis in dairy cattle, Proceedings of the Thirty-Third Annual Conference, American Association of Bovine Practitioners, Rapid City, South Dakota, USA, September 21-23, 2000. American Association of Bovine Practitioners, pp. 46-53.

Olson, J., 1991. Relationship of nutrition to abomasal displacement and parturient paresis. Bovine Practitioner 26, 88-91.

Penner, G.B., Oba, M., Gabel, G., Aschenbach, J.R., 2010. A single mild episode of subacute ruminal acidosis does not affect ruminal barrier function in the short term. Journal of dairy science 93, 4838-4845.

Perry, B., Wang, Y., 2012. Appetite regulation and weight control: the role of gut hormones. Nutrition & diabetes 2, e26.

Plaizier, J.C., Krause, D.O., Gozho, G.N., McBride, B.W., 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. Vet J 176, 21-31.

Razzuoli, E., Villa, R., Ferrari, A., Amadori, M., 2014. A pig tonsil cell culture model for evaluating oral, low-dose IFN-alpha treatments. Vet Immunol Immunopathol 160, 244-254.

Riva, F., Rahman, M.M., Turin, L., Ceciliani, F., Russo, S., Tribbioli, G., Lecchi, C., 2010. TIR8 receptor expression in bovine tissues. Vet Immunol Immunopathol 136, 65-70.

Sartin, J.L., Whitlock, B.K., Daniel, J.A., 2011. Triennial Growth Symposium: neural regulation of feed intake: modification by hormones, fasting, and disease. Journal of animal science 89, 1991-2003.

Sato, S., Ogimoto, K., Nakai, Y., 1990. Distribution of immunoglobulin-containing cells in calves inoculated orally with ruminal Bacteroides succinogenes and Selenomonas ruminantium. Nihon juigaku zasshi. The Japanese journal of veterinary science 52, 719-725.

Skinner, J., Brown, R., Roberts, L., 1991. Bovine haptoglobin response in clinically defined field conditions. The veterinary record 128, 147-149.

Suganami, T., Tanimoto-Koyama, K., Nishida, J., Itoh, M., Yuan, X., Mizuarai, S., Kotani, H., Yamaoka, S., Miyake, K., Aoe, S., 2007. Role of the Toll-like receptor 4/NF-κB pathway in saturated fatty acid–induced inflammatory changes in the interaction between adipocytes and macrophages. Arteriosclerosis, thrombosis, and vascular biology 27, 84-91.

Sunderman, F.W., Jr., Nomoto, S., 1970. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. Clinical chemistry 16, 903-910.

Tajima, K., Arai, S., Ogata, K., Nagamine, T., Matsui, H., Nakamura, M., Aminov, R.I., Benno, Y., 2000. Rumen Bacterial Community Transition During Adaptation to High-grain Diet. Anaerobe 6, 273-284.

Trevisi, E., Amadori, M., Bakudila, A., Bertoni, G., 2009. Metabolic changes in dairy cows induced by oral, low-dose interferon-alpha treatment. Journal of animal science 87, 3020-3029.

Trevisi, E., Amadori, M., Riva, F., Bertoni, G., Bani, P., 2014a. Evaluation of innate immune responses in bovine forestomachs. Research in veterinary science 96, 69-78.

Trevisi, E., Bertoni, G., Archetti, I., Amadori, M., Lacetera, N., 2011. Inflammatory response and acute phase proteins in the transition period of high-yielding dairy cows. INTECH Open Access Publisher.

Trevisi, E., Ferrari, A., Piccioli-Cappelli, F., Grossi, P., Bertoni, G., 2010. An additional study on the relationship between the inflammatory condition at calving time and net energy efficiency in dairy cows. Energy and Protein Metabolism and Nutrition. EAAP publication, 489-490.

Trevisi, E., Gubbiotti, A., Bertoni, G., 2007. Effects of inflammation in peripartum dairy cows on milk yield, energy balance and efficiency. PUBLICATION-EUROPEAN ASSOCIATION FOR ANIMAL PRODUCTION 124, 395.

Trevisi, E., Minuti, A., Cogrossi, S., Grossi, P., Ahmed, S., Bani, P., 2014b. Can a single rumen sample really diagnose SARA in commercial farms? Animal Production Science 54, 1268-1272.

Trevisi, E., Zecconi, A., Cogrossi, S., Razzuoli, E., Grossi, P., Amadori, M., 2014c. Strategies for reduced antibiotic usage in dairy cattle farms. Research in veterinary science 96, 229-233.

Van Knegsel, A., Remmelink, G., Jorjong, S., Fievez, V., Kemp, B., 2014. Effect of dry period length and dietary energy source on energy balance, milk yield, and milk composition of dairy cows. Journal of dairy science 97, 1499-1512.

Weimer, P.J., 2015. Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. Frontiers in microbiology 6, 296.

Yáñez-Ruiz, D.R., Abecia, L., Newbold, C.J., 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. Frontiers in microbiology 6.

Zebeli, Q., Metzler-Zebeli, B., 2012. Interplay between rumen digestive disorders and diet-induced inflammation in dairy cattle. Research in veterinary science 93, 1099-1108.

Zhou, Z., Loor, J.J., Piccioli-Cappelli, F., Librandi, F., Lobley, G.E., Trevisi, E., 2016. Circulating amino acids in blood plasma during the peripartal period in dairy cows with different liver functionality index. Journal of dairy science 99, 2257-2267.

FIGURE CAPTIONS

Figure 1. Presence of leukocytes in the saliva.

A) Cells isolated from the saliva of 3 cows were analyzed by flow cytometry to investigate CD45 expression. B) CD45, IGLC and KRT5 mRNA expression was analyzed by Real Time PCR in the saliva of 4 cows. The gene expression level of the target genes was normalized to GAPDH and the results are presented as Arbitrary Units (Delta Delta Ct x 10000). Results are expressed as mean ± 1 standard error in both panels.

Figure 2. Field survey of CD45 mRNA expression in rumen fluid.

CD45 mRNA expression was analyzed by Real Time PCR in 98 cows from 11 farms. The gene expression level of CD45 was normalized to GAPDH and the results are presented as mean value of our Arbitrary Units (Delta Delta Ct x 10000).

Figure 3. Mononuclear leukocyte population profile in rumen fluid.

Mononuclear leukocyte population profile was analyzed by flow cytometry in 93 cows from 12 farms. Results are shown as box and whiskers plots, as median values. The proportion of samples with zero counts for each cell type are the following: IL-A12+ (41%), IL-A29+ (42%), IL-A30+ (1%), IL-A51+ (15%) and IL-A24+ (26%).

Figure 1

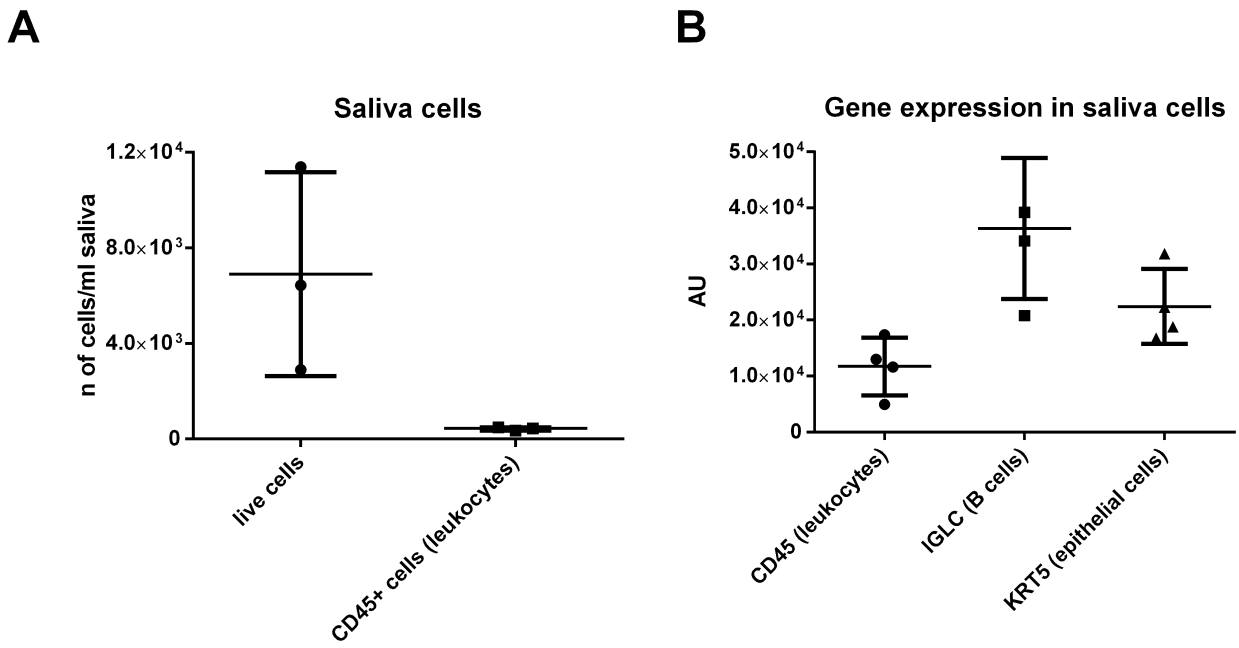

Figure 2

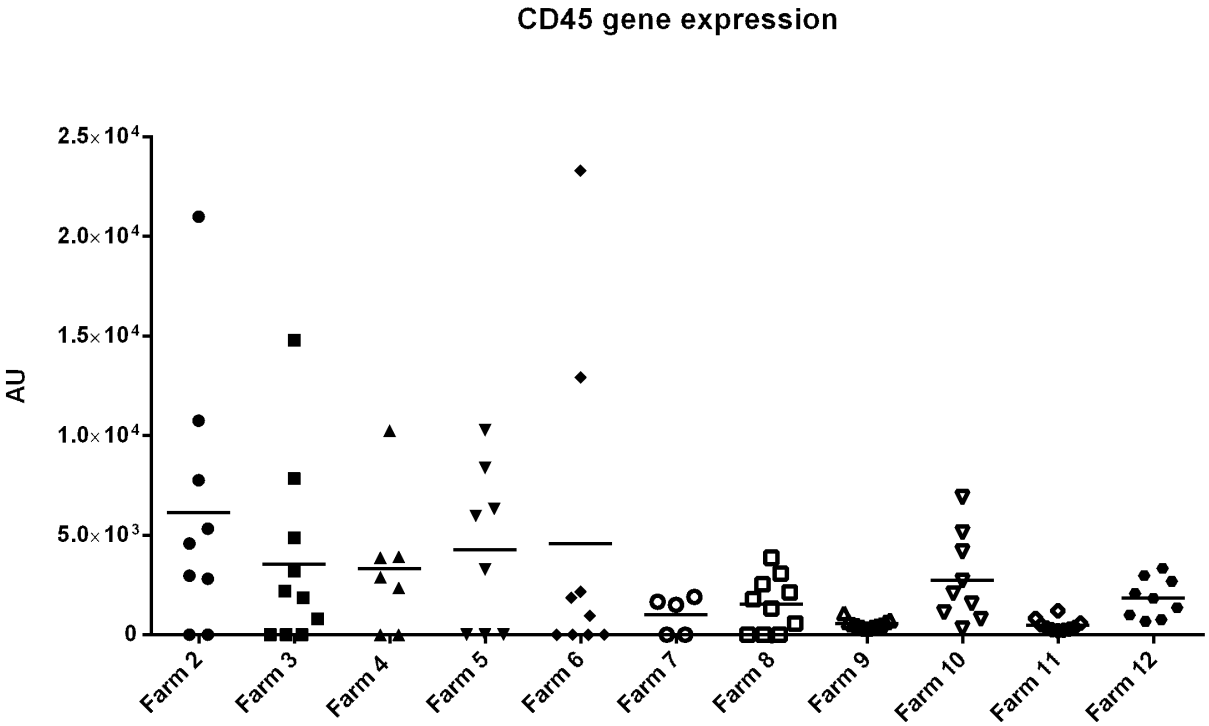

Figure 3

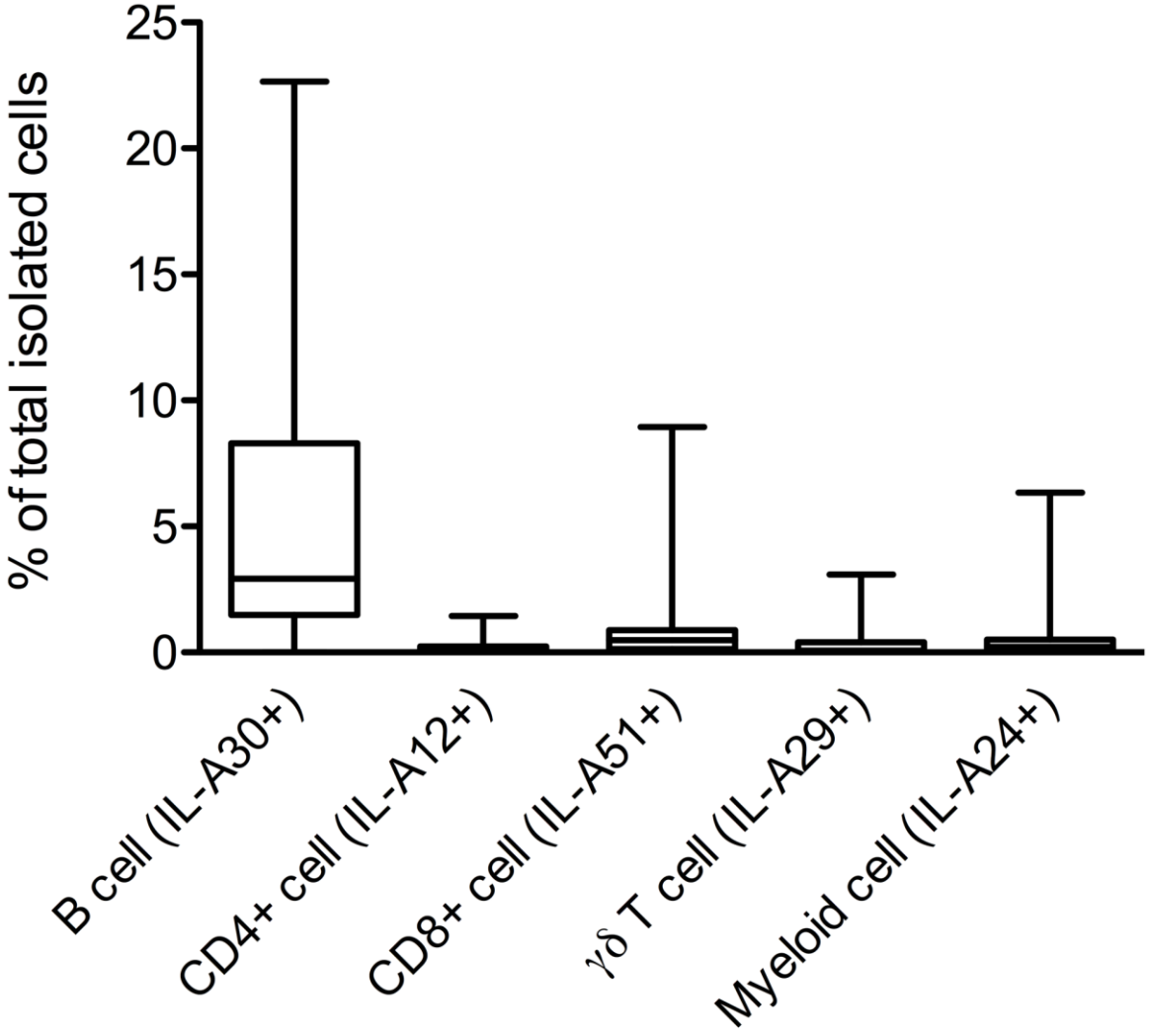

Table I. Oligonucleotide primer sequences for SYBR Green quantitative RT-polymerase chain reaction amplification.

Gene	*Protein*	*Sequence*	*Gene Bank accession 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
IGLC	Immunoglobulin light chain lambda	F: TCCTGGGTCAGCCCAAGTCC R: GGTGGTCTTCACGTTGCGG	GI:92096964
KRT5	Bovine keratin 5	F: CAAGGTCCTGGACACCAAGT R: TCCAGCTGTCTCCTGAGGTT	GI: 56710316

F = forward primer; R = reverse primer.

Reference

Trevisi, E., Amadori, M., Riva, F., Bertoni, G., Bani, P., 2014. Evaluation of innate immune responses in bovine forestomachs. Research in veterinary science 96, 69-78.

Table II. Bivarial linear correlations among CD45 gene expression in rumen fluids, pH, Ig and prevalence of some leukocyte populations.

	Parameter	Rho (Spearman)	Statistical significance	p	Sample source
CD45 gene expression (leukocytes)	Rumen pH	0.27	**	0.0068	Rumen
	IL-A29 (gamma delta T cells)	-0.29	*	0.024	Rumen
	IL-A24 (myeloid cells)	-0.27	*	0.042	Rumen
	Total immunoglobulins in rumen fluid	0.36	**	0.0092	Rumen

The bivarial linear correlations was performed between CD45 gene expression and one parameters at a time.

Table III. Bivarial linear correlations among leukocyte populations and biochemical or hematological parameters.

	Parameter	Rho (Spearman)	Statistical significance	p	Sample source
ILA12 (CD4+ cells)	ILA29 (gamma delta T cells)	0.48	****	<0.0001	Rumen
	ILA24 (myeloid cells)	0.50	****	<0.0001	Rumen

The bivarial linear correlations was performed between ILA12 expression and one parameters at a time.

	Parameter	Rho (Spearman)	Statistical significance	p	Sample source
ILA29 (gamma delta T cells)	Paraoxonase	0.37	***	0.0005	Plasma
	ILA12 (CD4+ cells)	0.48	****	<0.0001	Rumen
	ILA24 (myeloid cells)	0.48	****	<0.0001	Rumen

The bivarial linear correlations was performed between ILA29 expression and one parameters at a time.

	Parameter	Rho (Spearman)	Statistical significance	p	Samples source
ILA30 (B cells)	Fecal pH	0.40	****	<0.0001	Faeces
	Paraoxonase	-0.44	****	<0.0001	Plasma

The bivarial linear correlations was performed between ILA30 expression and one parameters at a time.

	Parameter	Rho (Spearman)	Statistical significance	p	Sample sources
ILA51 (CD8+	IgMCD21 (mature B	0.43	***	0.0002	Rumen

cells) cells)

The bivarial linear correlations was performed between ILA51 expression and one parameters at a time.

	Parameter	Rho (Spearman)	Statistical significance	p	Samples source
ILA24 (myeloid cells)	Bilirubinemia	0.39	***	0.0002	Plasma
	ILA12 (CD4+ cells)	0.50	****	<0.0001	Rumen
	ILA29 (gamma delta T cells)	0.48	****	<0.0001	Rumen
	IgMCD21 (mature B cells)	0.44	***	0.0001	Rumen

The bivarial linear correlations was performed between ILA24 expression and one parameters at a time.

Table IV. Bivarial linear correlations among immunoglobulin concentration in rumen fluid and biochemical or hematological parameters.

	Parameter	Rho (Spearman)	Statistical significance	p	Sample source
IgM in rumen fluid	Ruminal valeric acid	-0.45	****	<0.0001	Rumen
	Ruminal volatile fatty acids	-0.41	***	0.0004	Rumen
	ME[§] (Mature equivalent milk production)	-0.52	***	0.001	Rumen
	Na	0.47	****	<0.0001	Plasma
	Total immunoglobulins in rumen fluid	0.60	****	<0.0001	Rumen

[§] milk production records of each cow are standardized to predict their expected milk production potential as mature cows. The adjustment considers month of calving, age at calving, previous days open.

The bivarial linear correlations was performed between IgM and one parameters at a time.

	Parameter	Rho (Spearman)	Statistical significance	p	Sample source
Total immunoglobulins in rumen fluid	Ruminal pH	0.43	***	0.0002	Rumen
	Basophils (number)	0.38	***	0.0009	Plasma
	Basophils (%)	0.39	***	0.0006	Plasma
	Hematic urea	0.49	****	<0.0001	Plasma
	Na	0.43	***	0.0001	Plasma
	K	-0.40	***	0.0004	Plasma
	Paraoxonase	-0.39	***	0.0005	Plasma
	IgM in rumen fluid	0.60	****	<0.0001	Rumen

The bivarial linear correlations was performed between Total Ig and one parameters at a time.

Highlights

- Leukocytes of the rumen liquor partly derive from saliva
- B cells are the most frequent mononuclear leukocyte population in rumen liquor
- Rumen fluid analysis could contribute to identify subclinical disorders