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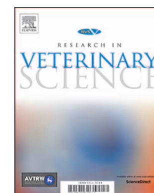

Innate immune responses to metabolic stress can be detected in rumen fluids

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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.

1. 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 et 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

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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.

2. Materials and methods

2.1. 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 1000 × *g*. Saliva was then processed for flow cytometry and molecular analyses.

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

2.2. 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 h after collection.

Rumen fluids were collected by rumenocentesis as previously described (Trevisi et al., 2014b) about 6 h 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 3000 × *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. (2013) and 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) 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 3500 × *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.

2.3. 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 1500 × *g* for 20 min 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 × *g*, 10 min, 5 °C), resuspended in 2 ml of PBS and centrifuged again through a 1-ml FCS layer at 300 × *g* for 10 min 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.

2.4. 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

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